

Different molecular techniques to examine the life cycles of phytoplankton or HAB species

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LIFEHAB
Life histories of microalgal species causing harmful blooms

Report of a European workshop organised jointly by the Fifth Framework Programme Energy, Environment and Sustainable Development of the European Commission, the Institut de Ciències del Mar, CMIMA-CSIC (Barcelona) and the Calvià Town Council (Majorca, Balearic Islands).

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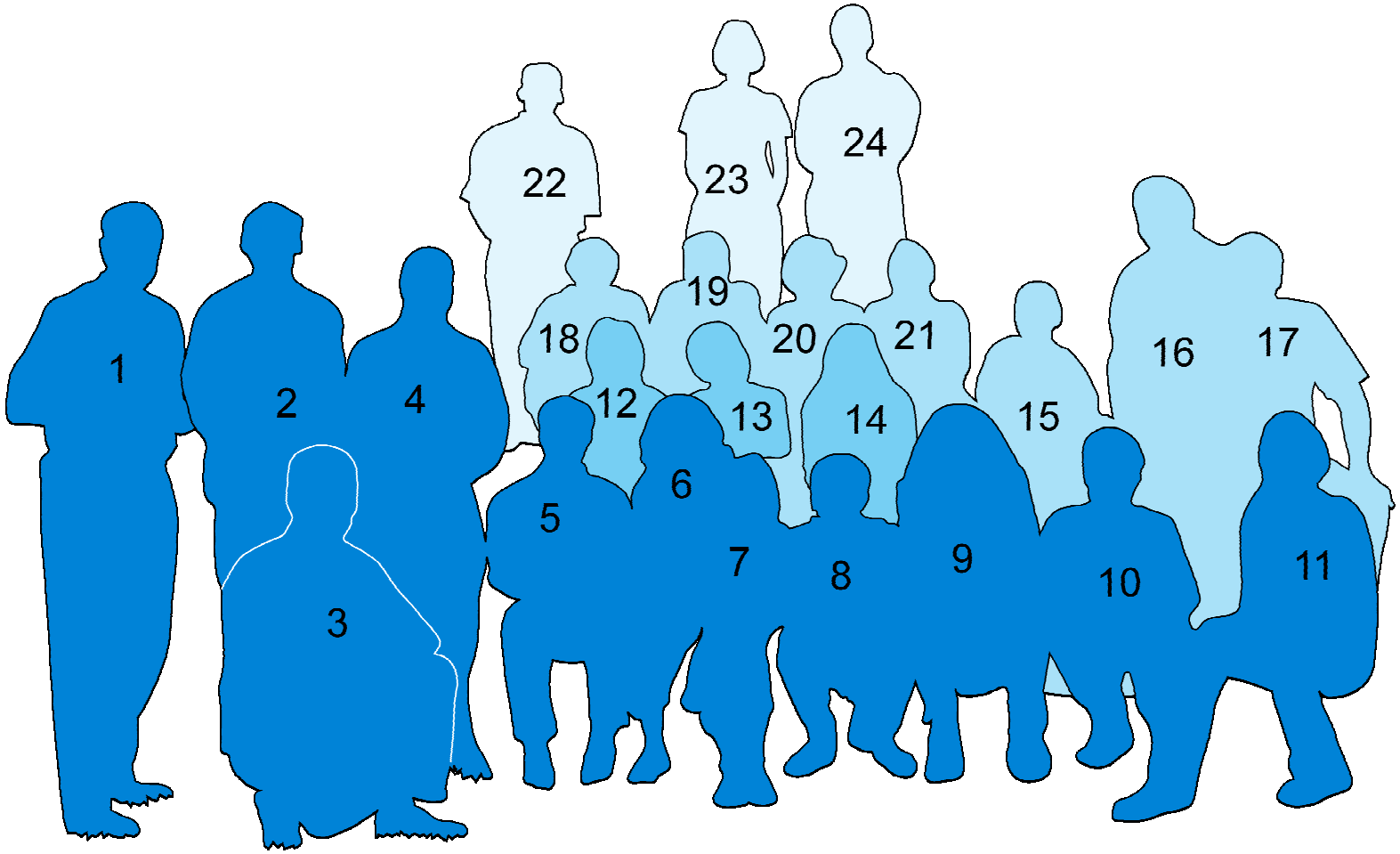
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EXECUTIVE SUMMARY

The LIFEHAB workshop was conceived as a forum for discussion among specialists on life histories of microalgal species. The main goals were to: 1) review current knowledge on the life-cycles of phytoplanktonic organisms, focusing on HAB species; 2) identify the role of heteromorphic life cycles in population dynamics; 3) define future HAB research directions to fill existing gaps in knowledge; 4) debate the most appropriate approaches and methods; and 5) promote the development of co-operative scientific initiatives.

The workshop included 25 presentations covering the most relevant aspects of life cycle studies in phytoplankton, ranging from an updated review of the information available for the major groups, to the role played by different life history stages in population dynamics, to the new tools available for addressing research questions. Discussion sessions focussed on the main gaps of knowledge and research needs.

Complex, heteromorphic life cycles are a general feature of the large majority of phytoplanktonic algae. Diatoms, dinoflagellates, haptophytes and raphidophytes are characterized by different life cycles, including stages with different ploidy levels, linked together by syngamy and meiotic events. There is increasing evidence that complex mating systems are a common feature among phytoplankton organisms. Understanding these is crucial for the taxonomic circumscription of species and for gaining insights into speciation mechanisms in these organisms.

Most taxa are characterized by an alternation between dormant/quiescent phases and growth phases. It is recognized that life history strategies (*sensu latu*, including formation of resistant/defense stages, colonies, encystment-excystment rates, vertical migrations, modulation of growth capabilities, etc), play a key role in the occurrence and dynamics of Harmful Algae Blooms (HABs). The alternation between different life history stages and their relationships with the physical, chemical and biological environment are very important for the success of harmful species.

In the course of the Workshop, the main gaps in knowledge and research priorities in this field were identified and discussed. Results of these discussions and recommendations are summarized in the following list of topics.

Life history complexity and alternating life cycle stages

- Identification of all the different stages included in the species' life histories (e.g. small cells, colonies, temporary cysts, coccoid stages, resting stages, auxospores). Processes that need investigation include vegetative division processes, syngamy, mating systems, and sexual reproduction. This information is required for a larger number of HAB species belonging to all taxonomic groups in order to understand their roles in harmful blooms.

- Application of a combination of morphological, molecular, genetic and reproductive compatibility approaches to better identify species boundaries and population structure of HAB species.

- Study of the sexual life cycle dynamics in natural populations and their genetic and ecological consequences. This implies the identification of sexual stages and the understanding of the mechanisms that initiate sexual reproduction.

- Investigations on mating mechanisms and strain compatibility, and assessment of the relative importance of homothallism versus heterothallism, of diploid versus haploid, or haplo-diploid life cycles.

- Identification and characterization of the morphological and physiological features of resting/dormant stages (including cysts, spores, and resting cells in bottom sediments), whose occurrence and role are presently underestimated by conventional plankton studies.

- Validation through *in situ* studies of results on life cycles obtained from laboratory investigations carried out on culture material (e.g. Where and when do the different flagellate stages produced by *Phaeocystis* spp. occur in the natural environment? Do *Chrysochromulina* and *Prymnesium* produce benthic stages?).

- The role of biological control in different phases of blooms should be explored at different levels. The presence of endogenous mechanisms potentially responsible for life-cycle transitions should be investigated, as well as the role of infochemicals, and algae/bacteria interactions in determining alternations between life cyle stages.

- Investigate inter- and intra-specific differences in life strategies, assess genetic *versus* biotic/abiotic control of sexual reproduction and life cycle transitions.

- Ascertain differences in toxin content among different life history stages.

- Investigate gene expression during the transition between different life cycle stages of HABs. Genetic markers for specific physiological conditions that can be related to life stage transitions should be identified. Genome sequence databases may provide key tools for the identification of functional genes and the study of genetic control at the cellular level. Protein identification and characterization (proteomics) may also be a valuable tool.

Ecological role of life histories

- Identify key areas affected by HAB events along European coasts to be used in ‘case studies' for population dynamics of species blooming in different environmental conditions. These areas would benefit from long-term physico-chemical and planktonic data sets, which represent the most useful information for detecting species timing and recurrences, and relating them with environmental and biotic factors.

- Field studies on HAB dynamics should focus on the role of life history stages at different phases of a bloom (initiation, maintenance and decline). These studies should concern all taxonomical groups, including cyanobacteria and freshwater dinoflagellates. The following questions need to be addressed: When are resting stages produced? How many? What is the proportion of the population undergoing sexual reproduction? What proportion of resting stages is viable in the sediments? How is the timing of their germination regulated? What are the sizes and locations of residual overwintering populations that can act as inocula for subsequent blooms? The unique conditions of the thin layers exploited by certain species (diminished shear stress, specific dissolved organic compounds, co-occurrence or absence of potential prey) should be ascertained.

- Reconstruct long-term trends of HAB species using fossilizable resting stages in sediment cores, integrated with the signals provided by other biological and chemical proxies. This will provide a tool for reconstructing species trends and abundances through changing environmental conditions (climate, eutrophication, etc.). These sites would provide optimal conditions for integrated multidisciplinary research (i.e. biologists, micropalaeontologists, sedimentologists, geochemists).

- Elucidate the role of life history stages of HAB species as means to: i) avoid predation or attack by viruses, bacteria and parasites; ii) preserve genetic diversity; iii) inoculate blooms; and iv) promote species dispersal.

- The effects of phytoplankton on environmental conditions, such as light field characteristics, nutrient availability, and rheological properties of sea water, should be taken into account as possible mechanisms that can trigger transitions and/or enhance blooms.

- Species-specific models are needed which integrate the population dynamics of life stages of harmful species into bloom dynamic models.

- The biogeographical distribution of HAB species along European coasts should be assessed. Mapping benthic stage distributions in sediments would allow the establishment of a baseline for the monitoring of spreading events, introduction of new species, and human-assisted dispersal of HAB species and enhancement of blooms.

Methods and sampling techniques

- *Identification probe*s. The identification of all life history stages (including rare stages in the water column and benthic stages) in the field is a prerequisite for the reconstruction of the life cycles of the different species. To obtain sequence data for a larger number of HAB species and to investigate genetic intraspecific variability, it is necessary to design molecular probes at different taxonomic levels (species, populations) and to validate available probes.

- *Functional probes*. Develop molecular probes to detect and selectively mark (e.g. gene expression markers) life-history stages of HAB species. Test the use of these probes coupled with flow cytometry, image analysis and other tools for fast screening of cultures and natural populations. Develop biochemical markers to distinguish physiologically dormant from actively dividing cells. Develop fingerprinting markers for population studies.

- Develop sensitive protocols to be used with single cells and/or with small samples (10-100 cells), for physiological analyses, ploidy identification, DNA sequencing, and toxin content.

- Develop techniques and technologies to sample life history stages in specific small-scale structures such as microlayers, sediment-water interfaces and other physical and biological discontinuities. Develop techniques to allow determination of *in situ* growth rates, resting stage production and germination rates.

- New interdisciplinary sampling procedures should be used in field studies. Multiscale physical-chemical-biological interactions in life-stages transitions require that the scale of the relevant biological processes dictate the scale of sampling for environmental parameters.

- Standardize methods for collection, detection and quantification of resting stages in sediments.

Infrastructure

- Organize workshops on culturing methods and techniques.

- Develop websites that include life cycle information.

- Organize advanced training courses.

- Develop taxonomic guides and/or websites for the identification of life stages (e.g. benthic resting stages, auxospores) and their correspondence with planktonic vegetative stages.

INTRODUCTION

Harmful algal blooms in European marine waters

Nuisance blooms of phytoplankton organisms are recurring events in European coastal waters. These harmful algal blooms (HABs) can be local phenomena or affect large areas. In either case they may harm human health, marine ecosystems and resources such as tourism, fisheries and aquaculture. The nature of HABs is very diverse across the European seas (Box 1), and may be due to different species (Table 1 and APPENDICES 1 - 4).

Box 1- Types of harmful algal blooms in Europe (Modified from EUROHAB).



Fish mortality in aqualture pools in Ebro Delta 1995. Photograpy by J. Camp. Accumulation of *Phaeocystis* mucus on Netherlnads beach. Photograpy by L. Peperzak. Water discoloration due to *Alexandrium taylori* in a catalan beach in summer 2001. Photograpy provide by Agència Catalana de l'Aigua, Generalitat Catalunya.

- Algal toxins accumulate in shellfish and can cause diarrhoea, amnesia and paralyses in humans, even when the microalgae are present at low cell concentrations in seawater.
- Algae produce water-soluble toxins which can cause mass mortalities of fish.
- Cyanobacteria that can produce toxins and accumulate to a high biomass are a permanent threat in the Baltic Sea.
- A number of non-toxic algae can (i) discolour seawater in many coastal areas, or (ii) produce enormous amounts of unpleasant foam (North Sea) or (iii) ugly mucilage (Adriatic Sea), which float and accumulate along beaches.

Table 1. Phytoplankton genera, including species that cause problems in European coastal waters.

Organism	Effect	Life history	Areas with major HAB problems
DINOFLAGELLATES			
Alexandrium	PSP, high biomass	Partly known, cyst-former	All European coasts, except Bay of Biscay, Southern North Sea and Baltic Sea
Dinophysis	DSP	Partly known, no benthic stages confirmed	Europe wide
Gymnodinium	PSP	cyst-former (*G. catenatum*)	Western Iberia, Western Mediterranean Sea
Gyrodinium	ichthyotoxic	Not known	Western Mediterranean coast
Karenia	ichthyotoxic	Partly known	Skagerrak, Kattegat, Celtic Sea, Western English Channel, Central and Northern North Sea, Bay of Biscay
Protoceratium	YTX	Cyst-former	Western Mediterranean, Adriatic Sea
Protoperidinium	AZP	Not known	Celtic Sea, Northern North Sea, Norwegian Sea
DIATOMS			
Pseudo-nitzschia	ASP	Sexual reproduction	Europe wide
Coscinodiscus	mucilage formation	Sexual reproduction	Western English Channel
HAPTOPHYTES			
Phaeocystis	high biomass, foam production	Polymorphic life cycle	North Sea, English Channel, northern Norwegian fjords
Prymnesium	icthyotoxic	Cyst former	Brackish waters and coastal lagoons in the Baltic Sea, Central and Southern North Sea
Chrysochromulina	ichthyotoxic	Partly known	Skagerrak, Kattegat, Lofoten Archipielago, Baltic Sea
RAPHIDOPHYTES			
Fibrocapsa	ichthyotoxic	Cyst former	Skagerrak, Kattegat, Southern North Sea
Heterosigma	ichthyotoxic	Cyst former	Western Iberia, Brittany, Western Scotland
Chattonella	ichthyotoxic	Cyst former	Skagerrak, North Sea coasts of Denmark and Germany

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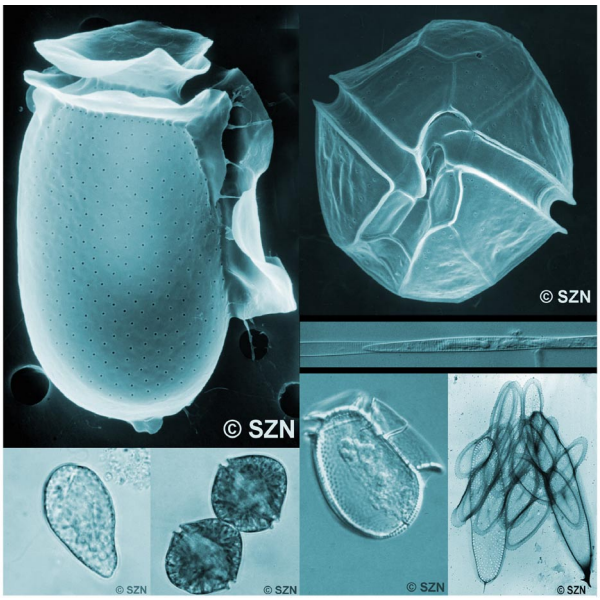
Organism	Effect	Life history	Areas with major HAB problems
HETEROKONTOPHYTES			
Dictyocha	Fish killer	Partly known	Kattegat, Brittany, Galicia
CYANOBACTERIA			
Nodularia	high biomass, hepatotoxic	akinetes, heterocysts	Baltic Sea
Aphanizomenon	high biomass, neurotoxins	akinetes, heterocysts	Baltic Sea
Microcystis	hepatotoxins	Unknown, planktonic colonies, benthic senescent stages	coastal waters with fresh-water influence

Why are the life cycles of HABs important?

The impact of HABs is a result of species-specific harmful properties, which in turn show up in relation to the occurrence and bloom dynamics of the species concerned.

The dynamics of harmful blooms varies from site to site, depending not only on specific hydrographic and topographic conditions, but also on the ecological and

Box 2 - The diversity of harmful species.



Photograpy provide by Stazione Zoologica 'A. Dohrn', Italy. *Pseudo-nitzschia* photo by S. Bates.

Harmful species belong to 6 algal groups (diatoms, dinoflagellates, haptophytes, raphidophytes, cyanophytes, pelagophytes) and differ greatly in terms of morphological, physiological and ecological characteristics:

- The size of harmful organisms spans from a few micrometres (*Aureococcus anophagefferens*) to a few millimetres (*Phaeocystis* colonies and *Pseudo-nitzschia* chains).
- Shape and size diversity is enhanced by the colonial habit, which is found in diatoms, dinoflagellates, haptophytes and cyanophytes.
- Harmful species include non-motile or scarcely motile species as well as swimmers, which can control their position in the water column over the range of several metres.
- Some species prevalently depend on light as the energy source, others can assimilate organic compounds, ingest small food particles, or even predate on organisms bigger than themselves.
- The requirement and affinity of harmful species for different nutrients (organic and inorganic) varies greatly among species.
- Harmful algal species are found in almost all kinds of habitats, including harbours, estuaries, eutrophic coastal waters, relatively pristine shelf waters, subsurface micro-layers, and benthic communities.
- The toxins and other nuisance mechanisms vary notably depending on the causative species.
- The alternation of different morphotypes (flagellate-coccoid, planktonic-benthic, active-resting, single cells-colonies) in the life cycle further increases the intrinsic diversity of harmful species.

biological characteristics of the causative organisms (Box 2). Many HAB species have complex life cycles, including stages with markedly different morphological and physiological characteristics, i.e. single cells and colonies, flagellate and coccoid stages, growth stages responsible for biomass increase and benthic cysts and other resting stages capable of withstanding hostile environmental conditions.

Life cycles have important implications for the occurrence and bloom dynamics of harmful species:

- Heteromorphic life cycles expand the range of environmental conditions under which a species can survive.

- The human-mediated and natural transport of vegetative and resting stages contributes to extend the geographic range of harmful species and to increase genetic diversity of regional populations.

- Microalgal life history often includes sexual events, which profoundly affect the genetic structure and diversity of populations, with clear implications for the plasticity, fitness and success of the species.

- Different life stages play important roles at different phases of harmful blooms. For example, the switch from non-motile resting cells to an actively dividing population initiate blooms, whereas massive encystment may be responsible for sudden bloom terminations.

- Some life stages can provide protection from viruses, grazers or parasite attacks.

It is thus evident that the interactions between life histories and the physical, chemical and biological environment have profound implications for species success, timing and recurrence of blooms and ultimately succession and structure of the pelagic communities.

Finally, it is important to note that life history studies can play a vital part in the description and circumscription of species:

- Phytoplankton taxonomy is presently based almost exclusively on the morphological features of one single stage, and different stages in the life cycle of one species have sometimes been wrongly classified as separate species. Information on the reproductive modalities and on alternative life stages is therefore needed in order to amalgamate some species descriptions, and provide a sounder basis for species classification in general.

- Not all microalgal species are morphologically distinct, nor do morphological and genetic similarity always reflect the ability to mate and exchange genetic material. Information on mating compatibility must be combined with morphological-molecular approaches to achieve a better understanding of the species in microalgae.

EUROHAB

Because of the social and economic importance of HABs in European seas, several European funding agencies have supported HAB investigations in the past few years, ranging from specific workshops to different research projects. The European Commission started the European Initiative on Harmful Algal Blooms (EUROHAB) in 1998. EUROHAB provides the framework to coordinate and share information on mechanisms, distribution and trends of harmful algal events at a European scale. This information will be a valuable contribution towards determining management options of the problem through a co-ordinated action of the different EU countries. The EUROHAB Science initiative was described in a publication of the European Commission (European Commission, Research in Enclosed Seas-5, EUR 18592, ISBN 92-828-6612-2, 1999). Ongoing European research on HABs was reviewed and compiled in the European workshop 'The research and infrastructural needs for the management of Harmful Algal Blooms in European Seas' (Brussels 16-17 October 2000), organized by the EU-MAST (DG XII) (European Commission, Research in Enclosed Seas-10, EUR 19434, 2001, in press).

EUROHAB includes projects such as: BIOHAB (Biological control of harmful algal blooms in European coastal waters: Role of eutrophication), HABES (Harmful Algal Bloom Expert System), Harmful Introductions by Ships (Test monitoring systems for risk assessment of harmful introduction by ships to European waters), STRATEGY (New strategy of monitoring and management of HABs in the Mediterranean Sea), DOMTOX (Importance of dissolved organic matter from terrestrial sources for the production, community structure and toxicity of phytoplankton of the European Atlantic and Baltic coastal waters; role of micro-predators for transmission of toxins to commercial shellfish and fish larvae), ALIENS (Algal introductions to European shores), FATE (Transfer and fate of Harmful Algal Bloom toxins in European marine waters), HABILE (Harmful Algae Blooms initiation and prediction in large European marine waters) and the herewith-outlined Workshop, LIFEHAB.

Why LIFEHAB?

One of the key topics identified by EUROHAB was the fragmentary information available about life history strategies of harmful algal species. Given the complexity and importance of the topic, improvements in basic knowledge are needed to estimate the impact of different life history stages in the population dynamics of HABs. In particular, the quantification of processes involved in the life cycles of HAB species is a fundamental step towards building reliable conceptual and predictive models (Box 3) as effective tools in HAB management and mitigation.

Box 3 - Modelling HAB Dynamics by William Silvert.

Modelling is an essential part of research on harmful algal blooms. Aside from their obvious use in predicting the occurrence, timing and severity of blooms, models play an important role in developing and testing hypotheses about the factors causing blooms and in exploring the effectiveness of possible mitigation strategies.

Harmful algal blooms are complex processes involving an interplay of many physical, chemical and biological factors. The number of variables and forcing functions, and the apparent sensitivity of blooms to small changes in the relative values of some of these variables, pose major obstacles to the development of models.

In addition to refining existing models, it is valuable to explore new modelling approaches and complementary model formulations of harmful algal blooms. One possibility is to develop rule-based expert systems, such as the EU HABES project (Harmful Algal Bloom Expert System) already under way, and to explore other phenomenological types of models to complement the mechanistic models widely used at the present time. Rule-based models provide a valuable means for integrating the available knowledge about complex systems and can provide a sound basis for development of more detailed simulation models.

There is a danger that as more information on life histories and alternative growth strategies is gathered, models that attempt to incorporate all of this information will become unwieldy and impractical. To avoid this, it is important to review the processes incorporated in models and to verify that the components play a significant role in determining the model output. In this regard, the identification of the most relevant life-history features of HAB species is of the utmost importance.

LIFEHAB was conceived as a forum of discussion among specialists of different fields (taxonomy, physiology, ecology, molecular biology, modelling) and of different harmful microalgae (diatoms, dinoflagellates, prymnesiophytes, raphidophytes). It was aimed at summarising current knowledge on the life history of harmful species, identifying the main gaps of knowledge, and discussing the most appropriate approaches and methods to address the role of life cycles in HAB dynamics.

Diatom life cycles

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Introduction

Harmful Algal Blooms (HABs) are caused by a variety of diatom lineages (APPENDIX 1). According to recent phylogenetic data from 18SrDNA (e.g. Medlin *et al.* 2000a) and the *rbc*L gene (Mann *et al.* 2001 and unpublished data), the genera involved span almost the whole of the Bacillariophyta and so the life histories of HAB diatoms will probably be correspondingly diverse. Of the HAB diatoms, *Pseudo-nitzschia*, *Chaetoceros* and *Coscinodiscus wailesii* have received most attention in recent years. The list of HAB species will probably grow in future, since environmental change (including climate change, eutrophication, alterations of oceanic circulation) and the erosion of biogeographical limits through human intervention (e.g. through shipping and aquaculture) will create unprecedented combinations of conditions for phytoplankton growth. *Pseudo-nitzschia* was not known to be a potential cause of HABs before the 1980s (Bates *et al.* 1989). In some cases, it may be possible to anticipate problems. For example, one might hazard to predict that *Thalassiothrix* and its allies (*Trichotoxon*, *Lioloma*: Hasle & Syvertsen 1996), with cells of extreme length (up to 5.7 mm) but narrow width (~ 10 μm), may (if present in high density) prove problematic to filter feeders or fish (through clogging or penetration of gill tissue).

Several key features of diatom life histories have been well documented for a long time (see summaries and illustrations by Drebes 1977; Round *et al.* 1990):

(1) Population cell size decreases during the phase of vegetative growth and mitotic cell division. In extreme cases, linear dimensions (e.g. length) can decrease to 6% of the original size, at least in culture (in *Tabularia*: Roshchin 1994, p. 93 ff.). The vegetative phase is diploid (hindering the development of genetic analysis through the generation and use of mutants).

(2) Shape and, to a lesser extent, pattern changes accompany size reduction, except in diatoms with circular valves; these changes complicate taxonomic judgments made during classification and identification.

(3) Cell size is restored via the development of a special growth stage, the auxospore.

(4) Meiosis, gametogenesis and fertilization immediately precede auxosporulation.

(5) Centric diatoms (diatoms with a ± radially organized pattern of ribs and pores, originating from a ring-like pattern centre) are oogamous (producing large egg cells and small, anteriorly uniflagellate sperm), with release of one or both types of gamete before fertilization. Pennate diatoms (diatoms with a bilaterally organized, feather-like pattern of ribs and pores) are morphological isogamous, with non-flagellate gametes, and often exhibit gametangiogamy (i.e. the recognition and apposition of gametangia during sexual reproduction). Both groups of diatoms are hologamous, i.e. the whole of the organism is used up in gametogenesis.

(6) The transition from vegetative cell to potentially sexual cell is controlled by cell size. There is a genetically determined permissive threshold in the life cycle, such that above a certain critical size, no sexual reproduction occurs, whereas below it,

sexual reproduction will occur if other constitutional or environmental conditions are met. Because of the existence of the size threshold and the existence also of a lower size limit, beyond which cells are non-viable or non-inducible, the life cycles of diatom clones (and hence to a lesser extent populations and species), exhibit characteristic ranges of sizes. It appears that cells become progressively easier to sexualize as they dip further below the permissive size threshold (V.A. Chepurnov, D.G. Mann, W. Vyverman, K. Sabbe & D.B. Danielidis, unpublished observations of *Seminavis*), until cells become moribund near their lower size limit (e.g. Mann *et al.* 1999; Hiltz *et al.* 2000).

(7) Often, there is 'no significant antagonism between factors promoting vegetative growth and those eliciting gametogenesis' (Drebes 1977, p. 274) and, in culture, species often become sexual while growing exponentially and not limited by any nutrient (e.g. Davidovich & Bates 1998a, b). However, in some cases, particularly in centric diatoms, sexuality has been found to be linked to particular nutrient or light regimes (Drebes 1977; Edlund & Stoermer 1997) or even the presence of particular bacteria (Nagai *et al.* 1999). Given the diversity of environments occupied by diatoms, it is very unwise to make *a priori* assumptions about what will elicit a sexual response in a particular diatom species or population, except in relation to controls exerted by size.

(8) In contrast to many other groups, the sexual phase is only very rarely a prelude to dormancy or the formation of a resistant resting stage (one exception is *Leptocylindrus*; others are listed by Edlund & Stoermer 1997). Instead, dormant stages are intercalated into the vegetative diploid phase. Resting stages may or may not be morphologically detectable.

In addition, it has often been assumed (9) that diatoms are homothallic, and (10) that size restitution occurs in one step.

The sexual phase

Certain of the generalizations listed above may require modification. For example, in some diatoms in culture there is an alternative pathway to auxosporulation, viz. vegetative enlargement (e.g. Roshchin 1994), although in this the restoration of size may be less complete, and the control of shape less well controlled, than with auxospores. Also, Roshchin and Chepurnov (e.g. in Roshchin 1994) have shown, again using cultured strains, that in some centric diatoms size restitution may take place in two or more steps, and it is clear that the 'window' in the life cycle within which cells can be sexualized is broader than was previously thought (e.g. review by Edlund & Stoermer 1997). Davidovich (1994; 2001) has demonstrated the dependence of final auxospore size on gametangium (hence gamete) size in raphid pennates, which shows the need for caution in interpreting size spectra of natural populations and of the 'cardinal points' of the diatom life cycle, although, because of the permissive size threshold, there is also an upper limit to auxospore size within a clone or clonal lineage (Mann *et al.* 1999). Furthermore, some diatoms do not get smaller (see Round *et al.* 1990, p. 82).

The diversity of sexual processes among diatoms may well be greater than we know because, despite the observation of allogamous sexual reproduction of pennate diatoms as early as the 1840s (Thwaites 1847) and the final demonstration of oogamy in

centric diatoms by von Stosch (1950), few diatoms have yet been observed through the whole of the vegetative life cycle; still fewer have been studied during the sexual phase itself and auxosporulation and immediately post-auxospore cells are very rarely observed in natural populations. Some diatom groups are especially poorly known, e.g. the araphid pennate lineages. Fortunately in the present context, few araphid pennates have yet been listed as HAB organisms, except *Tabularia* (see Bates & Davidovich, this volume, APPENDIX 1).

The reasons for lack of knowledge of the diatom life cycle are several. (1) The vegetative phase of the life cycle is long, relative to the sexual phase. The limited information available to date, mostly for freshwater diatoms, indicates that size reduction can take several years in nature (Mann 1988; Jewson 1992), supporting Lewis's (1984) idea that the form of the life cycle is adaptive, restricting the frequency of sexual reproduction to intervals of > 1 yr, for which environmental cues would be ineffective. (2) Through wastage of gametes, production of ill-adapted or non-viable genotypes, and interruption of synthesis during auxosporulation, the frequency of larger cells will always be less than that of smaller cells within an asynchronously sexualizing population (Mann 1988). (3) Some populations show evidence of non-synchronous, episodic auxosporulation, so that more or less discrete size classes are produced and sexual reproduction may be missed, except by chance observation during a sexual 'event' or through careful long-term observations. (4) Diatoms are often studied dead, after removal of the organic components of the cell; in such cases, sexual reproduction cannot be demonstrated, although initial cells (the cells formed within the auxospores) may be detectable by their larger size and distinctive morphology (e.g. Mann 1989). (5) Few phycologists have been trained in the observation and interpretation of sexual and auxospore stages in diatoms.

A full understanding of the dynamics of HAB populations cannot be obtained without data on the initiation, frequency and consequences of auxosporulation in nature. This is because, for example, (1) auxosporulation interrupts vegetative growth (Lewis 1983); (2) life cycle size changes will inevitably affect the cell surface area : volume ratio, with concomitant effects on physiology (Potapova & Snoeijs 1977); and (3) size is a primary factor in relation to grazing and parasitism (Reynolds 1984, Mann 1988). Life cycle information will not be easy to obtain without long-term time series (>> 1 yr) of observations at 2–4 week intervals at fixed stations, involving measurements of 500 or more cells. Such information is expensive, unless measurement can be automated.

RELEVANCE OF LIFE CYCLE STUDIES TO TAXONOMY: Studies of reproductive isolation give valuable and unique information in relation to diatom taxonomy, as first shown by Mann (1984, 1999) and more recently illustrated for *Pseudo-nitzschia* by Davidovich & Bates (1998a, b). Ecological and physiological studies and monitoring programmes are only as good as the underlying taxonomy allows. Fortunately, when Amnesic Shellfish Poisoning was discovered to be caused by *Pseudo-nitzschia* species (Bates *et al.* 1989), there was already a good taxonomic treatment, based on careful TEM studies made by Hasle (1965). However, even after further revision (e.g. Hasle & Syvertsen 1996) the taxonomy still needs refinement, as has been shown by recent molecular genetic studies (Miller & Scholin 1998; see Mann 1999). Only by a combination of careful morphological studies, molecular systematics, and breeding data will it be possible to provide a full taxonomic basis for HAB monitoring; none of these types of data are sufficient on their own. Besides targeted studies on known HAB species, however, it will be important to gain general insights into speciation, evolution

and biogeography in planktonic diatoms, through studies of different taxonomic groups and life forms, since the HAB species list is not definitive: other species will undoubtedly have to be added as the climate and oceanographic circulation change and near-shore eutrophication increases. Total diatom species numbers are unknown, but will rise considerably now that there are methods to detect cryptic and semicryptic species; the current best guess is Mann & Droop's (1996) estimate of ~ 200,000 species globally. The vast majority of these, however, are benthic.

HOMOTHALLISM VS HETEROTHALLISM: The assumption that diatoms are homothallic has been overturned by recent studies initiated by Roshchin (e.g. 1994) and pursued since by Chepurnov, Mann, Davidovich and Bates. Many pennate diatoms are not homothallic (e.g. Roshchin 1994; Davidovich & Bates 1998a, b; Mann *et al.* 1999; Roshchin & Chepurnov 1999) and various types of mating system exist, enforcing different degrees of outbreeding (e.g. Chepurnov & Mann 1997, 1999, 2000). However, the majority of taxa studied so far have been benthic and one might predict that planktonic diatoms, growing as they do in a more rarefied 3-dimensional environment, may differ from their benthic counterparts with respect to the life cycle and mating system. The situation in centric diatoms is especially unclear and requires much further study, despite the legacy of detailed culture studies by von Stosch and co-workers (summarized by Drebes 1977). Some data suggest that some centric species exhibit significant interclonal differences in the ability to produce male and female gametes (Drebes 1977; Roshchin & Chepurnov 1999).

Knowledge of reproductive biology provides an essential basis for managing culture collections. If a species is heterothallic, it will be unable to complete its life cycle in monoclonal culture and cells will either become so small as to be nonviable, or may accumulate near the minimum size, when they either stop getting smaller or exhibit a short cycle of vegetative enlargement and reduction (Roshchin 1994).

Resting stages

Many planktonic diatoms are known to form resting stages, which may or may not be morphologically distinct from the vegetative cells; if they are, they are referred to as 'resting spores'. A fairly recent review is available (McQuoid & Hobson 1996). Formation of resting stages can be induced by a variety of factors, but rapid deterioration in combined nitrogen levels or the light climate are particularly effective in many cases. Resting stages (McQuoid & Hobson 1996) and in some cases vegetative cells (Lewis *et al.* 1999) can remain viable for long periods in cool dark conditions, but many can germinate within a short period of formation. In coastal areas, some may function to maintain a population through periods when growth in the water column is impossible; the thickened walls of resting spores may be significant in survival (e.g. as protection against benthic grazers) or in providing for an enhanced rate of transport down to the sediments. Resting stages may reseed planktonic populations from the sediments or from the pycnocline, and their germination characteristics may account in part for seasonal succession of species. However, probably in no case do we know the relative importance of resting stages vs vegetative cells in maintaining populations between bloom events.

One of the most important HAB diatoms, *Pseudo-nitzschia*, is not known to produce resting cells. However, this may simply reflect lack of study, since another important HAB species, *Coscinodiscus wailesii*, was found to produce resting cells by Nagai *et al.* (1995), whereas no resting stages had previously been reported for any

Coscinodiscus species (e.g. McQuoid & Hobson 1996), despite the abundance of *Coscinodiscus* species in nature.

Final comments

Considerable advances have been made during the last 20 years in our knowledge of diatom life cycles. Ironically, however, this has undermined the validity of many generalizations – e.g. the near universality of homothallism, the obligate link between enlargement and sexuality and the auxospore, and restitution of size in a single-step – that we had previously thought were well established. It is unwise to assume very much about the life cycle of any diatom without direct observations, except that the vegetative cells are almost certainly diploid.

To what extent are dinoflagellate life histories important for HABs?

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Introduction

Many dinoflagellate species have more or less complex heteromorphic life cycles (Fig. 1) (Pfiester & Anderson 1987; Pfiester 1989). The most intriguing example is the ambush dinoflagellate *Pfiesteria piscicida*, where motile thecate stages of different size and ploidy, lobose amoebae and chrysophyte-like cysts are linked together by a network of possible pathways (Burkholder & Glasgow 1997). The whole range of morphological variability of a species is not always included in the taxonomic descriptions and, at times, different life stages of the same species have been described as different species (e.g. *Dinophysis* small cells). Fossiliseable dinoflagellate resting cysts even have a separate nomenclatural system within palaeontology, and cyst morphology has only recently begun to be included in biological species descriptions. Furthermore, oceanographers, ecologists and modellers most often relate phytoplankton species abundance only to the physical and chemical parameters of the water column, disregarding the role that different life stages, such as cysts, spores and resting cells, can play in determining the species annual timing, succession, and success.

From the perspective of HABs, we are mainly interested in explaining why and when these species bloom. We are thus interested in the intensity and duration of the 'growth' phase, related to the biomass increase of a population. Successful growth can be obtained by increasing division rates, but also by reducing loss factors (i.e. mortality, parasite and viral infections, grazing pressure). A heteromorphic life stage can represent an advantage, since it allows the allocation of the species biomass into stages of different size ranges, morphology and survival-defence capabilities. To unravel the role of life cycles in population dynamics, we should thus aim at understanding the adaptive significance of the different life stages and the different factors that trigger shifts among them during the life history.

The growth phase

Dinoflagellates can grow faster or slower depending on their species-specific growth capability, which can be regulated by environmental factors. Different division modalities have been described for dinoflagellates, e.g. binary fission, with or without shedding the parental theca, and formation of a division cyst. Besides metabolic considerations – shedding a theca at every cell division seems a waste of energy (does it?) - there should be evolutionary reasons for different division mechanisms. Some species (e.g. *Alexandrium taylori*) even have two possible division modalities: oblique fission of the motile cells and cell division within a 'division cyst'. What is the advantage? Does one type of division prevail under some specific environmental conditions? What is the most effective division mechanism in contributing to cell number increase? We could speculate that oblique fission is the fast division mechanism that accounts for cell number increase, whereas division within the cyst offers the advantage of a higher protection but at the expense of dividing more slowly.

Many (the majority? all of them?) dinoflagellates have a phased division cycle, regulated by the daily light/dark cycle. What is the evolutionary advantage of phased

division? Several species show migratory behaviour on a daily basis. Is this related to the division process? Does cell division take place in restricted layers of the water column where predatory pressure is lower?

Some dinoflagellates form colonial stages (i.e. *Alexandrium catenella*, *Gymnodinium catenatum*) but, at least in culture, they are also found as single cells of a different size. What are the advantages of forming long colonies? In what conditions do colonial stages prevail?

Some dinoflagellates are not grazed by their potential predators and can even negatively affect growth in predators (Matsuoka *et al.* 2000, Montresor, unpublished data). The presence of grazing-deterrent mechanisms can partially explain HAB species accumulation in the water column. To what extent are chemical defences present in HAB species? Are they present throughout the whole life cycle or only in selected life stages or physiological conditions?

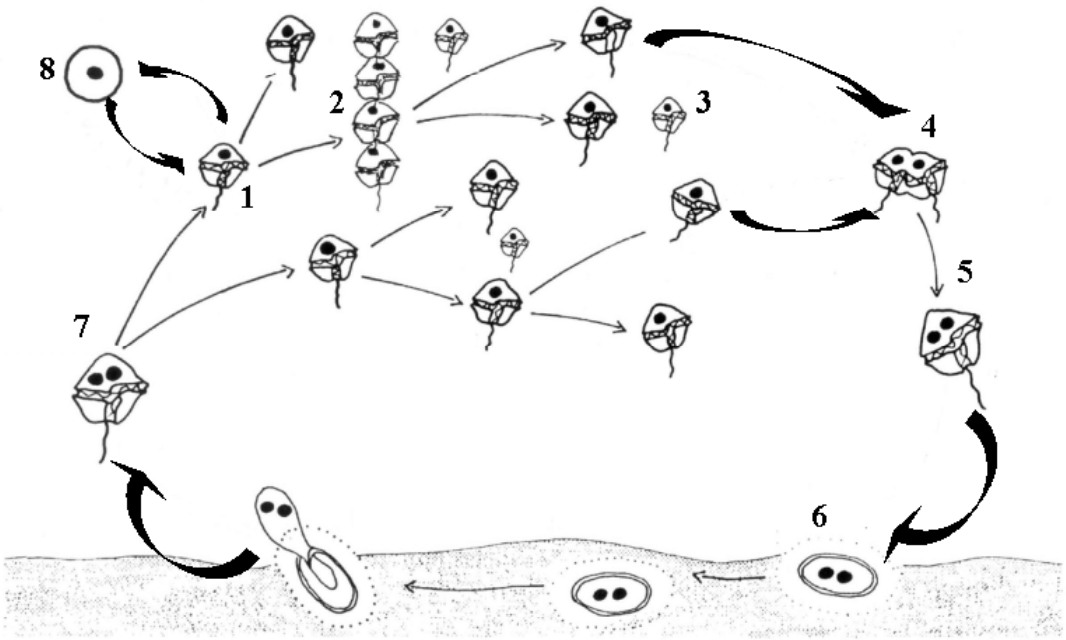


Fig. 1. Life history of *Alexandrium* (modified from Wyatt & Jenkinson 1997). Dinoflagellates have heteromorphic life cycles, including motile and non-motile stages: motile vegetative cell (1), multicellular colony (2), small cells (3), conjugating gametes (4), planozygote (5), non-motile resting cyst (6), planomeiocyte (7), temporary cyst (8). Thick arrows mark transition events among the different stages, which are important in population dynamics.

Small cells and temporary cysts

The formation of 'small cells' has been reported for several dinoflagellates. For some species (e.g. *Dinophysis*), there is enough evidence to interpret the small cells as gametes but this is not the general rule. Both normal and small cells have been described for *Karenia mikimotoi* (Partensky & Vaulot 1989). In this species, small cells show higher growth rates as compared to normal cells. The capability of differentiating

cell size within a population could thus contribute in explaining the success of *Karenia mikimotoi* as a bloom species.

Temporary cysts are considered as short-term resting stages. What is the advantage of shifting from a motile to a non-motile stage under apparently stressful conditions? The 'diablillo' parasite of *Alexandrium catenella* (Delgado 1999) does not infect the non-motile stages. Species capable of producing temporary cysts could have developed a defence system to protect at least a fraction of the population from parasite attacks.

Sexual reproduction and cyst formation

Dinoflagellates have a haplontic life cycle. Sexual reproduction implies the conjugation between two 'functional gametes' with the consequent formation of a planozygote. In some species, the planozygote can undergo morphological and physiological transformations, which lead to the formation of a resting cyst (hypnozygote). Some dinoflagellates (e.g. *Gymnodinium catenatum*, Blackburn *et al.* 2001) are heterothallic, i.e. sexual reproduction and cyst formation only occur when clones of different polarity are mixed together, whereas other species (e.g. *Peridinium* spp., *Scrippsiella trochoidea*) are homothallic and cyst formation occurs in monoclonal cultures.

Cyst formation is most often the only evidence of the occurrence of sexual reproduction, but probably not all planozygotes necessarily turn into cysts. The frequency of sexual events is important in determining the genetic structure of a population, but estimates of the incidence of sexual reproduction in dinoflagellates are completely lacking. For sexual reproduction to occur, a threshold concentration of gametes should be reached (Wyatt & Jenkinson 1997). A bloom would seem the necessary prerequisite, however, cells can also aggregate in thin, discrete layers of the water column and/or gamete encounters could be mediated by gamone-like compounds.

The ecological role of dinoflagellate cysts has often been compared with that of seeds in higher plants: cysts sink to the bottom where they undergo a dormancy/maturation period of different species- (population-?) specific length. When maturation is completed, cysts can germinate and inoculate the water column with motile stages, or remain in a quiescent stage for years. An understanding of the factors that regulate the switch between motile cells and cysts is extremely important for comprehending bloom dynamics. Laboratory experiments suggest that cysts are produced when nutrients are limiting (e.g. Anderson & Lindquist 1985), but *in situ* investigations do not support this hypothesis. Other factors have been investigated to explain the formation of resting stages: different photoperiod regimes (Sgrosso *et al.* 2001), interactions with peculiar cyst-inducing bacteria (Adachi *et al.* 1999), defence from predators (Rengefors *et al.* 1998), and population density-mediated mechanisms (Uchida 2001). Cysts have morphological characters that increase their resistance, i.e. tough walls and mucous layers, but there are most probably specialized predators that can manage in breaking their defences (Persson 2000). Moreover, anoxia, toxic compounds from human pollution, interactions with bacteria, fungi, etc. could alter their viability, thus affecting germination rates and success. All the above-mentioned topics need to be investigated if we aim at assessing the germination potential of cyst beds.

Cyst germination

Germination timing is another important factor for explaining the onset of a bloom. The size of cyst beds, the length of dormancy period, the mechanisms regulating germination of quiescent cysts, and the role of resuspension events, are all crucial factors for understanding the timing of a bloom. Dormancy/maturation lengths for the different species have generally been estimated with laboratory experiments carried out on a single culture in the laboratory. What is the variability of cyst dormancy length within a population and across the species biogeographic range? Cysts remain viable for years in the sediments and their germination potential is thus extended over time. What are the environmental signals that regulate the germination of quiescent cysts? Is cyst germination strictly timed? Is the inoculum of vegetative stages in the water column spread over time? For these latter species, and for the species that do not produce resting cysts, the timing of the bloom will probably depend on the survival capabilities of a few motile cells in the water column.

The presence of an endogenous circannual clock was hypothesized to regulate germination timing of *Alexandrium tamarense* cysts collected offshore from the US east coast (Anderson & Keafer 1987), and an apparent annual rhythm in the growth rate of vegetative cells was indeed described for the same species (Yentsch & Mague 1980). Although very challenging, these hypotheses have not been further investigated and the possible mechanisms underlying these observations are unknown.

Life cycles of HAB forming haptophytes

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HAB forming haptophytes

The algal division Haptophyta is composed of nanoplanktonic cells characterized, at least in some stages, by two flagella and a third filiform organelle, the haptonema. Coccoid, colonial, amoeboid or filamentous stages are also observed in haptophytes (Edvardsen *et al.* 2000a). Haptophytes are widespread and may compose a significant part of the nanophytoplanktonic community. The taxonomy of the Haptophyta is based on morphometric details of cell scale coverage, flagella, haptonema and/or thread-like material (Jordan & Green 1994). This algal division and more particularly, the class Prymnesiophyceae Hibberd, includes some well known HAB forming species, either ichthyotoxic such as *Chrysochromulina* spp., *Prymnesium* spp. or high-biomass forming species such as members of the genus *Phaeocystis*. The latter includes six species: *P. globosa, P. pouchetii, P. antarctica, P. scrobiculata, P. cordata* and *P. jahnii* (Zingone *et al.* 1999), but colony forms were only reported for *P. globosa, P. pouchetii, P. antarctica* and *P. jahnii*. Haptophytes also include coccolithophorids that are generally not considered as HAB species but are seen as important species for climate regulation (e.g. *Emiliana huxleyi*). Some non-blooming coccolithophorid species (e.g. within the genera *Pleurochrysis* and *Ochrosphaera*) are suspected to be capable of producing toxins (Probert, unpublished results).

Occurrence of these haptophytes has caused serious damage to the environment. In May-June 1988, a bloom of *C. polylepis* caused the death of 900 tons of farmed fish (salmon, trout) in the Kattegat and Skagerrak area of the North Atlantic, as well as lethal effects on populations of invertebrates, macroalgae, zooplankton and bacteria due to haemolytic compounds (Rosenberg *et al.* 1988; Gjøsæter *et al.* 2000). In 1991, *C. leadbeateri* killed 600 tons of farmed salmonids in Lofoten, northern Norway (Johnsen *et al.* 1999). Toxic blooms of *P. parvum* have frequently been reported from coastal waters and brackish lakes and pounds worldwide and are often associated with fish mortalities due to haemolytic toxins, the prymnesins, acting on the permeability of biological membranes (Edvardsen & Paasche 1998). *Phaeocystis* blooms of mucilaginous colonies recur in the Barents Sea, Norwegian fjords, the Southern Ocean and the continental coastal waters of the North Sea (Lancelot *et al.* 1998). The most visible harmful effect related to these blooms, mainly those of *P. globosa* in the Southern Bight of the North Sea, is the deposition of thick layers of odorous foam on the beaches, thus affecting tourism and recreational activities. *Phaeocystis* colony blooms were also reported as responsible for clogging fishing nets, repulsing fish, and possibly for having negative impacts on benthic life. The species *P. pouchetii* was also shown to be responsible for toxin production that affected cod larvae development (Aanesen *et al.* 1998).

Current knowledge of the life cycle of haptophytes

Despite the significance of these haptophyte species, the life cycle of most species is still not completely elucidated. This is partly due to the taxonomic confusion and uncertainties about the identity of species or strains, as well as to difficulties with

species identification, which requires electron microscopy. The recent implementation of new techniques, such as nucleotide sequencing and flow cytometry used for the genetic characterization and ploidy level determination, respectively, has nevertheless allowed serious progress in this field. For example, *P. parvum* and *P. patelliferum* were considered distinct species on the basis of taxonomic criteria (the organic scale morphology) until the recent demonstration that these were two stages of the same life history (Larsen & Edvardsen 1998). Investigations of life cycles also suffer from the inability to reproduce complete life cycles under laboratory conditions and to culture some stages (e.g. the microflagellates of *P. globosa*). On the other hand, interpretation of field observations is sometimes difficult due to the presence of different species or several types of cells, and ignorance of the overwintering and seeding forms.

Alternation of different morphological stages (e.g. free-living cells, colonies, amoebae, presence of various scale coverings) and motility capability seem to be characteristic of the life cycle of haptophytes (Hibberd 1980; Billard 1994). Both *C. polylepis* and *P. parvum* exist as two types of flagellates characterized by different organic scales and one non-motile cell stage (amoeboid, cyst) in culture (Edvardsen this issue). Coccolithophorids also alternate between holococcolithophorids and heterococcolithophorids (Thomsen *et al.* 1991) or coccolithophorids and scale-bearing cells (Billard 1994; Green *et al.* 1996). Some *Phaeocystis* species exhibit an alternation between several types of free-living cells (motile and non-motile of various size) and gelatinous colonies (Rousseau *et al.* 1994; Peperzak *et al.* 2000). This has been clearly demonstrated for *P. globosa*, which is referred to in most field and culture investigations (Fig. 2). The function of the various cell types within the life cycle of *P. globosa* life cycle, and in particular their involvement in colony formation, is not yet fully understood. Colonies are composed of cells that are deprived of flagella, haptonema, and organic scales, and are embedded in a mucilaginous matrix secreted by the cells themselves. Their size varies considerably from 10 µm for a 2-cell colony to several mm with several thousands of cells (Rousseau *et al.* 1990). The termination of the *P. globosa* colonial stage is characterized by colonial lysis (Brussaard *et al*. 1995; Rousseau *et al*. 2000), aggregate formation, or by cell motility development and subsequent emigration from the colonies, leaving behind ghost colonies (Peperzak *et al*. 2000).

Haplo-diplontic life cycles have been demonstrated in some haptophytes, e.g. in *Emiliana huxleyi* (Green *et al.* 1996; Medlin *et al.* 1996), *Chrysochromulina* spp. (Edvardsen & Vaulot 1996; Edvardsen, this issue) and *Prymnesium parvum* (Larsen & Edvardsen 1998). The existence of two ploidy levels and haploid microzoospores released from colonies gives some support to the existence of a haplo-diplontic life cycle in *P. globosa* as well (Rousseau *et al.* 1994; Vaulot *et al.* 1994). Observation of syngamy and meiosis is, however, restricted to a few genera such as the coccolithophorid *Pleurochrysis*.

The nature of the benthic stages is also still unclear for most of the haptophytes. Non-motile amoeboid or walled cells of *Chrysochromulina* spp. and cysts of *Prymnesium* spp. could possibly function as a resting stage. However, only limited information is available on the abundance, role in bloom formation, and survival conditions in nature of the benthic stages. The role of sediment as a seeding reservoir for the water column also deserves more attention.

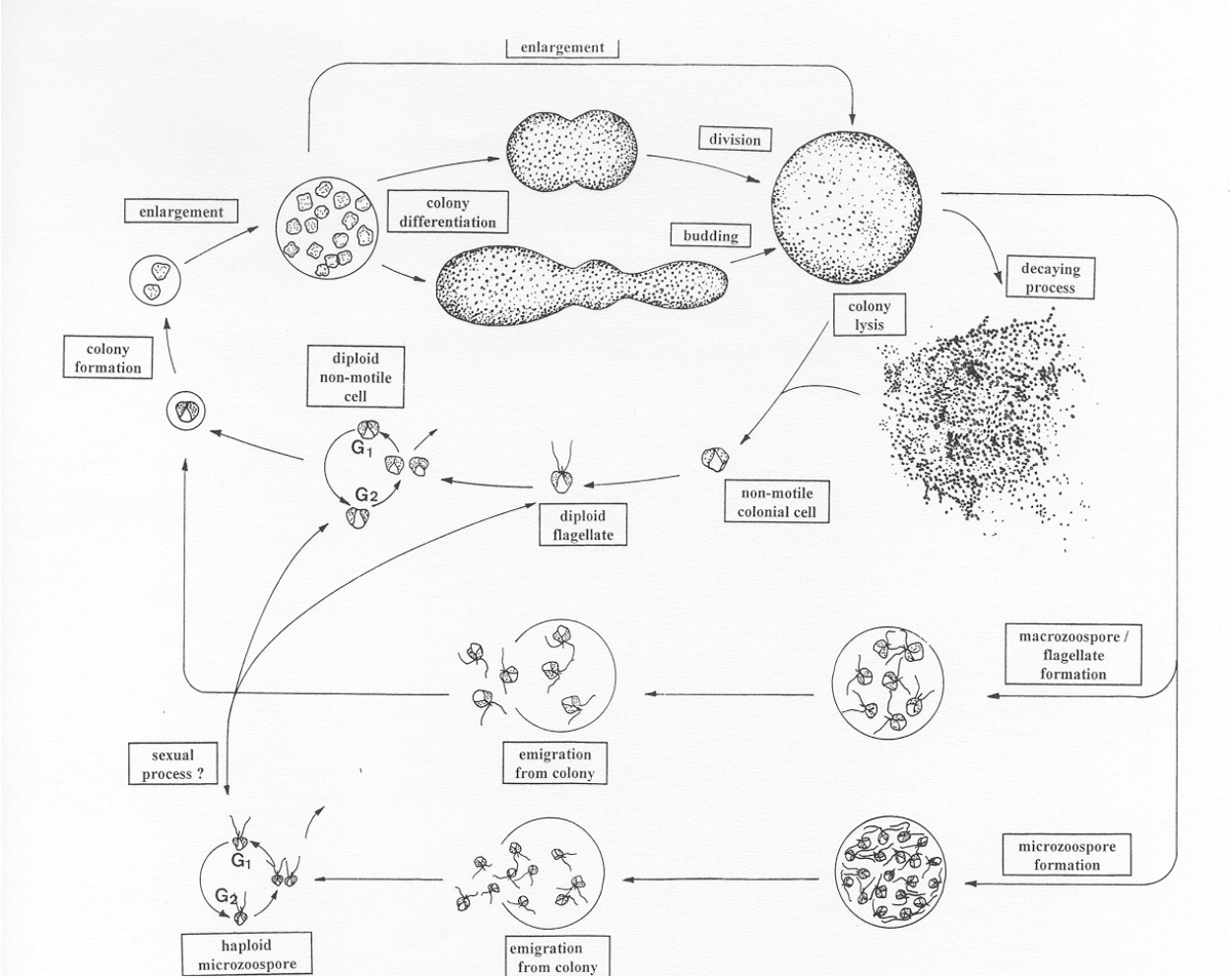


Fig. 2. The life cycle of *Phaeocystis globosa*, as compiled from culture and field observations, illustrates the complexity of haptophyte life cycle events. Based on microscopic observations and flow cytometric DNA analysis, 3 different types of free-living cells were shown to alternate with mucilaginous colonies composed of non-motile cells deprived of flagella, haptonema and scale coverage (from Rousseau *et al.* 1994).

The transition between the life stages in haptophytes is presumably controlled by the interplay of endogenous and environmental factors, but the role and the relative importance of these factors are poorly known. Such information is, however, essential for understanding how and why blooms form and species survive during non-bloom periods. The lack of knowledge is at least partly due to difficulties in distinguishing between factors affecting the transition between the different life stages and those affecting growth of a particular stage. This is clearly illustrated in the case of *P. globosa*, for which several factors have been suggested to play a role in triggering the passage to the colonial stage from a free-living cell. Among them, the requirement of a solid substrate for cell attachment has been suggested from the observation of small

colonies attached to the setae of *Chaetoceros* spp. at the early stage of the bloom (Boalch 1987; Rousseau *et al.* 1994). Phosphate concentration has also been suspected to be important for colony formation (Veldhuis *et al.* 1986; Veldhuis & Admiraal 1987; Cariou *et al.* 1994), but it is still unclear if phosphate deficiency triggers the colonial form, or if this form outcompetes free-living cells due to its higher ability to use organically bound phosphate (Veldhuis & Admiraal 1987). Chemical substances derived from the vernal diatom bloom have also been suspected to be important for colony formation (Weisse *et al.* 1986; Rousseau *et al.* 1994), but have never been demonstrated. Flagellate formation into colonies at the end of a *P. globosa* bloom is presumably linked to unfavourable conditions for colony survival, e.g. nutrient stress and light limitation, subsequent to vertical transport (Peperzak *et al.* 2000).

The ecological importance of life cycle events for haptophytes

Some features of the life cycle of haptophytes appear to be important for the dynamics of these species. The difference in autecology, with various light and temperature tolerances, of the authentic and alternate cells of *C. polylepis* would allow this species to occupy different ecological niches (Edvardsen, this volume). The occurrence of a cyst in some *Prymnesium* species would represent a seeding stage for blooms (Edvardsen, this volume). The alternation of *Phaeocystis* life forms, i.e. free-living nanoplanktonic cells and large gelatinous colonies, have different implications for planktonic and benthic ecosystem structure and function. The success of *Phaeocystis* and its impacts on the marine ecosystem have been linked to their capacity to form large gelatinous colonies (Lancelot *et al.*, this volume). The colony matrix structure does indeed provide a competitive advantage to this alga for a limiting resource, but it also protects *Phaeocystis* from grazing and viral and/or bacterial infection (Lancelot *et al.*, this volume). Possibly, the suspected common occurrence of sexuality in haptophytes, resulting in high genetic plasticity, could well be an explanation for their widespread distribution.

Pseudo-nitzschia life cycle and the sexual diversity of clones in diatom populations

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Introduction

The causes of Harmful Algal Blooms (HABs) are diverse, and may involve several different species. Diatoms are common in marine communities and play a significant role as members of HABs (APPENDIX 1). For example, among 25 marine species causing blooms in the Black Sea, diatoms amount to 80 % (Zaitsev & Alexandrov 1998, Table 27). Although not all of them are harmful, several, such as *Pseudo-nitzschia pseudodelicatissima* and *Pseudo-nitzschia seriata*, are known to be deleterious elsewhere in the world (Bates *et al*. 1998).

The principles of cell size diminution during the vegetative phase and the restoration of the original size as a result of auxospore formation during the generative phase of the life cycle are well developed (Pfitzer 1871; Geitler 1932; Drebes 1977). An accepted classification of patterns of sexual reproduction has also been elaborated (Geitler 1935; Mann 1993). Essential developmental work has been carried out to elucidate breeding systems in diatoms (Roshchin & Chepurnov 1999). However, to date the life cycle of an overwhelming majority of diatoms has not been investigated, and information on the life history of most harmful species is fragmentary (Mann, this volume). At least 20,000 species of diatoms are recognised today; with a narrower species concept, this would rise to 200,000 (Mann & Droop 1996), but only slightly more than 200 species have had significant aspects of their life history reported (Mann 1988; Edlund & Stoermer 1997). Thus, there is a requirement to study the reproductive biology of algae, including the following aspects:

- schemes of the life cycles;
- patterns of the sexual reproduction process (mating behaviour);
- breeding systems.

Even after over 150 years of observation by microscopy, we still have much to learn about the life strategy and life history of diatoms.

Sexual reproduction of *Pseudo-nitzschia*

At the laboratory of Dr. Stephen Bates (Moncton, Canada), we studied the sexual reproduction in two species from the genus *Pseudo-nitzschia*, i.e. *Pseudo-nitzschia pseudodelicatissima* (Hasle) Hasle and *P. multiseries* (Hasle) Hasle. Briefly, the pattern of sexual reproduction in these two species is as follows (for details see Davidovich & Bates 1998a, b; Kaczmarska *et al.* 2000; 2001). Two cells belonging to the opposite sex line up side-by-side (either valve-to-valve or valve-to-girdle; the exact configuration is difficult to view by light microscopy) (Fig. 3A-B). Each gametangium produces two gametes, accomplished by the rearrangement of the cellular content (Fig. 3C). Although the gametes in a gametangial pair are morphologically identical, they differ in behaviour; gametes in one gametangium are active, whereas they are passive in the another. Active gametes move by amoeboid action towards the passive ones (Fig. 3D). There is no predetermined direction (cis- or trans-) of the fusion; as a rule, gametes

situated most closely to each other fuse first. Normally, two zygotes arise as a result of gamete fusion (Fig. 3E). The zygotes are weakly connected to one of the frustules of the "mother" cell. Auxospores grow perpendicularly to the frustule if the connection is not lost (Fig. 3F-G), eventually producing a large initial cell (Fig. 3H) which exits the auxospore and divides, forming a chain of large cells (Fig. 3I). The described scheme corresponds to Type **IA2** of Geitler's classification (Geitler 1932; 1935).

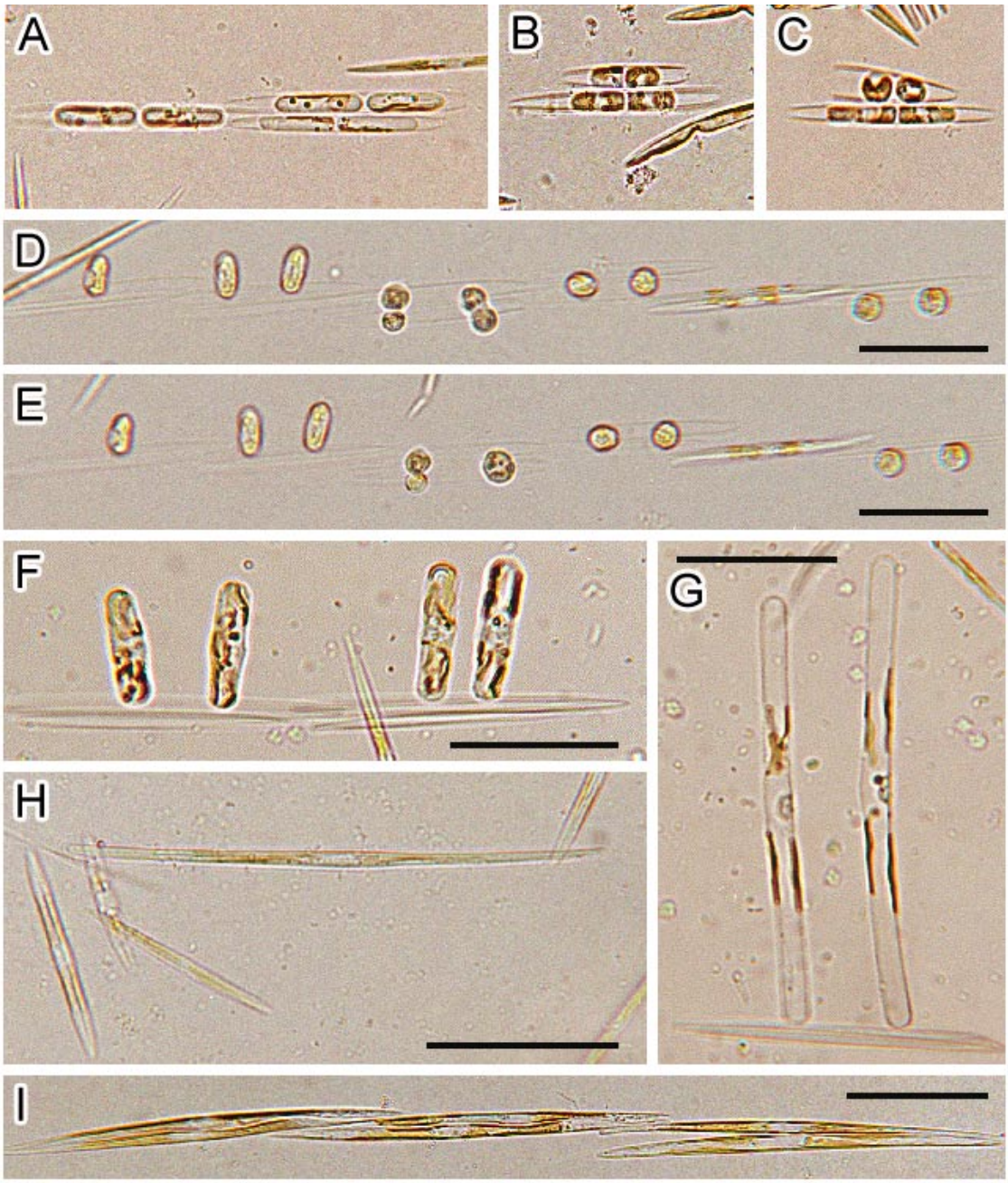


Fig. 3. Sexual stages of *Pseudo-nitzschia multiseries* and *P. pseudodelicatissima*. See text for details. Images from Davidovich & Bates (1998a). Scale bars = 100 µm.

The allogamous sexual reproduction that we observed in *P. pseudodelicatissima* and *P. multiseries* appears to be typical for all pennate diatoms (Rosowski *et al.* 1992). The success of this investigation was based on the presumption that these species have a heterothallic sex distribution (dioecism). This means that two sexually compatible clones of opposite sex, when mixed together, should give rise to auxospores and then initial cells. However, some enigmatic facts were revealed. For example, on one occasion with *P. pseudodelicatissima*, initial cells were produced when a mixture was made of two clones of the same sex (Davidovich & Bates 1998b). There was no proper explanation for this finding, if strict dioecy of the species is believed.

Diversity of sexual reproduction behaviour in diatom clones

Meanwhile, a diversity of sexual behaviour was shown to be common in clones of other diatom species derived from natural populations, as well as in those resulting from inbred mating (Chepurnov & Mann 1997; 1999).

In addition, some efforts have been undertaken to investigate the variety of sexual behaviour in clones of the diatom *Nitzschia longissima* (Breb.) Ralfs. To date, more than 50 clones have been randomly isolated from field populations near Karadag, Ukraine. Mutual mating gave more than 350 pairs of combinations. According to their mating compatibility, all the clones were arranged into four groups (Fig. 4).

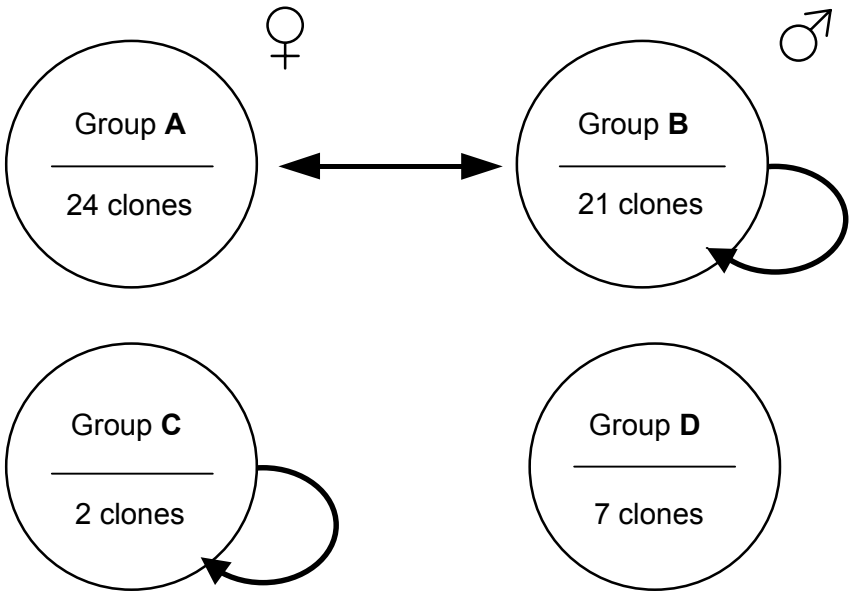


Fig. 4. Sexual compatibility of clones of the diatom *Nitzschia longissima*. See text for details.

Clones of Group **A** were able to mutually mate with clones of Group **B**. The latter were also able to reproduce intraclonally, but not as vigorously as in the case of interclonal reproduction. The tendency to reproduce intraclonally may in part be explained by the fact that members of Group **B** belong to a "male" type, as determined by the specific morphology and activity of the gametes that they produced. The third group (Group **C**) reproduced exclusively intraclonally. Within Groups **B** and **C,** the frequency of intraclonal reproduction varied varyfrom clone to clone. The last group

(Group **D**) was unique. We tried to mix those clones with others during almost an entire year. All attempts ended in failure until we isolated two new clones which were able to interbreed each other; one of them was also found to be a sexual partner for all the clones of Group **D**. According to the biological concept of "species", one should therefore recognise this sexually isolated group as a species separate from other investigated clones of *N. longissima*. The assumption has recently been confirmed by the peculiarities of the frustule structure, as detected by scanning electron microscopy. Based on the above biological concept, one might consider the clones in Group **C** as a separate species, as well. However, the morphology of these clones in essence corresponds to that of Groups **A** and **B**. The sexual characteristics of the descendants of the intraclonal lineage now remain to be determined.

These examples indicate the great complexity of the sexual structure in diatom populations. The clonal variety of sexual behaviour that seems to be under genetic control should be taken into account when the life cycle is considered. It was previously reported that separate phenodemes, which are possibly true species, exhibit different life cycles (Mann & Droop 1996). We are dealing here with the sexual diversity of individuals whose status is lower than species level. Therefore, depending on the sexual structure of a population (clonal composition), one may expect diversity in the way the life cycle is manifested.

In view of the goals of the LIFEHAB meeting, one might consider several questions designed to elevate our knowledge of the reproductive biology and life strategy of diatoms. What is the mechanism of genetic control of the sexual status in a clone? Can the sexual status change in the next generations? What is the ratio of male to female clones in natural populations of dioecious algae? What is the proportion in the population of clones with alternative (bisexual, monoecious) behaviour? And for all that, what is the sexual structure of diatom populations?

Factors affecting the sexual reproduction of diatoms, with emphasis on *Pseudo-nitzschia* spp.

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Introduction

Diatoms have the peculiar predicament that every time they divide vegetatively, they decrease in cell size. This is due to their unique construction of having two rigid silicon thecae that compose the diatom frustule. When the diatom cell reaches a threshold size, it becomes physiologically capable of undergoing sexual reproduction to produce auxospores which then develop into large initial cells (Geitler 1932, 1935; Drebes 1977; Round *et al*. 1990; Mann 1993). Most diatoms rejuvenate their original large cell size in this way, according to the scheme developed independently by MacDonald (1869) and Pfitzer (1869) in 1869 (Fig. 5). If the cells do not undergo auxosporulation, they will continue to decrease in size until they eventually die. Details of how sexual reproduction is carried out are different, depending on if the diatom is pennate or centric (Drebes 1977; Round *et al*. 1990).

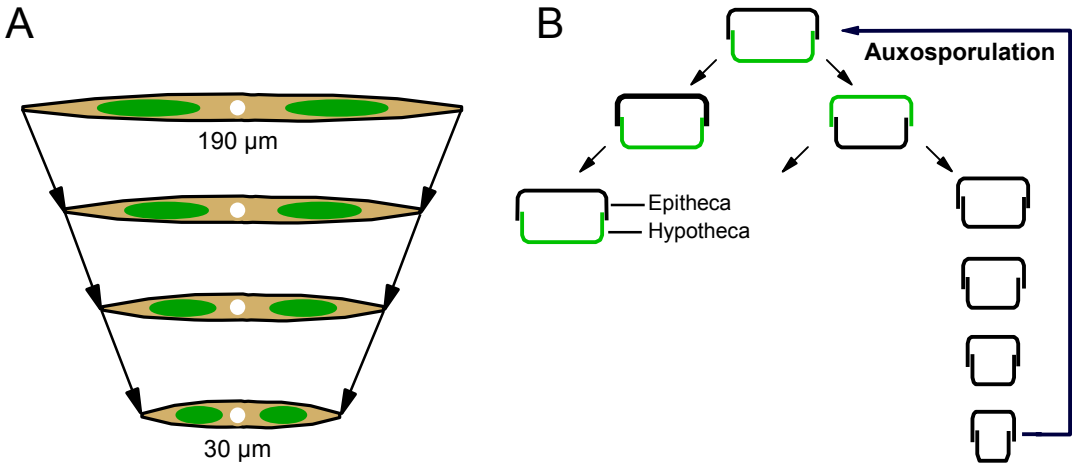


Fig. 5. (A) Decrease in cell apical length of *Pseudo-nitzschia.* (B) MacDonald-Pfitzer scheme for cell size reduction, and the restitution of large cell size via auxosporulation, for a generic centric diatom.

This presentation outlines the main factors that are responsible for affecting the sexual reproduction of diatoms. It will focus mainly on pennate diatoms of the genus *Pseudo-nitzschia* (Bates 2000). In 1987, *P. multiseries* was identified as the source of the neurotoxin, domoic acid, that contaminated blue mussels at aquaculture sites in eastern Prince Edward Island, Canada (Bates 1998; Bates *et al*. 1998). It was the first time that a diatom was shown to produce a phycotoxin. Indeed, diatoms form only a

small minority of harmful or toxigenic phytoplankton species, the majority belonging to the Dinophyceae. Several species of toxigenic *Pseudo-nitzschia* are present in European waters (e.g. Lundholm *et al*. 1994; 1997; Fraga *et al*. 1998; Amzil *et al*. 2001; Sarno & Dahlman 2000; Gallacher *et al*. 2001).

Toxic and harmful diatoms

All of the diatoms known to have produced a toxin or to have caused harm are shown in (APPENDIX 1) (cf. Hasle & Fryxell 1995; Fryxell & Hasle 2002). Curiously, many of the harmful, but non-toxic, diatoms are all fish-killers; they are also all centrics. Two species of *Chaetoceros* are characterized by barbed setae which can become lodged between the secondary lamellae of the gills of salmonids, causing mucus overproduction and eventual death by suffocation (Rensel 1993). Another diatom, that resembled *Corethron* sp., was associated with the mortality of coho salmon smolts (Speare *et al*. 1989). The cell has an apical corona of spiny setae that may become embedded in the fishes' gills. A centric, *Leptocylindrus minimus*, was implicated in mortalities of aquacultured salmonids in southern Chile (Clément & Lembeye 1993); the mode of death is not certain. In Asia, *Skeletonema costatum* and *Coscinodiscus wailesii* are considered "red tide" species (Yamochi & Joh 1986; Nagai & Imai 1999). They form intense blooms which can deplete the water of nutrients and decrease the light available for the cultivation of the red macroalga "nori" (*Porphyra*) for human consumption. The epiphytic *Tabularia affinis* causes problems by forming ribbon colonies that adhere to the surface of cultured *Porphyra* in Japan (Nagai *et al*. 1996).

Except for *Pseudo-nitzschia multiseries*, *P. pseudodelicatissima*, and *Coscinodiscus wailesii*, the sexual reproduction of the other harmful diatoms has not been studied specifically. It is possible that the centric diatoms are monoecious, each clone being able to produce both "male" and "female" gametes, since this is the pattern that has often been assumed to be characteristic of centrics (Drebes 1977); but this needs to be checked for each species. The resting stage is another aspect of the life history of diatoms for which there is little information (Mann; Diatom Discussion Group Report, this volume). Resting stages are known for some of the centrics listed in APPENDIX 1 (see also Hargraves & French 1975; French & Hargraves 1980; 1985; Garrison 1984; McQuoid & Hobson 1996; Nagai & Imai 1999).

Interest in the mode of sexual reproduction of *Pseudo-nitzschia* arose because clonal cultures continued to diminish in size and eventually died, without undergoing sexualization. Experiments that mixed together different clones documented that these pennate species are dioecious, i.e., are characterized by cells that produce either "male" or "female" gametes by separate gametangia in different clones (Davidovich & Bates 1998a, b; Kaczmarska *et al.* 2000). Originally, the sexual reproduction of *P. multiseries* was incorrectly described by Subba Rao *et al*. (1991), for the reasons given in Fryxell *et al.* (1991) and Rosowski *et al.* (1992). Clonal cultures of toxigenic *Pseudo-nitzschia* sp. cf. *pseudodelicatissima* were recently described as undergoing "enlargement", without producing any sexual cells (Pan *et al.* 2001).

Factors inducing sexualization

Cell size. A key condition to be met before diatoms become sexualized is that they must first decrease to a threshold size, known as the first cardinal point (Geitler 1932, 1935). It has sometimes been suggested that "only relatively small cells, measuring generally about 30-40% of maximal valve diameter within a species-specific size range, prove to

be capable of sexualization" (e.g. Drebes 1977, p. 271). However, our experience with *Pseudo-nitzschia multiseries*, at least, is that the largest sexually inducible cells thus far are about 120 µm long and that the largest vegetative cells are about 190 µm long (Fig. 6), which is 63% of the largest cells. A value of 70% was found in other experiments (Hiltz *et al.* 2000). The range of 30-40% is therefore clearly an underestimation; the window of opportunity for sexualization is larger than previously reported. Such information is relevant for determining how long it takes for *P. multiseries* cells to decrease to the size allowing for sexual reproduction. Given a size reduction of ~2.5 µm per month, or ~30 µm per year under constant growth conditions

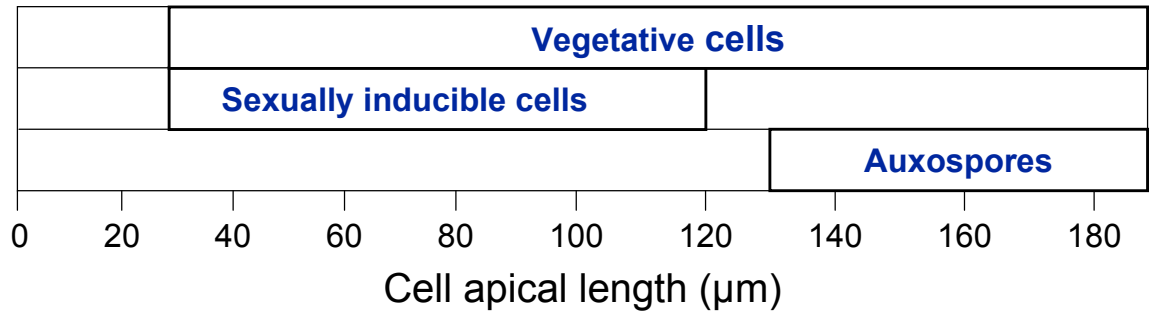


Fig. 6. Cell apical length of *Pseudo-nitzschia multiseries* at various stages in the life cycle (data from pers. observ.; Davidovich & Bates 1998b; Hiltz *et al*. 2000).

in culture, it was estimated that initial cells must grow for ~3 years to reach the sexually inducible size, assuming that the suitable length corresponds to 40% of the maximal size (Davidovich & Bates 1998b). In reality, recent experiments showed that one pair of clones produced initial cells after only 7 months, when they were ~120 µm long (pers. observ.). No information is available about the rate of cell size reduction of *Pseudo-nitzschia* spp. in the field, but changes in cell division rate over the long term must be taken into account. A knowledge of cell size may also be important if there is a relationship between cell size and toxicity, as has been observed in most of our cultures of *P. multiseries* (Bates *et al*. 1999).

Growth phase. Once in the appropriate size window, it is essential that *Pseudo-nitzschia* spp. cells be in good physiological condition for them to be capable of reproducing sexually. This means that they must be in the exponential growth phase (e.g. days 3-6 after inoculation in batch culture). In nature, one would therefore expect to find sexualized cells only when conditions are favourable for growth, although this may not have to be during a bloom.

Irradiance and photoperiod. Being in "good physiological condition" also implies that the cells are exposed to sufficient light energy during a 24-h period. The absolute irradiance level required to initiate sexual reproduction has not been studied for *P. multiseries*. However, a study of different photoperiods at an irradiance level of ~100 µmol photons m^{-2} s^{-1} showed an increased production of gametes per vegetative cell with an increase in photoperiod length, up to the maximum studied of 16:8 h Light:Dark (Hiltz *et al*. 2000). Davidovich (1998) likewise found that the production of gametes, auxospores, and initial cells of *Nitzschia lanceolata* increased with the total light energy

received by the parent cells. Thus, photosynthesis provides energy for sexual reproduction; stored energy is not sufficient to complete all the stages of sexual reproduction, including initial cell formation.

Water motion. All phytoplankton cells experience some movement due to currents within the water column. Such water motion may be important for spreading vegetative cells to new locations. For sexually reproducing cells, it may be both helpful and detrimental. Pennate diatom cells of opposite sex must come into contact with each other in order to mate. This may occur by the bi-directional movement of the cells over a substrate (e.g. suspended particulate material or directly on the sediments). Wind- or heat-induced water motion may also increase the chance of cell-to-cell encounter. Above a certain threshold, however, water motion may become a disadvantage. For example, even if the cells manage to touch one other, the hydrodynamic force may be too great for them to remain attached long enough to initiate the sexual process. Also, such forces may cause already formed gametes to become detached from the "mother" gametangial cell. Once they lose contact with the gametangium, it is unlikely that they will find another gamete with which to fuse to form a zygote.

A preliminary experiment showed that a mixture of *P. multiseries* clones of opposite sex grown in duplicate flasks containing 1.5 L of f/2 medium failed to produce gametes for four days while on an orbital shaker at 170 rpm; stationary control cultures produced gametes as usual during that same period. Once the water movement was stopped, however, there was a rapid, massive production of gametes and the subsequent formation of initial cells. A second experiment employed small volume (5 mL) cultures in petri plates (Gordon 2001). Results showed a significant effect of water motion (again, created by orbital shakers at 170 rpm), in both delaying and decreasing zygote production. In contrast to the large volume flask experiment, however, gametes were produced in the shaken treatments, a finding that may be related to the different culture volumes used and to the amount of water movement thus created. One may nevertheless conclude that still conditions favour *Pseudo-nitzschia* sexual reproduction. In high water motion, fertilization may still be successful if the cells reach a high density, when cell-to-cell contact is facilitated. If this were to be followed by a period of calm, then one may expect the highest success for producing auxospores.

Stimulants of gametogenesis (Pheromones?). Pennate diatom cells of opposite sex have the challenge of finding each other in order to mate. Although this is made easier when the cell number is high, the presence of a chemical signal (a pheromone) would be a further advantage. Because pennates are motile, they may take advantage of changes in chemical gradients of any such pheromone, in order to find a mate. Pheromones have been found in brown macroalgae for gamete attraction (Maier 1995). However, it has also been debated whether or not pheromones would be advantageous for mate location in microalgae (Dusenbery & Snell 1995).

To look for possible chemical signals, pairs of clones of opposite sex were exposed to filtrates that may contain such a signal (Haché 2000). The filtrates were derived from cultures in which a pair of clones was allowed to grow for 48 h in order to initiate the sexual process, and in so doing, release possible pheromones. Different proportions of each test clone were used in order to determine if any effect on gametogenesis would be more apparent in clone mixtures that contained more "male" or more "female" cells. Control cultures contained the clone mixtures at these same

proportions, but made in fresh f/2 medium instead of filtrates derived from sexually reproducing mixtures. The results showed a significantly greater production of gametes when the clone mixtures were made in filtrates compared to fresh f/2 medium. Moreover, the effect was most pronounced when the proportion of "female" cells was greatest. In that case, some "female" cells were also observed to produce gametes, even when they were not in contact with a "male" cell. The results suggest that the filtrates contained some type of compound (a pheromone?) that stimulated gametogenesis, but did not necessarily aid in mate location.

Bacteria. Bacterial-phytoplankton interactions are an intrinsic component of harmful algal bloom (HAB) ecology and physiology (Doucette *et al.* 1998). Bacteria have been shown to directly or indirectly take part in biotoxin production, promote or inhibit the growth of HAB species, and stimulate or inhibit phytoplankton sexual reproduction (perhaps via excretion of certain "promoter" organics). For example, the bacterium *Alcaligenes* sp. promoted spermatogenesis (but not oogenesis) in *Coscinodiscus wailesii* (Nagai *et al*. 1999). Experiments were therefore carried out to investigate the possible role of bacteria in mediating the sexual reproduction of *P. multiseries*, by mating pairs of axenic or non-axenic clones (Thompson 2000). A mixture of bacteria, isolated from each pair of non-axenic clones, was also reintroduced into the corresponding mixture of axenic clones. The control non-axenic clones retained their natural composition of bacteria. Results showed no sexual reproduction (i.e. no gametes or auxospores) in mixtures of one pair of axenic clones. Moreover, the reintroduction of bacteria to axenic mixtures restored sexual activity. In contrast, sexual reproduction did occur in axenic mixtures of another pair of clones. Both gametes and auxospores were observed in the axenic mixtures of this pair, as well as in the mixtures to which bacteria were added (as expected). It is possible that viable extracellular or intracellular bacteria remained in this particular pair of antibiotic-treated clones, accounting for the observed sexual activity. Additional experiments are required to unambiguously determine whether or not bacteria are required for *P. multiseries* sexual reproduction.

Clonal variability in domoic acid toxicity

Our ability to mate different clones of *P. multiseries* and to isolate individual initial cells into clonal culture has enabled us to look at differences in toxicity among sibling clones derived from the same parents. We have found both considerable variability among clones, as well as a decrease in toxicity as the clonal cultures age and the cells become smaller. The variations among sibling clones from common parents are caused in part by genetic variability. However, it is also possible that the presence of different types and numbers of bacteria in the individual cultures also accounts for these differences (see Bates [1998] for a summary of differences in toxicity between axenic and non-axenic cultures). Free-living as well as attached bacteria have been found in the diatom cultures (Kaczmarska *et al.* 2000). The diversity and numbers of bacteria attached to *P. multiseries* cells increase over time after inoculation in batch culture. How the bacterial composition and concentration change over time after the diatom cells are first isolated has not yet been established. The extent to which the different types and numbers of bacteria affect toxicity and sexual reproduction also remains to be determined.

Remaining questions and gaps

Research on diatom sexuality will improve our ability to predict the occurrence, periodicity, and toxicity of *Pseudo-nitzschia* blooms. For example, years in which blooms are most intense and toxic may be associated with a particular stage in the cells' life history. However, the following gaps must first be filled before we can successfully apply our knowledge about the sexual reproduction of diatoms:

- Identification of the sexual stages of harmful and toxic diatoms other other than those mentioned in this report.
- Molecular techniques to identify sexual stages.
- Time required for *Pseudo-nitzschia* cells to reach the sexually inducible size in nature.
- Location of overwintering cells.
- Information on resting stages, if they exist, or of heavily silicified "winter growth stages".
- Information on asexual vegetative cell enlargement, especially for pennate diatoms.
- Genetic variability vs. biotic/abiotic factors in controlling sexual reproduction and toxicity.
- Location of mating (suspended within the water column, on the surface of a particle, on the bottom sediments?).
- Relationship between cell size and toxicity.
- Bloom intensity and toxicity in relation to the timing of sexual reproduction.
- Proportion of the population undergoing sexual reproduction at one time.
- Information on mating systems in and compatibility among morphologically distinct or cryptic *Pseudo-nitzschia* species.
- Study of cell cycle progression, including volumetric growth (and girdle development) and the mechanism by which the characteristic stepped colony is formed.

Life cycle variation in PST content and cell toxicity in PST-producing dinoflagellates

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Introduction

A sexual life cycle that includes the production of a resting stage (cyst or hypnozygote) is a common feature of many dinoflagellates and can have a major influence on initiation, maintenance and decline of bloom populations. The paralytic shellfish toxin (PST)-producing dinoflagellates (*Alexandrium*, *Pyrodinium* and *Gymnodinium catenatum*) all possess the same basic haploid-diploid life cycle: a haploid vegetative phase; initiation of gamete formation and fusion leading to the production of a resting cyst; and germination and meiosis to re-establish the haploid vegetative phase (Fig. 7).

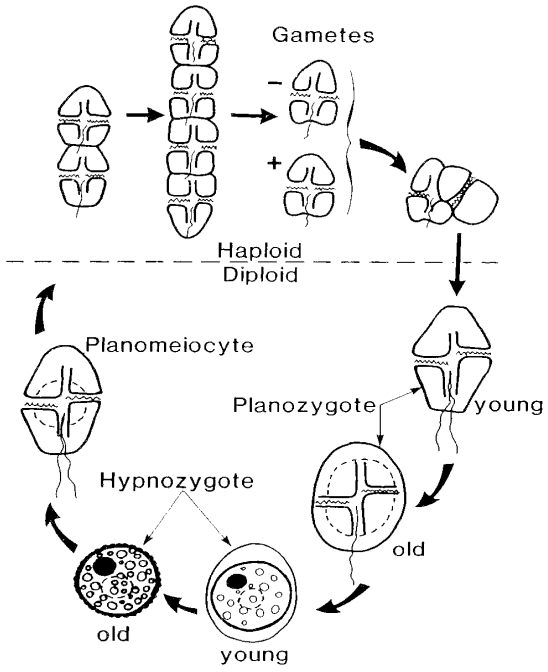


Fig. 7. The sexual life cycle of the toxic dinoflagellate *Gymnodinium catenatum*. (Blackburn *et al*. 1989).

A range of morphological, physiological and biochemical changes are associated with the sexual life cycle. This process involves the expression or up-regulation of many genes, e.g. the production of membrane-bound factors and the excretion of soluble compounds that control gamete recognition, and the cellular machinery that co-ordinates nuclear fusion in the planozygote stage. Potentially the most marked changes are those associated with cyst formation and cyst dormancy, which involve the

synthesis of a chemically complex and resistant cyst-wall, in preparation for cyst dormancy and maintenance of quiescence. We know little about the biochemistry of cyst formation. However, studies of *Scrippsiella trochoidea* cysts indicate that the latter process involves the reduction of a wide range of physiological and biochemical activities within the cell to a "basal maintenance" level during cyst dormancy (Lirdwitayaprasit *et al*. 1990), during which the cysts may continue to take up and utilize nutrients (Rengefors *et al*. 1996).

PST-producing species have similar life cycles and undergo similar, equally dramatic changes; not surprisingly, these changes alter the production of a range of secondary metabolites including PST production. Due to their well understood and relatively easily manipulated life cycles, the relatively few published studies of PST variation in dinoflagellate life cycle stages are confined to *Alexandrium tamarense* and *Gymnodinium catenatum*.

Vegetative cells

The toxicity of vegetative cells of *A. tamarense* and *G. catenatum* can vary considerably, usually ranging between 10 - 100 fmoles·cell^{-1} in *A. tamarense*, and 10 - 250 fmoles·cell^{-1} in *G. catenatum* (e.g. Oshima *et al*. 1992; Oshima *et al*. 1993; Negri *et al*. 2001; Yoshida *et al*. 2001). From published data, it is possible to draw some general observations about variation in vegetative cell PST-content:

1. Non-toxic strains are known but, in *A. tamarense* at least, these are particular regional genotypes (e.g. European, Tasmanian). The basis for their lack of toxicity is currently unknown.
2. Toxin content and PST profile vary among populations, but are relatively consistent within a population.
3. Natural vegetative cells produce more PSTs than cultures established from blooms.
4. Cultures established from blooms produce more PSTs than cultures established from cysts.
5. The per-cell toxin content and PST profile of cultured cells vary more than those of harvested natural bloom cells (Kim *et al*. 1993; Oshima *et al.* 1992).

The existence of genetic variation among populations is well established (Scholin *et al*. 1995; Adachi *et al*. 1996a). The PST profile of *A. tamarense* is a heritable character. Therefore, points 1 and 2 most likely represent genetic differentiation among regional populations. The reasons for the pattern of PST variation noted in points 3, 4 and 5 are less clear. It is possible these changes are associated with changes in major- or micro-nutrient status when cells are cultured in the laboratory. Alternatively, more recent evidence suggests that the increasing removal of biological factors prevalent in natural blooms (e.g. viruses, bacteria, grazers and their biochemical influences) has a significant effect on algal cell physiology and PST production. Changes in toxin content of semi-synchronized cultures of *A. fundyense* demonstrate that the PST content of cells also varies over the vegetative cell cycle. This variation shows that PST synthesis, measured as specific toxin production rate, is discontinuous, and associated with a light-induced G1 phase (Taroncher-Oldenburg *et al*. 1997).

Gametes

Knowledge of the toxin content and PST profile of gametes has been hampered firstly by the difficulty of determining which cells are gametes (e.g. swimming behaviour or evidence of cell fusion) as opposed to vegetative cells, and secondly, physically separating them from other cell types present in the mating cross. Studies of putative gametes of *G. catenatum* by Bravo *et al*. (1998) obtained partial PST profile data but did not report PST content per gamete cell. Their data showed a shift in C1/C2 ratios, with gametes showing a relative increase in C1 compared to C2 (Table 1).

Table 1. C1/C2 ratio of vegetative cells, putative gametes and resting cysts (Bravo et al. 1998).

Life cycle stage	PST content (pg·cell^{-1})	C2/C1 ratio (SD)
Vegetative cells	40, 45	5.42-11.27 (0.1-1.3)
Gametes	-	0.72 (0.23)
Cysts	12, 32	0.25 (0.01)

Resting cysts

The relative PST content of resting cysts compared, to vegetative cells, varies in literature reports. The data have also been confounded by comparison of wild cysts with cultured cells, which do not provide a representative estimate of wild cell toxin content. Several reports indicate much higher PST content per cell ranging, from 5 - 10 times more toxin than vegetative cells (e.g. Dale *et al*. 1978; Hurst *et al*. 1985; Oshima *et al*. 1992). However, these studies were not corrected to account for the larger cell volume of cysts. Once corrected, the relative differences would be reduced to perhaps near equal the PST content as vegetative cells. Cembella *et al*. (1990) corrected for cyst/cell volumes, yet in contrast found a considerably lower PST content of resting cysts. These differences may be indicative of population-specific differences caused by differences in the age or state of dormancy, or in the physiological history of the cells at the time they entered the sexual life cycle. In *G. catenatum*, the data are restricted to studies of cultures and laboratory-induced resting cysts by Bravo *et al*. (1998). In this case, the PST content of resting cysts was found to be less (uncorrected for volume) than that found in the parental cultured cells (Table 2).

A common feature from all studies of both species is that the ratios of PST 11-α-epimers (GTX1, GT2, C1) compared to PST 11-β-epimers (GTX4, GTX3, C2) is usually higher in resting cysts than in vegetative cells. It is thought that only the β-epimers are the first to be biosynthesised, followed by gradual epimerisation to the more stable α-epimers (either *in-vivo* or *in-vitro*) (Oshima *et al*. 1993, Cembella *et al*. 1998). These changes are thought to indicate:

- A low biosynthetic/metabolic activity in overwintering resting cysts (Cembella 1998).
- That PST synthesis has been significantly reduced early in the process of cyst production (Oshima *et al*. 1992) (Fig. 8), a hypothesis which is supported by the decreased C2/C1 ratio noted in *G. catenatum* gametes (Bravo *et al*. 1998).

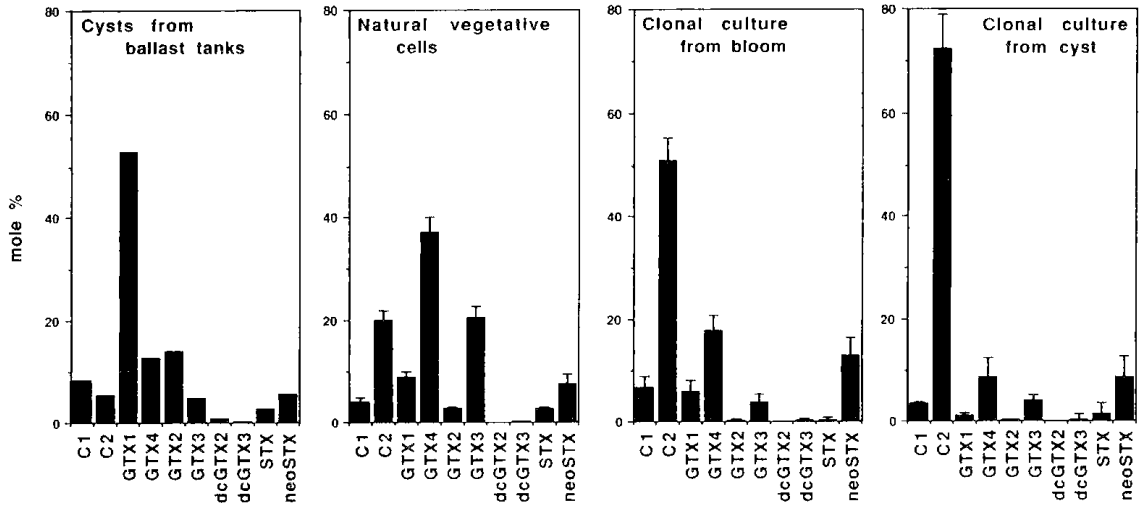


Fig. 8. PST profiles (mol% of cysts, natural vegetative cells and cultures of *Alexandrium tamarense* (from Oshima *et al*. 1992). The increase in ratios of PST 11-α-epimers (GTX1, GT2, C1) compared to PST 11-β-epimers (GTX4, GTX3, C2) is a common finding in all studies of cyst toxcin profiles and indicates reduced synthesis of PST compounds and /or a generally low biosynthetic activity of resting stages.

Post-germination

There are yet no reliable PST content and profile data available from planomeiocyte stages to assess whether/when PST synthesis starts after germination. We can presume that in natural populations, germination re-establishes or replenishes the water column with cells expressing PST production similar to that of the existing vegetative population. However, recent work has shown that cultures established from laboratory-germinated *G. catenatum* cysts show unusual and depauperate PST profiles (Negri *et al*. 2001). Additional unpublished studies have also determined that cyst-germinated cultures from lab-germinated cysts have a significantly reduced PST content (Negri *et al*. 2001). Comparative studies of *Alexandrium* spp. demonstrate that these species do not exhibit the same degree of PST synthesis "suppression" under laboratory germination (Bolch & Negri, unpubl. data). These data suggest that not only is PST synthesis most likely halted during cyst formation and dormancy, but that PST synthesis may be partially or completely impaired if germination is affected under controlled laboratory conditions.

Bacterial interaction with the life cycle and PST production in *Gymnodinium catenatum*

Phytoplankton influence bacterial communities by providing an increased surface area to which surface-associated bacterial groups can attach (Bidle & Fletcher 1995) as well as exuding DOM (e.g. complex polysaccharides) which stimulate and alter the resident bacterial community (e.g. Fukami *et al*. 1985; Janse *et al*. 2000; Riemann *et al*. 2000). Bacteria have in turn been shown to affect phytoplankton by producing: 1) growth promoters that favour particular groups or species (e.g. Furuki & Koboyashi 1991); 2) inhibitory/algicidal proteins or other substances (e.g. Imai *et al*. 1993; 1995; Yoshinaga *et al*. 1995; Lovejoy *et al*. 1999; Doucette *et al*. 1999; Kitaguchi *et al*. 2001; Mitsutani *et al*. 2001); or 3) compounds that interfere with or promote sexual reproduction (e.g. Sawayama *et al*. 1993; Adachi *et al*. 1999). There is ample evidence that similar interactions occur between marine bacteria and harmful algal bloom (HAB) species and that the algal and bacterial partners and their interactions can be highly specific (see Doucette *et al*. 1998 for a comprehensive review).

Gymnodinium catenatum is the only naked dinoflagellate known to produce PSTs. Field populations and cultures produce a wide array of the four structural classes of PSTs dominated by the sulphocarbamyl (C-toxins) (Oshima *et al*. 1993). Toxin composition (PST profile) is generally stable and consistent within a population, however different global populations exhibit minor PST profile differences (Oshima *et al*. 1993; Negri *et al*. 2000). Non-toxic strains have been described but, to date, have been considered to be spontaneous mutations deficient in some essential part of the PST synthetic pathway (Oshima *et al*. 1993). Work in our laboratories has, however, shed new light on the nature of these "non-toxic" strains and the apparent stability of the PST profile produced by *G. catenatum.* Careful cleaning and washing of resting cysts, followed by germination in the laboratory to establish a growing culture, results in strains with no detectable PSTs, or a significantly reduced PST content (>40-fold reduction in STX·cell^{-1}) and PST profile (Negri *et al*. 2000; Bolch & Negri, unpubl. data) (Fig 9).

Furthermore, all previous instances of "non-toxic mutants" can be traced back to a similar cyst isolation process by other workers (Bolch, unpubl. data). The same response is not evident in cultures established from cysts incubated with small amounts of marine sediment. The process of cyst-wall formation in *G. catenatum* (Blackburn *et al*. 1989) most likely excludes extracellular bacteria and, due to their peripheral location, possibly the majority of the few intra-cellular bacteria. We hypothesize that the bacteria may be directly or indirectly important for re-establishing PST production after germination and, in a natural marine sediment environment, may re-establish their relationship with the algal cell at germination. In the laboratory, this process may be interrupted.

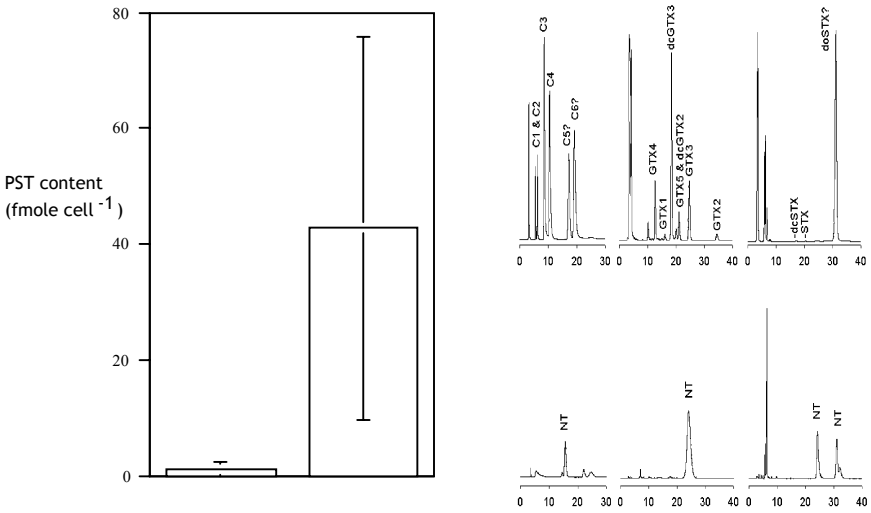


Fig. 9A. Total PST content (fmoles·cell^{-1}) of cultures established from: laboratory germinated cysts (left) and cultures isolated as single vegetative cell chains (right). Note: 8 of the14 cyst strains contained no detectable PSTs. **9B**. HPLC paralytic shellfish toxin profiles from a plankton cell culture of *G. catenatum* (above) and a cleaned resting cyst culture (below). (NT) denotes product peaks which are not PST compounds.

Among our collection of *G. catenatum* cultures, we have a number isolated from different regions that express typical PST –production, and several laboratory cyst-germinated cultures that produce little or no PSTs. We are in the process of characterizing the culturable bacterial flora of these cultures using standard microbiological methods complemented by molecular approaches, utilizing 16S rRNA gene cloning. Preliminary results indicate a diverse bacterial flora in *G. catenatum* cultures and that the majority of bacterial 16S genotypes are represented among our cultured bacterial isolates, suggesting that most, if not all, of the bacterial types are culturable. The bacterial diversity present in typical PST-producing cultures also appears higher than that of the PST-depauperate and "non-toxic" strains, supporting the hypothesis that laboratory isolation and germination may exclude some bacterial types that are important for PST synthesis and "normal" cell physiology (Green & Bolch, unpubl. data). In addition, by surface sterilizing resting cysts prior to germination (H_20_2, Bolch & Hallegraeff 1993), we can remove all extra-cellular bacteria and germinate the cell under sterile conditions. Bacterial re-addition experiments with these sterilized cysts indicate that certain members of the bacterial assemblage must be present at germination for germing cell survival and PST production of resulting cultures.

Dinoflagellate cysts in sediments: some insights into biogeography and past, present, and future blooms

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Introduction

Approximately 10% of marine dinoflagellate species produce resting cysts. Many have cyst walls incorporating calcareous or sporopollenin-like material that is extremely resistant to biological and chemical degradation. This allows the empty cysts to accumulate in bottom sediments, ultimately providing the fossil record of the group. Dinoflagellate cysts are one of the most important groups of microfossils used for dating, well-correlation, and palaeoenvironmental interpretation of rock samples in geological exploration (e.g. for oil and gas). This has stimulated the need to understand more about the ecology of living cysts, e.g. as a basis for developing the use of fossil cysts as more precise palaeoenvironmental indicators.

Biogeography

Global studies of the distribution of living and recent cysts have shown these to be particularly useful indicators of biogeography. The cyst assemblages in the upper few cm of surface sediments represent an integrated record from several years of plankton. As such, they provide biogeographic information for these species that would otherwise be difficult or practically impossible to obtain by conventional plankton studies, given the large amount of sample coverage needed. The results of cyst surveys show that at least many of the cyst-forming species occupy the standard biogeographic zones in the world ocean previously established largely for benthic organisms. This is presumed to reflect a life cycle in these species incorporating both a planktonic phase and a benthic phase (cyst) that effectively "anchors" the species to a preferred environment, comparable to mollusks or attached macro-algae, also with planktonic life cycle stages (Dale 1983; 1996).

Ecological signals

Recent cyst distributions have also allowed us to identify ecological signals (i.e. recognition from the cyst assemblages of certain environmental parameters) potentially useful for palaeoenvironmental interpretation. These include cyst signals (summarized by Dale 1996) of: seawater temperature, salinity, coastal versus oceanic waters, and productivity (e.g. ocean upwelling and coastal eutrophication). In well-dated cored bottom sediments, this in turn allows us to apply cyst analysis to investigate the history of marine pollution and eutrophication on time scales of tens to hundreds of years (Saetre *et al*. 1997; Dale *et al*. 1999; Matsuoka 1999).

History of past blooms

The important HAB species of dinoflagellates producing fossiliseable cysts include: *Gymnodinium catenatum*, *Pyrodinium bahamense, Gonyaulax polyedra*, *Protoceratium reticulatum*, and a species of *Protoperidinium*. For these and closely related species, studies of cysts archived in sediments provide insights into the history of blooms (e.g. the *G. catenatum*-like *G. nolleri*, currently found as a small remnant population in the Kattegat, but with previous massive "blooms" around 1000 and 6000 years ago (Dale *et*

al. 1993; Dale & Nordberg 1993; Thorsen *et al*. 1995). Similarly, they have been used to develop supportive evidence of recent introduction of HAB species into regions (e.g. *G. catenatum* in Australia presumed to be introduced from ships' ballast water, McMinn *et al*. 1997). In joint research between our group in Oslo and Dr. R.V. Azanza's group in Manila, The Philippines, we are currently using cyst analysis in sediment cores to explore the possible links between cultural eutrophication and HABs of *P. bahamense* in Manila Bay (Azanza *et al*., work in progress).

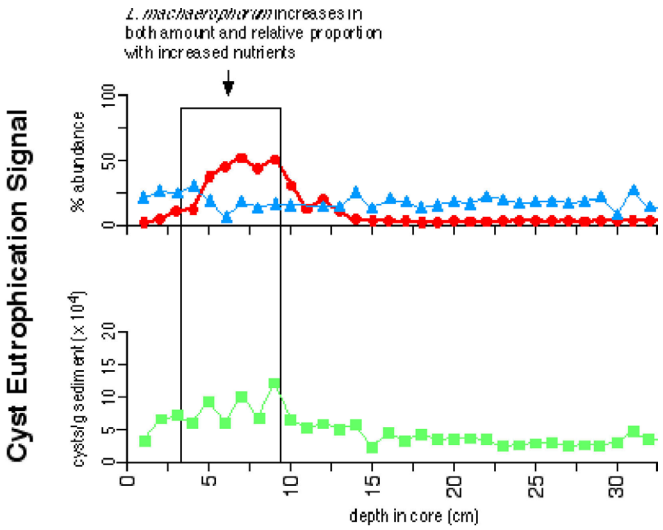


Fig. 10. Diagramatic representation of the cyst eutrophication signal from the Oslofjord (Dale *et al*. 1999); box shows period of eutrophication from roughly 1900 to 1980. *L. machaerophorum* (circle symbol) is the palaeontological name for the cyst of *Gonyaulax polyedra*, the species showing marked increase accounting for much of the increase in total cysts/g sediment with eutrophication. In contrast, *Pentapharsodinium dalei* (triangle symbol) showed no particular increase. This difference is considered to reflect the fact that the Oslofjord is naturally a nutrient limited system in which the summer blooming species such as *G. polyedra* are limited to the nutrients remaining after the spring bloom of diatoms and colder water dinoflagellates such as *P. dalei*. The effect of nutrients from sewage effluent added throughout the year therefore increased possibilities for larger blooms of the summer species, while the spring/early summer species which were previously not limited by nutrients remained unaffected.

Future blooms

Information from the sedimentary record on the history of blooms, together with the ecological information from recent sediments, provides the most comprehensive basis for estimating some aspects of future blooms (e.g. in response to expected increases in cultural eutrophication and climatic warming). Work on the Oslofjord (Dale *et al*. 1999) suggests that cultural eutrophication could cause increased blooms in the future, particularly of summer blooming species in nutrient-limited systems of the temperate zones or higher latitudes. The species most affected in the Oslofjord was *Lingulodinium*

polyedrum, suspected to produce YTX toxins, but other species may be expected to increase their blooms in other regions (Dale 2001).

Knowledge of the temperature signals identified from recent cyst distributions helps us to estimate the predicted changes to be expected from global warming (Dale 2001). These most likely will involve marked increases particularly in the warmer-water species occurring towards their colder limits in temperate zones and higher latitudes (e.g. comparable to the historic and prehistoric "blooms" documented in the Kattegat). Knowledge of the biogeographic zones mapped out from recent sediment studies should allow us to identify the cyst forming species likely to increase. One of the most pronounced responses to be expected from environmental change, including climatic change, is increased blooms of the cosmopolitan *P. reticulatum* (considered to be an opportunistic response), a species known to produce YTX toxins.

Our current work from the Norwegian coast shows that we may also expect some *unexpected* effects. The first response seen from the past decade of warming was a marked increase in the colder water species *Pentapharsodinium dalei.* This almost certainly reflects the fact that the measured warming in sea surface temperatures from the southern coast of Norway during the past decade involved only an increased *winter* temperature. Summer temperatures are so far not markedly affected. This in effect extends the temperature window for the colder water spring blooming species such as *P. dalei*, allowing more extensive blooms of the vegetative cells, and a correspondingly larger contribution of cysts to the sediments (Dale 2001).

Temporary cysts in dinoflagellates

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Introduction

Temporary cysts have been considered non-motile stages formed when motile, vegetative cells are exposed to unfavourable conditions such as mechanical shock, or sudden changes of temperature (Table 2). Alternative terms can be found in the literature to define these stages, such as pellicle cysts, ecdysal cysts (Dale 1983). They have frequently been observed in laboratory cultures and also occasionally recorded in natural plankton samples, although it is difficult to ascertain whether the cysts were naturally present in the sample, or were formed following the stresses derived from sampling and/or fixation procedures.

Studies on temporary cyst/stages are very scarce and there are several open questions concerning their role in dinoflagellate population dynamics: what are the morphological and physiological characteristics of a temporary cyst? Are we using the same name for functionally different stages? How can we properly plan *in situ* studies to better understand the role of temporary cysts? What are the factors triggering transitions between motile cells and temporary cysts? Consequently, the role of temporary cysts in population dynamics has not even been taken into account.

In the framework of a monitoring programme aimed at the detection of HAB species, we had the opportunity to study *in situ* temporary cyst formation by *Alexandrium taylori* and *Alexandrium catenella*, and to get insights into the possible role that these stages have in the species life cycles.

Alexandrium taylori

We studied recurrent blooms of *Alexandrium taylori* that occur every summer along the Spanish coasts since 1995, reaching cell concentrations up to 10^7 cells·L^{-1}. *A. taylori* showed *in situ* daily vertical migration, shifting from a motile stage at the water surface to a non-motile stage in the sediments. Most vegetative cells lose their thecae and flagella and thus motility, turning into temporary cysts that settle on the bottom in the early evening. The number of temporary cysts in the water column rose in the evening and at night. The temporary cysts gave rise to motile cells the following morning. In this species, temporary cysts have the function of 'division cysts' that is the stage in which vegetative division occurs.

Temporary cyst formation in this species can represent a means for reducing population losses. High concentrations of temporary cysts (10^3-10^4 cysts g^{-1}) were recorded in the sediments during the bloom maintenance period and the formation of clusters of cysts, reaching concentrations up to 10^6 cells·L^{-1}, was often observed in the water layer close to the sediment. Encystment probably assists *A. taylori* to withstand short or low-intensity perturbations, for instance storms or swells, and restore the population densities after a few days of calm weather, during the beginning and maintenance of the bloom.

Table 2. Dinoflagellate species for which the formation of temporary cysts or pellicle cyst is reported.

Genus	Species	Field/Culture	Surrounding conditions	Reference
Alexandrium	*catenella*	F/C	Parasite attack	Delgado 1999 Vila pers. comm.
	hiranoi	F	Stage of the life cycle	Kita *et al*. 1985; Kita *et al*. 1993
	minutum	F/C		Garcés per. comm.
	pseudogonyaulax	C		Montresor 1995
	ostenfeldii	C	Ageing of cultures	Jensen & Moestrup 1997 Østergaard & Moestrup 1997
	tamarense	C	Deficiencies in specific nutrients, Changes in temperature	Anderson & Wall 1978 Fritz *et al*. 1989 Doucette *et al*. 1989 Schmitter 1979
	taylori	F	Stage of the life cycle	Garcés *et al*. 1998; Garcés *et al*. 1999; Giacobbe & Xang 1999
Amphidinium	*carterae*	C		Sampayo 1985
	klebssi			Sampayo 1985; Barlow & Triemer 1988
Ceratium	*hirundinella*			Chapman 1982
Coolia	*monotis*	C	Induction by bioactive compound from the green alga *Bryopsis* sp.	Sakamoto *et al*. 2000
Disodinium				Elbrächter & Drebes 1978
Gambierdiscus	*toxicus*	C	Induction by bioactive compound from the green alga *Bryopsis* sp.	Taylor 1979 Sakamoto *et al*. 2000
Glenodinium	*foliaceum*	C		Bricheux *et al*. 1992
Gymnodinium	*catenatum*		Stage of the life cycle	Blackburn *et al*. 1989
Gonyaulax		F		Alldredge *et al*. 1998
	polyedra	C	Lower temperature and light	Behrmann & Hardeland 1995
Heterocapsa	*circularisquama*	C	Bacterial attack	Nagasaki *et al*. 2000
		C	Contact cells in bialgal cultures	Uchida *et al*. 1996; Uchida *et al*. 1999; Nagasaki *et al*. 2000
		C	Virus attack	Tarutani *et al*. 2001
	triqueta	C		Olli pers. comm.
Peridinium	*quinquecorne*			Hallegraeff *et al*. 1995
	volzii		Environmental stress	Manjarres & Fritz 1999
	inconspicuim		Environmental stress	Manjarres & Fritz 1999
Prorocentrum	*lima*	C	Induction by bioactive compound from the green alga *Bryopsis* sp.	Sakamoto *et al*. 2000
Prorocentrum	*minimum*	C	Changes in temperature	Grzebyk & Berland 1996
Pyrodinium	*bahamense* v. *compressum*			Usup & Azanza 1998
Pyrocystis				Elbrächter & Drebes 1978

The formation of temporary cyst aggregates could further contribute to avoid population losses from the area as Margalef (1997) has suggested for cell aggregations. The production of temporary cysts can be an advantage since in this way a stock of the population is stored in the sediments. In fact, about 30% of the temporary cyst population in the sediment did not divide within a 24-hour period. We demonstrated that temporary cysts remain viable for one month from *in situ* samples. We still do not know if temporary cysts could survive for longer in the sediments and constitute the inoculum for the following bloom. Temporary cysts are a good strategy to overcome short-term fluctuations in environmental conditions and their role has probably been underestimated due to the lack of sampling.

The relatively short 'lag' period between temporary cyst formation and formation of vegetative cells allows for a rapid shift between benthic and planktonic stages of *A. taylori*. Preliminary data on temporal variability of temporary cyst concentrations in the sediments indicate that encystment and excystment rates vary considerably during the different bloom phases (beginning, maintenance and end) (Garcés, in press). However, there is a notable methodological problem in correctly measuring encystment and excystment fluxes and in estimating the spatial heterogeneity of temporary cyst distribution in the sediments. Probably environmental conditions modify encystment and excystment fluxes, but in the same sense that they modify cellular division, since daily encystment in *A. taylori* is part of its cellular growth.

Temporary cysts could also act as dispersal vectors for the population. Floating plastic debris has frequently been observed at La Fosca during the summer and the fragments of plastic were covered by vegetative cells and temporary cysts of *A. taylori.* These temporary cysts are sticky, and this characteristic facilitates both the formation of aggregates and the attachment to surfaces that could act as dispersal vectors.

Alexandrium catenella

In *Alexandrium catenella,* the formation of temporary cysts was observed in natural samples infected by the parasite *Parvilucifera infectans* in Barcelona Harbour during summer 1998. Three different stages were observed in the population: vegetative motile cells, temporary cysts and parasitized cells. Temporary cysts accounted for 66% of the total population of *A. catenella* reaching concentrations up to 6.5×10^4 cells·L^{-1}. Three weeks later, a bloom of this species was recorded in the harbour and no parasitised cells were detected. During the bloom, temporary cysts accounted only for 17% of the total population (Vila, pers. comm.). Parasite infection was also observed in cultured *A. catenella* cells. In culture experiments, it was confirmed that the parasite does not infect temporary cysts Delgado (1999) and the dinoflagellate *Alexandrium taylori* remained resistant to infection after several attempts in cultures. In the case of *A. taylori,* we do not have evidence that the parasite triggers the formation of temporary cysts but its formation could be an advantage. In the case of *A. catenella*, it is tempting to speculate that the formation of temporary cysts can be triggered by external biological factors such as parasites. Cell contact and virus attack is a possible mechanism to cause temporary cyst formation, as Uchida *et al.* (1999) and Tarutani *et al.* (2001) showed, respectively, in the dinoflagellate *Heterocapsa circularisquama.*

Data on encystment and excystment rates in dinoflagellates.

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Introduction

The life cycles of some dinoflagellates involve sexual reproduction with the formation of resting cysts (Dale 1983). Cyst formation and cyst germination (encystment and excystment) in dinoflagellates are not simple, single step processes. I believe there is merit in deconstructing these processes and considering data on each of the elements. We might use such data to improve our understanding of the processes involved in an individual species, to provide comparative information across species and ultimately to generate and parameterise models. Each of these purposes requires a different standard of rigour. For example, if we are merely concerned with one species, we can generate data in any form that is consistent across our dataset, for that species. However, when we wish to compare species and formulate models, in order to generate a deeper understanding of the wider implications of the processes, then data has to be in the same form across the species addressed. Therefore in this review, I aim to define the different steps in the encystment, excystment processes; to consider the data available for each of these and to generate discussion regarding standardization of methodology and calculation which I believe will facilitate comparison and modelling across the field.

If we break down the processes of encystment and excystment fully, the following represent the detailed data required to describe the process:

Gametes

- Factors that induce gamete formation
- Proportion of the population forming gametes*
- Sinking/Swimming rate of gametes*
- Survival time of gametes*
- Factors affecting mating (e.g. including behaviour to form aggregations, gametic recognition)*
- Proportion of gametes that mate successfully (including consideration of reversion to vegetative state)*
- Time taken to mate*

Planozygotes

- Survival time of planozygotes
- Sinking rate of planozygotes*
- Factors affecting encystment
- Proportion of planozygotes that encyst*
- Time taken to encyst*

Cysts

- Sinking rate
- Dispersal/transport/burial*

- Factors that affect length of mandatory dormancy period
- Mandatory dormancy period
- Predation rates*
- Cyst mortality/survival
- Factors that affect excystment (including endogenous rhythms)
- Proportion of cysts that excyst

Planomeiocytes

- Behaviour (including consideration of swimming/sinking rates, phototaxis)*
- Factors affecting successful vegetative transformation*
- Proportion of successful conversion to vegetative cells*

In the literature, the availability of such detailed data is patchy; those marked by asterisks above have little or no information reported for them. Most often several of these steps are conflated and we are provided with data that do not pick out the subtleties of the process. It may be that such a detailed analysis is too demanding (or impractical with current techniques) but it may, nevertheless, be important.

So these are the details we might like to have – what are we faced with in reality? I will briefly run through some of the data available, largely from laboratory studies. In order to generate this overview, I used some 70 papers, not all of which are reported here – more detailed analysis will be presented elsewhere.

Investigations of encystment rarely pass any comment about gametes. In some species, gametes are not easily distinguished from vegetative cells and it seems possible that gametogenesis may merely be effected by an internal switch within vegetative cells (for examples of detailed studies see Coats *et al.* 1984; Xiaoping *et al*. 1989). Times taken for gamete fusion are rarely reported. However, there are reports of times taken for planozygotes to proceed to encystment. These are usually of the order of 1-2 weeks (Anderson *et al.* 1983; Turpin *et al*. 1984; Sako *et al*. 1984; Anderson & Lindquist 1985; Blackburn *et al*. 1989) and rarely as long as 4 weeks (Park & Hayashi 1992). The formation of cysts, as opposed to reversion to vegetative cells, may be dependent on cell density (Uchida 2001). Actual cyst formation has rarely been observed but has been documented to take as little as 10-20 minutes (Kokinos & Anderson 1995; Lewis & Hallett 1997).

Data are available on factors that "induce encystment"; the most frequently cited factors are nutrient (N and P) deficiency (Pfiester & Anderson 1987). However, there is 'conflicting' evidence from laboratory and field studies that probably reflects methodological problems centred around the scale of nutrient measurements, the difficulty of determining the nutrient status of cells and identification of gametes (Probert, this volume). In one instance, iron depletion was cited as a trigger for encystment (Blanco 1995a). Other factors have also been documented to influence the process: temperature and salinity (e.g. Wall *et al*. 1970); day length and temperature (e.g. Sgrosso *et al*. 2001); bacteria (Adachi *et al*. 1999); density of cells (Uchida 2001). One can hypothesize that nutrient stress triggers gamete formation and, once formed, gamete survival, mating success and so on are modified by the conditions in which gametes find themselves. Investigations of encystment have been carried out on a variety of species. Most are autotrophic species that are of interest because of their potential as HAB formers or are those species that are easy to manipulate and culture in the laboratory. It is worth noting that there is information concerning only one

heterotrophic species that apparently encysted spontaneously (Morey-Gaines & Ruse 1980).

Another parameter that has been reported regularly is "cyst yield" (the proportion of the vegetative population that forms cysts). There is more than one way of calculating this (using data on vegetative cell population and cysts produced). A straightforward proportion of cysts to vegetative cells can be used (Ichimi *et al*. 2001). However, a more realistic calculation takes into account that two cells fuse to form a cyst ($2N_{cysts}$ / $2N_{cysts}$ + $N_{veg.\ cells}$) (e.g. Anderson *et al*. 1984). A further difficulty is that some authors do not report how they have calculated their data. Cyst yields of up to 100% have been reported (Sgrosso *et al*. 2001; Olli, this volume).

Cyst dormancy periods have been investigated for a range of species in the laboratory. Of the possible factors that might affect this, temperature has been the most frequently investigated. Intriguingly, it would seem from these results that the length of dormancy of one species varies with the geographic population under consideration (Table 3; Hallegraeff *et al*. 1998). Likewise, where temperature effects are observed, they were often linked to the environment from where the population originated. There is another point of standardization that can usefully be considered here - most investigators report length of dormancy to the point where the first cyst germinates. Binder and Anderson (1987) introduced the use of a median germination time (the time required for 50% germination to occur), which was endorsed by Hallegraeff *et al*. (1998), and is perhaps a better quantitative measure of this parameter.

Table 3. Excystment results for *Alexandrium tamarense* from different areas of the world. F = field-collected material; C = cultured cysts; nd = no data.

Source	Storage	Dormancy (months)	% Germination	Reference
Hiroshima Bay	5°C (D)	1	85-98	Adachi *et al*. 1999
Cape Cod (F)	5°C (D)	4-6	50-70	Anderson 1980
	22°C (D)	1-3	65-90	
Gulf of Maine (F)	4-6°C (D)	4		Dale *et al*. 1978
St. Lawrence Estuary (F)	4°C (D)	12	82-99	Perez *et al*. 1998
British Columbia (C)	17°C (L)	2		Turpin *et al*. 1978
Seto Inland Sea (C)	13°C (L)	10		Yoshimatsu 1984

Sporadic records are available concerning cyst survival (reviewed by Lewis *et al*. 1999). These survival times have to be regarded as minima, as they reflect the time period of the experiment. Most are from laboratory material and it is not clear that these survival times will be the same in the environment. Indeed, two records from field samples are among the highest recorded (Huber & Nipkow 1923). Indirect estimates from sedimentological data support field survival times of at least these values (Keafer *et al*. 1992). It may be more appropriate to use a median survival time (the time required for 50% mortality of cysts) in order to provide data on the rate of decay of the population.

Germination conditions have been investigated for some 20 species. Where there has been detailed investigation, it seems that some oxygen is required for cyst germination (Anderson *et al.* 1987; Rengefors & Anderson 1998; Kremp & Anderson 2000). Temperature also plays a key role and is the most frequently investigated variable. Studies have shown that light (e.g. Anderson *et al*. 1987), presence of predators (Rengefors *et al.* 1998), and endogenous rhythms (e.g. Anderson & Keafer 1987) can affect excystment. Considering these data, and relating them to the natural environment, it is clear that opportunity for many species to excyst exists at times of the year when they do not occur in the water column. Endogenous rhythms seem likely to be important in this respect and we can deduce that there must geographic variability in populations of widespread species (Rengefors & Anderson 1998).

The proportion of cysts that germinate in laboratory studies is variable. Many reports have high final excystment rates (80-100%). However, in the field these rates will be tempered by the age of the cysts (and hence their viability) and their environmental opportunity (e.g. if they are buried in anoxic layers of the sediment they will not germinate).

Very few studies have considered the conditions affecting the survival or success of the planomeiocyte. Anderson & Wall (1978) showed for *Alexandrium tamarense* that light and increased chelation improved survival. More recently, Kremp (2001) also showed that for *Scrippsiella hangoei* and *Peridiniella catenata*, light was important in survival and, counter intuitively, for *P. catenata* turbulence improved survival. This is an important but neglected area. In my experience in the laboratory, germination is no guarantee of success; cysts will excyst under conditions where subsequently the planomeiocyte will perish.

Conclusion

To summarize – much remains to be done! It is clear looking at the literature, that it is dangerous to generalize across species. Data need to be collected for each species under consideration and possibly for each geographic population under consideration (e.g. Table 3). I also believe more attention needs to be addressed to the detail of the processes. I look forward to new molecular techniques giving us a better handle on determination of life cycle stage and physiological status of cells, to move our understanding forwards.

High encystment of a dinoflagellate in batch culture

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Introduction

Sexual cysts have been reported in many dinoflagellate species (Pfiester & Anderson 1987). It is likely that the list will continue to grow as more observations are made, both from cultures and natural waters. One of the obscure aspects of dinoflagellate (and other protists) encystment has been the quantitative importance of sexuality in a population. The majority of the studies has shown the maximum encystment rate to be ca. 10 - 20% (e.g. Anderson *et al*. 1984; Binder & Anderson 1987; Montresor & Marino 1996).

Recently, I started a culture from a single resting cyst of *Scrippsiella* cf. *lachrymosa*, where after a vigorous vegetative growth almost all the cells encysted. Final cyst yield in batch cultures with f/2 medium (880 µM NO_3; 36 µM PO_4) exceeded 10^5 cysts·mL^{-1} and hardly any other cell stages were found.

This finding raises numerous questions. If a dinoflagellate population can completely encyst in a culture, can we assume a similar high encystment potential in natural conditions? Was the particular species/culture extraordinary, or is 100% encystment common place in nature? There is no definitive answer at present, but 100% encystment potential of natural dinoflagellate populations is feasible in the light of my finding. Possibly, this high rate can only be approximated in nature, as other losses from the vegetative population (e.g. grazing, viral lysis) cannot be excluded.

If this is true, what is the reason for the hitherto reported low encystment success, both in natural waters (Kremp & Heiskanen 1999), and in cultures? First, estimating encystment success (i.e. relating cyst production to the abundance of the vegetative population) is notoriously difficult in natural conditions, largely because of logistical and methodological constraints (sampling design). On the other hand, even a population with 100% encystment potential may only be able to fulfil a fraction of this because of environmental conditions.

The recent history of success with cultured dinoflagellate encystment experiments is largely a history of developments in culture techniques. Thus, my high encystment rate might be a sign of a perfect match between the culture conditions and the requirements of the particular species, rather than a sign of an "unusual" strain.

High encystment was achieved in cultures started from f/2 medium, not in low nutrient "encystment medium", as commonly used to induce cyst production (von Stosch 1973; Coats *et al*. 1984). Did encystment occur in high nutrient conditions? The evidence does not support this. Mineral nutrient measurements from aged cultures revealed only low nutrient concentrations (0.02 - 0.5 µM PO_4 and NO_3). Dividing the initially available nutrient amount to the final yield of cells revealed low cellular nutrient quota of 4.6 pmol N and 0.19 pmol P, in extreme cases (when a specific nutrient was supplied in shortage), even as low as 2.5 pmol N and 0.09 pmol N. These quota are indirect estimates, and probably reflect upper values, as we cannot exclude the possibility that significant amounts of N and P were bound to the dissolved organic fraction. Compared to the few published papers on nutrient quota of dinoflagellates and cysts (Anderson & Lindquist 1985; Lirdwitayaprasit *et al*. 1990; Rengefors *et al*. 1996; Rengefors *et al*. 1999), these indirect estimates are in the lower range. Although not

conclusive alone, low residual nutrient concentration and low cellular nutrient quota suggest that encystment proceeded in nutrient deplete conditions.

This leads to yet another puzzling question: if gametes are formed when nutrients are depleted in batch cultures, how then does the planozygote obtain sufficient resources to complete the transition to cysts, to support prolonged dormancy, quiescence, germination and growth? Anderson (1998) has proposed that possibly only the first planozygotes to form are able to complete the transition to cysts, perhaps because they are able to take up additional nutrients before concentrations become too low in the batch culture to permit significant uptake. Only those with adequate nutrients were then able to complete the transition to cysts. This could explain the often observed large planozygote population in cultures, the majority of which fail to encyst (Coats *et al*. 1984; Anderson *et al*. 1985). Encystment was completed by all the planozygotes in our study; perhaps the nutrient depletion dilemma was bypassed by forming smaller sized cysts in later stages of the batch culture.

We found a clear morphological difference in cysts produced at different stages of the culture. Cysts produced at earlier stage were larger (2500 μm^3) and with a strong calcareous cover (Fig. 11). Cysts produced at a later stage had a thinner calcareous cover and, ultimately, no distinct cover at all. Parallel to the disappearance of the calcareous cover, cysts produced at later stage of the culture were smaller in size (700 μm^3; see also Table 4).

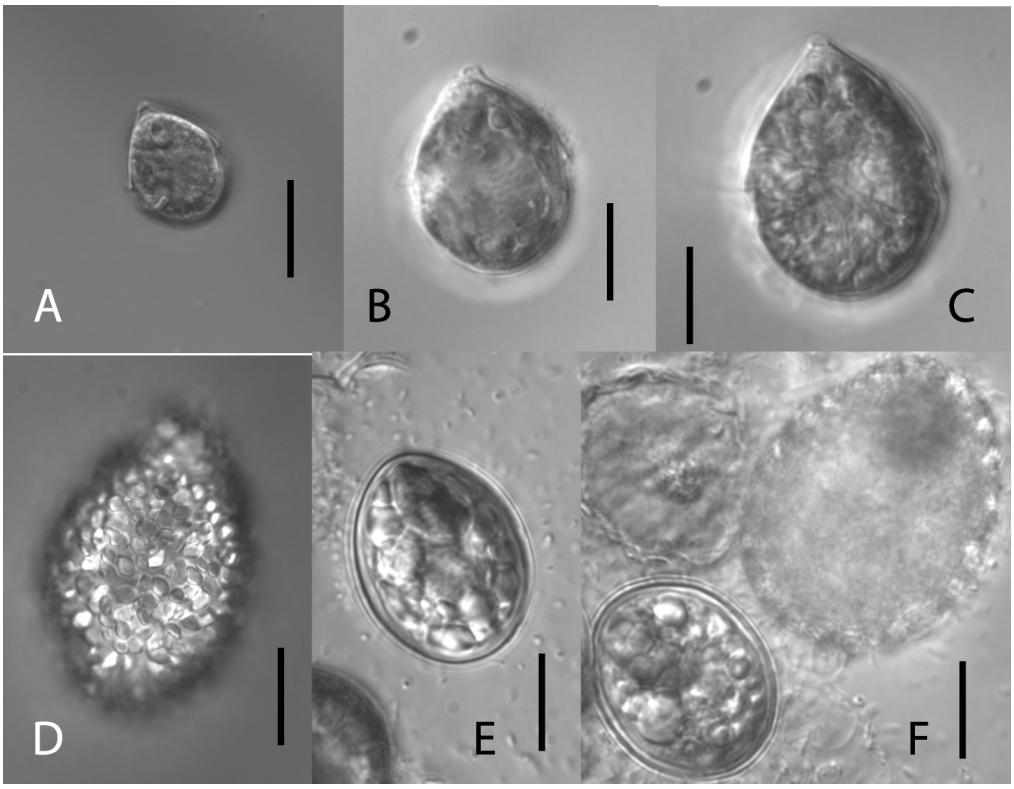


Fig. 11. Cultured cells of *Scrippsiella* cf. *lachrymosa*. (A) A small cell, probably a gamete; (B) Vegetative cell; (C) A large cell, probably a planozygote; (D) Calcareous cyst formed in the early phase of the culture growth; (E) Cyst formed in a later phase of the culture growth, no calcareous cover; (F) Two cysts demonstrating the variability in size.

Table 4. Change of the cyst length and breadth (median; range in parenthesis) from the first day (Day 4) when cysts appeared in batch culture (25 mL volume in 30 mL borosilicate glass tubes) to late phase with complete encystment (Day 52).

Day	Cyst length (µm)	Cyst breadth (µm)	n
4	46 (36 – 53)	30 (23-37)	59
52	30 (23 – 35)	21 (16-26)	94

It is tempting to speculate that the smaller size of the cysts reflects nutrient (N, P) depletion during encystment; i.e. as the nutrient concentration became too low in late phase of batch culture to permit significant uptake, the cells were still able to complete the transition to cysts, but not able to retain the original cyst size. Similarly, the concentration of Ca ions in natural marine waters, not considered limiting for phytoplankton growth in general, might run low in cultures where high concentrations of calcareous cysts are produced. Interestingly, germination experiments revealed that the small and "naked" cysts produced at later stages did not have lower germination rate compared to larger cysts with strong calcareous covers. Also, the flagellated cells of *S.* cf. *lachrymosa* were polymorphic. Gametes were notably smaller (Fig. 11), exhibited a different, "restless" swimming pattern, and were often present in considerable numbers (Fig. 12). Planozygotes were larger (Fig. 11), but never present in large numbers, suggesting that this stage had a short duration.

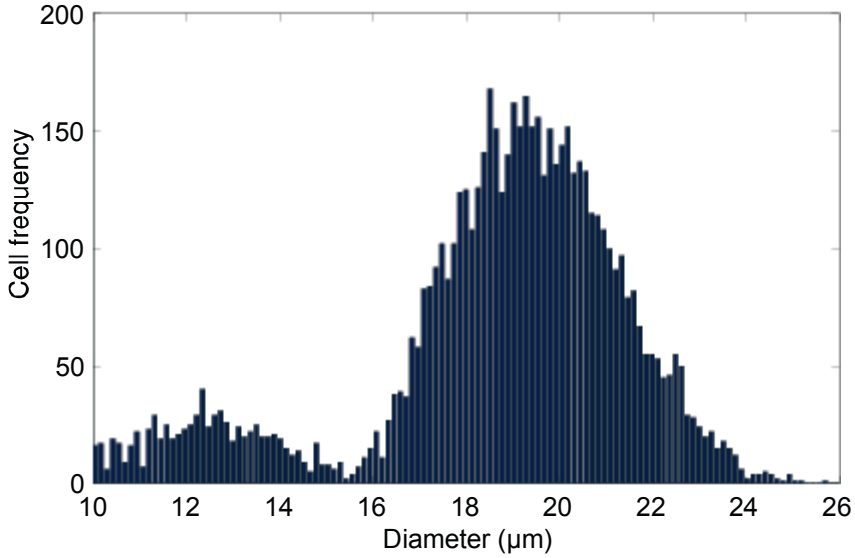


Fig. 12. A typical size distribution (measured with a Coulter Counter as equivalent spherical diameter) of motile cells in a batch culture during gamete formation. The lower peak centered around 12 µm depicts the gametes; the larger peak vegetative cells.

Conclusion

If high encystment success could take place in culture, it is tempting to suggest that it can also be commonplace in nature. The ability of any particular culture to produce cysts in a high nutrient medium might depend on its potential to grow to very high densities that inevitably lead to nutrient depletion, not on its ability to reproduce sexually at high nutrient conditions. Some morphological traits of cysts, e.g. calcareous cover and cyst size, are not conservative attributes. Cyst volume can change by a factor of 3.5, with no decrease in germination success.

The induction of sexual reproduction in dinoflagellates: culture studies and field surveys

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Introduction

The majority of research relating to specific dinoflagellate sexual stages concerns the control of excystment and thus understanding of bloom initiation (e.g. Anderson & Morel 1979; Anderson *et al.* 1983). Despite an increasing realization of the significance of the onset of sexuality and the subsequent cyst formation process in contributing to bloom decline, the controlling factors and physiological mechanisms involved have not been clearly defined and conflicting information exists from laboratory and field observations.

The majority of evidence from published accounts of laboratory culture experiments suggests that dinoflagellate sexuality is induced under the relatively specific condition of limitation of one or both of the major micronutrients, nitrogen (N) and phosphorus (P), with initial indications suggesting that gametogenesis may be triggered when the intracellular concentration of the limiting nutrient falls to a threshold level (Anderson 1983; Anderson & Lindquist 1985; Anderson *et al.* 1985). Dinoflagellate sexual stages have, however, been observed without any apparent nutrient stress in several culture studies and in a number of field observations (e.g. Anderson & Morel 1979; Anderson *et al.* 1983). This contribution gives an overview of our investigations into the link between internal nutrient physiology and the induction of sexual reproduction in the toxic dinoflagellate *Alexandrium minutum*, and highlights some of the important questions which have arisen in the course of this study.

Culture studies

Detailed observations revealed that *A. minutum* gametes are morphologically and morphogenetically indistinguishable from vegetative cells, but planozygotes can be identified on the basis of size. The timing and extent of gamete formation was estimated by calculation of the 'gamete index', an extrapolation based on measured planozygote abundances and knowledge of the average duration of gamete fusion. The problem of accurately and efficiently identifying different life stages is common to the study of most microalgal classes. Our observations suggest that gamete attachment in *A. minutum* is initiated by a flagellar attachment process similar to that observed in *Scrippsiella trochoidea* by Gao *et al*. (1989), suggesting the presence of a cellular recognition system involving agglutins similar to those found in *Chlamydomonas* (reviewed by Mitchell 2000), and thus highlighting potential lines of research involving the application of molecular and flow cytometric techniques. Tests in culture of potential promoters or inhibitors of dinoflagellate gamete formation, attachment or mating may provide further insights into the sexual induction process. Our experimental culture strain of *A. minutum* is homothallic and crossing experiments did not reveal any evidence for heterothallism (i.e. no significant increase in zygote formation in crossed clones), although other *Alexandrium* species have been reported to exhibit heterothallism (Destombe & Cembella 1990). Mating systems are probably much more complex phenomena than we realize at present, and detailed studies are

required across the phylogeny of dinoflagellates to determine the mechanisms, the evolutionary pattern and the ecological relevance of mating system types. Only by obtaining such information will we be able to address intriguing questions such as whether, for example, homothallism may have been selected for in species which inhabit environments in which blooms cannot (for whatever reason) be sustained for extended periods, requiring a high ratio of gamete encounter / successful fusion in order to complete the life cycle.

As in previous reports, evidence from our batch and semi-continuous culture experiments suggests sexual induction was linked to intracellular nutrient status declining to a threshold level. P stress was always accompanied by N stress, even in the presence of excess N nutrients, suggesting that in this species sexuality may be induced by a single mechanism linked to the depletion of internal N, or one of its components, to the threshold level. None of the classical nutrient stress indices employed proved to be reliable markers of a particular nutrient status at which sexuality might be induced, possibly due to the concomitant effects of other nutrient stresses.

Whereas internal nutrient physiology clearly plays a role in triggering the sexual process, the response to this trigger is not a simple one. For example, overall metabolic state apparently influenced the sexual response, high growth rates in recent metabolic history favouring high rates of sexual reproduction. In addition, gamete activity seems to be a reversible process, being inhibited by prolonged internal nutrient starvation, and deactivated upon alleviation of internal nutrient-stress above the threshold level. Significant progress in our understanding of the molecular and biochemical pathways involved in gamete formation and fusion is needed in order to understand the basis of these variable responses to the induction trigger.

Bloom dynamics

The two *A. minutum* blooms surveyed in the Aber Wrac'h and Penzé estuaries (northern Brittany, France), in 1995 and 1997, respectively, were initiated by *in situ* excystment around the time of large spring tides. Based on cell counts early in the blooms and enumeration of viable cysts in surface sediment layers, inoculum motile cell concentrations were concluded to have been low. This conclusion, of particular relevance to our interpretation of the ecology of this species which forms concentrated beds of long-lived cysts, requires more detailed experimental validation. Technological advances towards the accurate measurement of *in situ* excystment rates would be of outstanding value in addressing the actual role of cyst beds in the ecology of cyst-forming organisms.

The competitive ability of *A. minutum* during early population development is dependent largely on the extent of water column stratification (and thus water temperature and river-flow velocity), with grazing and advection both potentially important limiting factors. In bloom years, calculated *A. minutum* growth rates in the estuaries exceeded maximal growth rates in nutrient-replete culture (all growth rates calculated from daily cell counts). Since estimations of growth rates in open-system natural conditions are open to error, sampling strategies including the possible use of alternative techniques for measurement of growth rates must be closely evaluated. In order to more accurately simulate natural conditions, advances in culture techniques are also clearly required.

Both active (behavioural) and passive (associated with hydrology) concentration of populations contributed to extremely rapid accumulation of cells leading up to the

bloom maxima. The induction of gametes (estimated by calculation of the gamete index), linked to declining absolute intracellular N status, occurred relatively early in population development, presumably due to physiological nutrient stress (the reduction in cellular nutrient quota caused by cell division not being fully compensated by nutrient uptake between divisions). In this context, the problem of accurately defining physiological state of a particular species early in bloom development in a mixed population needs to be addressed. According to our theory, in the presence of excess aqueous nutrients, the reversibility of gamete induction allows continued vegetative growth, the continuing effects of physiological nutrient stress keeping cellular N levels close to the gamete induction threshold level. Upon concentration of cells, gametes are thus present at the time when inter-cell encounters will be highest. Despite very high population densities, the threshold density for gamete encounters (Wyatt & Jenkinson 1997) was not reached; micro-scale aggregation of cells undoubtedly occurs, but how can we accurately measure this phenomenon and is it a result of inter-cell chemical signalling? In the context of the use of indirect estimations of gamete numbers based on planozygote counts, further investigation of possible differential migration patterns between different life stages is essential.

Grazing and advection are not considered to have contributed greatly to the very rapid decline in *A. minutum* cell numbers following the bloom maxima. A previously unknown parasite of *A. minutum*, capable of very high efficiency of host infection in culture conditions, was discovered (Erard-Le Denn *et al.* 2000). The parasite was not thought to have had a major influence on bloom decline, but future *in situ* investigations are necessary to validate this conclusion. Estimations based on cumulative zygote formation rates indicate that the transfer to sexual reproduction and subsequent encystment were the dominant factors causing rapid termination of both blooms surveyed. Improved technology for accurate measurement of cyst deposition would be useful in this context.

Since in *A. minutum* gamete induction may be considered to be a consequence of prolonged rapid asexual growth, and rapid growth is itself a prerequisite for proliferations of bloom proportions, the motile phase can be seen to employ a highly effective strategy which maximizes the chances of success in a difficult environment, and hence ensures continued survival of the species in the area. The dinoflagellates are recognized to be in many aspects a highly diverse group, and hence detailed physiological life history studies of other dinoflagellate genera are required before generalizations about the link between nutrient physiology and life cycle transitions can be made.

Small cells in *Dinophysis* spp: a life cycle strategy for phytoplankters with a holoplanktonic way of living?

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Introduction

"Small cells" have been reported for several species of *Dinophysis*. They are easily discriminated from larger vegetative cells and have often been described as different species. Studies on *Dinophysis* life cycles have been hindered by the usual low numbers occurring in field populations and the lack of established cultures, but information has been gathered from intensive *in situ* sampling during proliferations, incubations of concentrated natural populations, and microplate incubations of isolated cells (Reguera & González-Gil 2001). In the field, small cells represent a very low percentage (1-10%) of the total population with the exception of the observations of MacKenzie (1992) in Bay of Plenty (New Zealand), where they made up to 50% of the total, and recent observations in Ria de Vigo (Reguera, unpubl. data) at specific depths and times of the day. They have been detected at the end of the exponential growth phase, or during downwelling events that may stress the cells (Delgado *et al*. 2000; Moita & Sampayo 1993; Peperzak *et al*. 1996; Reguera *et al*. 1990; 1995); but high numbers of vegetative cells are not always associated with small cells (Pazos *et al*. 2001).

Comments on the spatio-temporal distribution of *Dinophysis* spp.

Dinophysis can be detected the whole year round in the plankton if appropriate ways of sampling (nets, concentration of large volumes of water, etc.) are used. Recent protocols allow purification of DNA from one or several cells (Edvardsen *et al*. 2000b; Marín *et al*. 2001a, b) and molecular probes for *Dinophysis* species (Guillou *et al*. submitted) will constitute a valuable tool to improve detection limits of low density planktonic populations. Increases in cell numbers of *D. acuminata* are usually associated with cell aggregation in thin layers, at specific depths stratification, and co-occur with blooms of diatoms (*Pseudo-nitzschia* spp.) or dinoflagellates (*Prorocentrum micans*). Aggregation seems to trigger increases in number, vertical migration in some cases, and formation of small cells in later stages. But it is difficult to localize the precise depth of these aggregations with conventional sampling (hoses, bottles, nets, etc.), and more sophisticated devices, e.g. vertical particle profilers (Gentien *et al*. 1995) or multi-syringe vertical samplers (Gentien pers. com.; Blanco pers. com.) are essential to identify the environmental conditions where the populations thrive. It seems that active division can proceed only when scarce populations of *Dinophysis* form "high concentration layers" by interactions between the physical conditions and behaviour.

Observations from laboratory incubations

Incubations in culture plate wells (0.2 mL) of groups (10-20) of isolated cells of *D. acuminata* (Reguera & González-Gil 2001), *D. acuta* and *D. caudata* (Reguera unpubl. data) support a hypothesis based on field observations that small cells of these species (*D. skagii, D. dens* and *D. diegensis*, respectively) are the result of "depauperating divisions" *sensu* von Stoch. Several rapid divisions occur only if cells are incubated in

groups in the wells. This, and field observations, suggest *Dinophysis* cells experience an Allee effect, i.e. a threshold concentration of cells (or of their extracellular products) is required before division can proceed. Eventually, the whole population in a well would comprise small cells (up to 100-120 cells in 0.2 mL wells). Some small cells may form couplets with larger cells and undergo a conjugation-engulfment and cell fusion process (see review by Reguera & González-Gil 2001), but the scarcity of couplets in nature and in incubations do not strongly suggest that sexuality plays a major role in the life cycles of these species (Fig. 13). On the other hand, small cells can grow again to form intermediate and normal-sized vegetative cells, i.e. if they are not used in sexual reproduction, they can rejoin the vegetative population without wasting energy in cyst formation, sedimentation, germination during the next growing season and other processes. These small cells must have very different physiological requirements, and do exhibit very different swimming behaviour.

Putative planozygotes and/or hypnozygotes have been described for *D. acuminata*, *D. sacculus, D. acuta* and *D. tripos* (reviewed by Reguera & González-Gil 2001), in extremely low numbers (20 – 40 cyst·L^{-1}), during exceptional blooms of these species, but germination of hypnozygotes has not been demonstrated, nor have they been found in nearshore sediments. Occasionally, high concentrations of *Dinophysis* cells, with reddish pigmentation, are found (e.g. in Reloncavi Fjord, Chile (Clément pers. com.), near the bottom in the Baltic Sea (Granéli pers. com.), and off Brittany (Gentien pers. com.) at the end of the growth season. There are suggestions that these pigmented cells represent some kind of "resting population", but there is no information on the specific characteristics (ploidy, pigment composition, etc.) of these cryptic populations. A life cycle has been proposed, but there are some hypothetical steps that need experimental confirmation (Fig. 13).

Considering our present knowledge of *Dinophysis* spp. life cycles, it seems reasonable to ignore (as not very relevant to their population dynamics), the "putative cyst-stage" and proceed as if *Dinophysis* species are truly holoplanktonic, using aggregation in thin layers and small cell formation as their peculiar strategy to exploit optimal environmental conditions, or to withstand adverse conditions, respectively. Scarce field data (MacKenzie 1992; Reguera unpubl. data) suggest that these subpopulations of small cells can be spatially segregated from the vegetative cell populations.

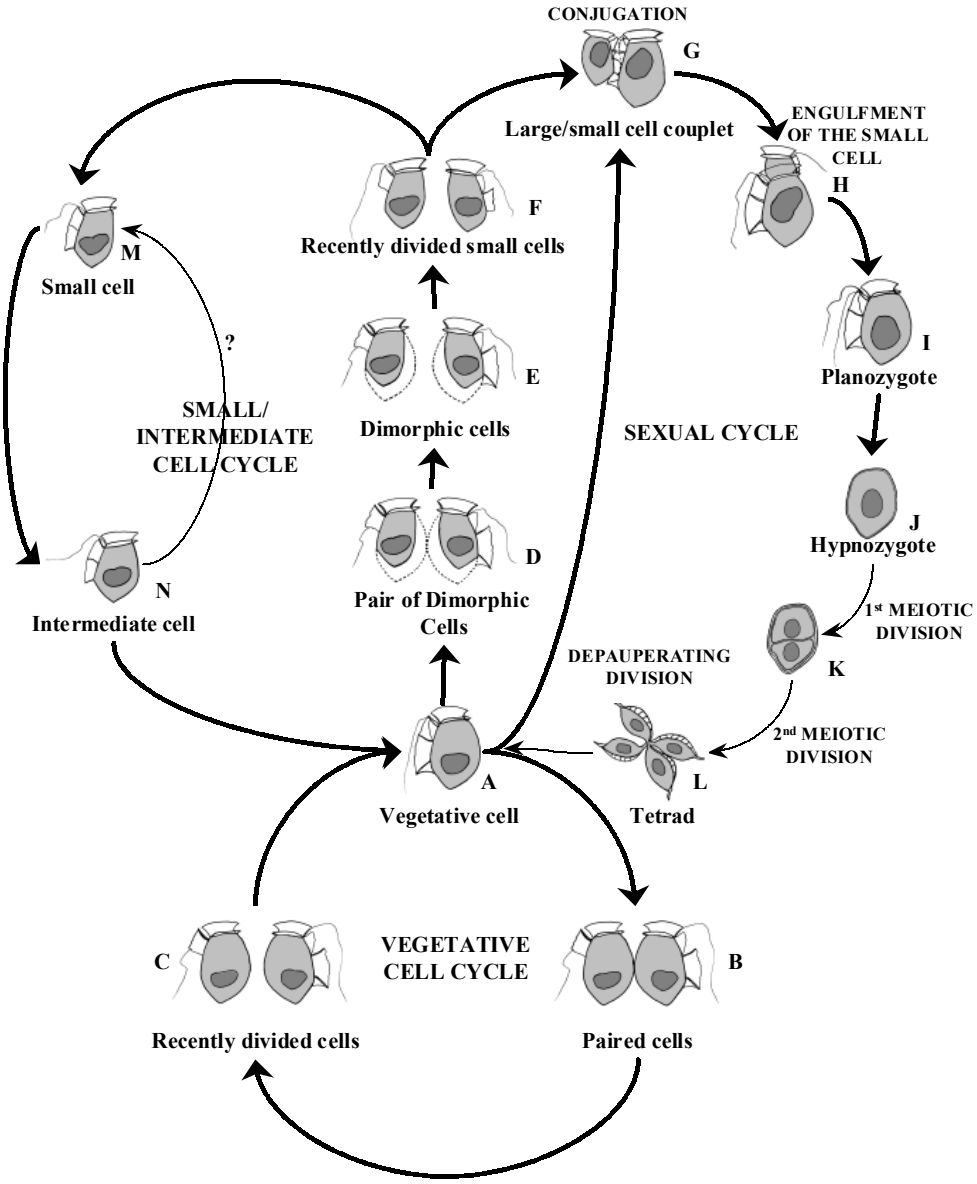


Fig. 13. Diagram of confirmed (solid lines) and hypothetical stages (dotted lines) in the life history of *Dinophysis* spp. (A-C) Vegetative cell cycle: (A) Fully developed vegetative cell, (B) paired, and (C) recently divided cells showing incomplete development of the left sulcal list (LSL). (A-L) Sexual cycle: (D) Pair of dimorphic cells resulting from a depauperating division and (E) recently separated dimorphic cells (dotted lines indicate the contour of the maternal hypothecal plates). (F) Recently divided small cells still with incomplete development of the LSL. (G) Small cell (acting as putative plus anisogamous gamete) and large cell (acting as putative negative anisogamous gamete), with nuclei migrated to anterior positions, firmly attached by the ventral margins in apparent conjugation. (H) Engulfment of the small cell by the large cell through the apical end of the sulcus. (I) Planozygote with two trailing flagella. (J) Suspected double-walled hypnozygote. (K) Suspected first meiotic division. (L) Tetrad. Simplified small/intermediate cell cycle. (From Reguera & González-Gil 2001).

Main gaps in our knowledge concerning *Dinophysis* spp. are:

- Is there a minimum-sized offshore residual population of *Dinophysis* that will act as the inoculum for next year's proliferation? What would be the role of the potential cysts of *Dinophysis* spp.? Can we improve our predictive capabilities by mapping the residual populations?
- Will the size of next year's population be influenced by the size of the residual stock?
- What are the physiological characteristics of the residual populations (biochemical markers), allowing us to distinguish them from actively dividing vegetative cells?
- Are toxin production and division rates correlated? If not, would this explain isolated observations of winter populations with extremely high content of toxin per cell?
- What are the unique conditions of the thin layers exploited by certain mixotrophic species of *Dinophysis* (co-occurring potential prey, certain dissolved organic compounds, diminished shear stress.)?

Freshwater dinoflagellates – life history and HAB potential

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Introduction

Freshwater dinoflagellates are generally not considered as toxic or harmful algal bloom species. However, there is evidence suggesting that this view should be questioned. First of all, dinoflagellates are common in many freshwater ecosystems, and often form dense blooms. Secondly, there are toxin-producing freshwater dinoflagellates, often related to fish kills. Furthermore, freshwater dinoflagellates serve as ideal model organisms for studies of life cycles and population dynamics, as sampling and field experiments are much easier in lakes than oceans.

Toxins in freshwater dinoflagellates

In the literature, there are several references of toxic outbreaks caused by freshwater dinoflagellates (Jurgens 1953). The available evidence suggests that dinoflagellates within the *Peridinium* and *Peridiniopsis* genera can produce toxins. *Peridinium* (*Peridiniopsis*) *polonicum* caused fish kills in a reservoir in Japan (Adachi 1965). This toxin was later identified as a tetrahydropyridine, comparable to brevetoxins in ichthyotoxic potency (Oshima *et al.* 1989). Another species, *Peridinium bipes*, was shown to have an algicidal effect on the cyanobacterium *Microcystis aeruginosa* (Wu *et al.* 1998). Further evidence was provided by Mills *et al.* (1995),who reported suspected fish kills due to algal toxins released by dinoflagellates in an artificially acidified lake. Most recently, Rengefors & Legrand (2001) showed that the winter/spring blooming *Peridinium aciculiferum* produces a toxin(s) which kills *Artemia* larvae, shows haemolytic activity and has an algicidal effect on other phytoplankton species.

Toxic blooms of freshwater dinoflagellates probably occur more frequently than reported, and may have a major effect on the biota in those habitats. These toxins may have an effect on the food web either through their algicidal action, or through direct or vectorial toxicity, as proposed by Rengefors & Legrand (2001). There is evidence suggesting that toxin production by *P. aciculiferum* could be the cause of large larval mortality of the commercially important vendace (*Coregonus albula*) in Lake Mälaren, Sweden. In addition to potential ichthyotoxicity, dinoflagellate toxicity should be of concern for water quality management, as many lakes serve as drinking water supplies.

Life cycle

The life cycle of freshwater photosynthetic dinoflagellates in temperate regions (e.g. Europe) typically involves an alternation between a motile planktonic stage and a non-motile resting cyst. There are, however, more complicated life cycles to be found, including thecate *versus* non-thecate stages, amoeboid stages, etc. (Popovsky & Pfiester 1990). In northern temperate freshwater ecosystems, phototrophic dinoflagellates rest as cysts during most of the year (7-9 months) (Rengefors 1998). This is true both for summer species (which rest during fall and winter) and winter species (which rest during the summer months). The benthic resting stage is thus an important part of the freshwater dinoflagellate life cycle, and is tightly coupled to bloom initiation. Rengefors (1997) showed that the presence of a cyst stage, and the regulation of

germination, allows for a seasonal succession of dinoflagellates, thereby reducing the competition among related species

Vegetative phase

A majority of the photosynthetic freshwater dinoflagellates are not perennial, but their occurrence appears as yearly events following excystment from the benthic resting cyst. In temperate regions, they have their population maxima in summer or late winter (Rengefors 1998), whereas in subtropical regions these occur at the end of the mixed water period (Pollingher 1988). In the tropical Amazonian flood-plain lakes, the motile phase occurs during low-water, stagnant period, whereas cyst formation appears to occur during the inflowing lotic period (Meyer *et al*. 1997).

Sexual reproduction and encystment

The sexual life history of dinoflagellates has been described for about 30 species, but sexuality is probably present in all species (Popovsky & Pfiester 1990). Sexual reproduction was first described in *Peridinium cinctum* (Pfiester 1975), and was induced by nitrogen deficiency (Pfiester 1975), which leads to gamete production. Gamete fusion precedes the formation of a biflagellated planozygote, which eventually loses its motility and develops into a hypnozygote or resting cyst.

As in marine dinoflagellates, freshwater species are generally believed to undergo cyst formation in nature as a response to unfavourable environmental conditions. However, it is also postulated that sexuality and cyst formation are induced when cell concentration is high, with correspondingly increased possibilities for contact between gametes in sexual reproduction (Dale 1983). *Peridinium willei,* like *Peridinium cinctum* in culture, was shown to encyst when it was nitrogen stressed (Chapman & Pfiester 1995). In the field, Heaney & Talling (1980) showed that encystment of *Ceratium hirundinella* coincided with the depletion of phosphate, ammonium and nitrate in the water column. It also appears that turbulence, perhaps in combination with temperature, may be an important factor as observed with cyst formation of *Peridinium aciculiferum* and *C. hirundinella* in Lake Erken (Rengefors 1998). Encystment of *P. aciculifeum* immediately precedes ice-out in Lake Erken, whereas *C. hirundinella* encysts in conjunction with a major turbulence event followed by a sharp drop in temperature.

The resting cyst

Most cysts are smooth walled and only occasionally have small bristles (Popovsky & Pfiester 1990; Rengefors 1998; Rengefors & Meyer 1998), but are resistant to enzymes and acids. Cysts have been described for most genera, including *Amphidinium*, *Ceratium, Gymnodinium, Peridinium*, *Peridiniopsis*, and *Woloszynskia*. Rengefors & Anderson (1998) showed experimentally that the maturation/dormancy period was several months in *P. aciculiferum* and *C. hirundinella*, irrespective of storage temperature. The cyst viability for some species appears to extend over many years, as shown by the only study available reporting at least 15.5 years for *P. cinctu*m and 6.5 years for *C. hirundinella* cysts (Huber & Nipkow 1923).

Excystment

A number of factors are involved in order to time and trigger the excystment of freshwater dinoflagellates. First, it is necessary for the cysts to have undergone dormancy, and entered the quiescent phase of their resting stage. Secondly, an endogenous biological clock appears to be involved (Rengefors & Anderson 1998). This clock determines when during a year cysts can potentially germinate, and it cannot be overridden by any other factor. Presumably, the purpose of this clock is to prevent germination during the wrong time of the year when other environmental factors may appear favourable. Third, temperature sets the germination window when the actual excystment can take place. Light may or may not be necessary for germination (von Stosch 1973; Rengefors & Anderson 1998), depending on the species. One environmental factor which can prevent excystment is anoxia (Rengefors & Anderson 1998).

A recent finding was that the presence of a benthic resting stage may be a predator-avoidance adaptation in dinoflagellates. In the presence of zooplankton exudates, the germination of *P. aciculiferum* cysts was inhibited, whereas that of *C. hirundinella* cysts was not (Rengefors *et al*. 1998). *Peridinium aciculiferum* is of ideal size and of high nutritional value to copepods, thus cyst formation may be an adaptation to avoid predators, and can explain why this species grows during winter (when few grazers are present) and rests during summer (when copepods are prevalent). *Ceratium hirundinella*, in contrast, is not grazed by copepods. These findings suggest that cyst formation is not exclusively a response to poor environmental conditions, but can also represent an escape from biological threats.

Life cycle strategies in the haptophyte genera *Chrysochromulina* and *Prymnesium*

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Introduction

At present, about 55 *Chrysochromulina* and 10 *Prymnesium* species have been described (most are listed in Jordan & Green 1994). A phylogeny inferred from 18S rDNA sequences and available morphological data of haptophytes indicate that *Chrysochromulina* is not a monophyletic group and can be divided into two main clades (Edvardsen *et al.* 2000a). Some species (*C. hirta, C. kappa* and *C. polylepis*) appear more closely related to *Prymnesium* species than to other *Chrysochromulina* species. A taxonomic revision of the two genera is underway (Eikrem *et al*. in prep.).

Whereas *Chrysochromulina* species form a regular component of the marine plankton (with a handful of representatives in freshwater), most *Prymnesium* records are from inshore localities and brackish-water lakes and ponds. Harmful blooms of *Chrysochromulina* seem to be exceptional events and have been recorded mainly from coastal waters adjacent to the North Atlantic, whereas *Prymnesium* blooms are recurrent in many parts of the world (review by Edvardsen & Paasche 1998). Both types of blooms may result in large fish kills causing great economic losses. All species of *Chrysochromulina* and *Prymnesium* may be considered potentially toxic in nature, but clear and sustained toxicity in cultures has been demonstrated only in *C. polylepis, P. parvum* (f. *parvum* and f. *patelliferum*) and *P. calathiferum*. Several *Chrysochromulina* species, e.g. *C. leadbeateri,* have caused large fish kills in nature, but were non-toxic when kept in laboratory culture (Edvardsen & Paasche 1998).

The aim of this report is to review available information on the life cycles of members in the genera *Chrysochromulina* and *Prymnesium* and to interpret this to assess the ecological role of alternate stages and the ecological significance of a sexual life cycle in these algae. It also aims at identifying gaps in our knowledge.

Current knowledge on life cycles

Within the Haptophyta, life cycles with alternating morphologically distinct generations are frequent. Yet, sexual reproduction has been demonstrated only in a small number of cases. Syngamy and meiosis have been observed in a few genera only (see review by Billard 1994). Syngamy and meiosis in haptophytes are probably very infrequent and short-lived events and may occur under special conditions that in most cases are unknown. The presence of haploid and diploid cells in unialgal cultures that divide vegetatively indicates a sexual haplo-diploid life cycle.

Life cycles in *Chrysochromulina*

Generally, haptophyte life cycles embrace an alternation between flagellated and non-flagellated stages. In *C. polylepis*, however, two motile cell types have been described, termed authentic and alternate (Paasche *et al.* 1990, Fig. 14). The cell types have identical nucleotide sequences in coding and non-coding regions of ribosomal DNA (18S and ITS 1, Edvardsen & Medlin 1998), but differed in body scale morphology and in cell size (Fig. 14). Flow cytometric ploidy analyses indicated that the authentic cells were haploid (n) and the alternate cells either haploid or diploid (n or 2n, Fig. 15;

Edvardsen & Vaulot 1996). The haploid alternate and authentic cell types possibly function as sexual stages (gametes) representing different mating types, the diploid alternate cells being the result of syngamy. However, syngamy and meiosis have never been observed (Edvardsen & Vaulot 1996). Environmental factors that promote the transition between cell types remain unresolved (however, see Edvardsen & Paasche 1992).

To investigate whether more *Chrysochromulina* species possess alternate stages in a sexual life cycle, Edvardsen (1998) examined the genome size, nucleotide sequences (18S and ITS 1 rDNA) and morphology in strains representing 17 different species. Two ploidy levels, assumed to represent haploid and diploid cells, were found in three species in addition to *C. polylepis*: *C. ericina, C. hirta* and *C. kappa*. The haploid and diploid forms differed in body scale morphology in *C. hirta*, but not clearly in *C. kappa* and *C. ericina*. In 13 other *Chrysochromulina* species, only one ploidy level was found. One may ask if the capability to reproduce sexually has been lost in these species, or if it is retained, but not evident in the investigated strains.

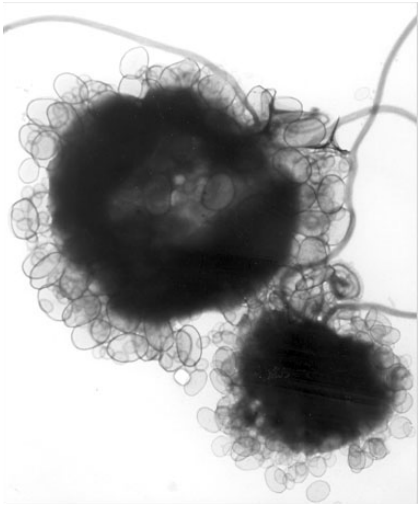


Fig. 14. Electron micrographs of *Chrysochromulina polylepis*, haploid or diploid alternate (upper) and haploid authentic (lower) cell (from Edvardsen & Paasche 1992).

In old cultures of *Chrysochromulina ephippium*, Parke *et al.* (1956) observed large, naked, non-motile, amoeboid cells on the bottom of the culture flasks. These cells could then form 2 or 4 non-motile, walled cells from which motile cells were released. Similar observations were made on *C. alifera, C. brevifilum, C. chiton, C. ericina, C. kappa, C. minor* and *C. strobilus* (Park *et al.* 1955; 1956; 1958; 1959). Cysts have never been reported in *Chrysochromulina*. However, the walled cells could possibly be a homologous cell type. All information on life cycles in *Chrysochromulina* has arisen from studies of unialgal cultures. It is unknown whether non-motile amoeboid or walled cells also occur in nature. Alternate cells of *C. polylepis* have been found in nature on a few occasions only, suggesting that *C. polylepis* has a life cycle in which the haploid authentic stage dominates.

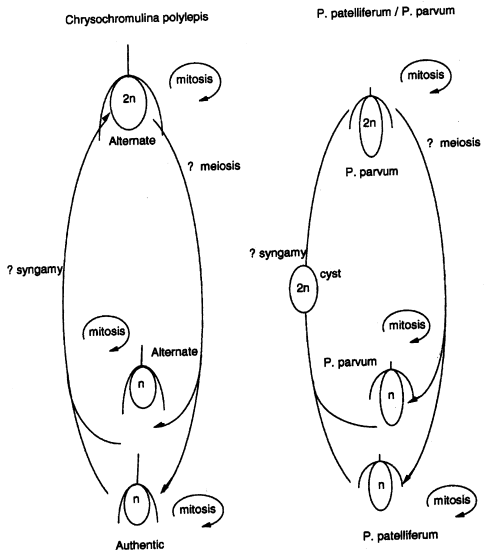


Fig. 15. Model of the life cycle, linking the authentic and alternate cell types of *C. polylepis* (left) and *P. parvum* f. *parvum* and *P. parvum* f. *patelliferum* (right). Syngamy and meiosis have never been observed. The role and placement of benthic stages in the life cycle of *Chrysochromulina* and *Prymnesium* are uncertain. (From Medlin *et al*. 2000b).

Life cycles in *Prymnesium*

Prymnesium parvum and *P. patelliferum*, which can be distinguished only by minor details in scale morphology seen in TEM, were until recently considered as two separate species (Green *et al.* 1982). However, Larsen & Edvardsen (1998) provided some evidence indicating that the two 'species' were joined in a haplo-diploid life cycle, and the names were emended to *P. parvum* f. *parvum* and *P. parvum* f. *patelliferum* (Larsen 1999). The two forms from western Norway were genetically identical in coding and non-coding DNA regions (18S and ITS 1 rDNA, Larsen & Medlin 1997). Flow cytometric ploidy analyses indicated that *P. parvum* f. *patelliferum* was haploid and *P. parvum* f. *parvum* could be either haploid or diploid (Larsen & Edvardsen 1998). The two forms often co-occur in nature. Billard (1994) put forward the hypothesis that the morphology of the organic body scales could indicate ploidy level in Haptophyta. Haptophytes bearing body scales with radial microfibrils on the proximal face and concentric ridges on the distal face are expected to be haploid, whereas species with radiating microfibrils on both sides may be diploid. This hypothesis is in accordance with life cycles of some coccolithophorids and *Prymnesium parvum*.

In *Prymnesium,* cysts with walls composed of layers of scales have been observed in *P. parvum* (f. *parvum* and f. *patelliferum*), *P. minutum*, *P. saltans* (e.g. Pienaar 1980; Green *et al.* 1982; T. Johnsen pers. com.) and *P. nemamethecum* (I. Probert pers. com.). When investigated in TEM, the scales of the cysts were found to be overlaid by electron-dense, siliceous oval structures (Pienaar 1980; Green *et al*. 1982). A change between flagellated and non-motile cells of *Prymnesium* was observed when

different types of growth media were tested (Padan *et al*. 1967). Fig. 15 shows a model of the life cycle of *P. parvum* linking the two forms.

Differences between life cycle stages in autecology and toxin production, and their possible ecological role

The two stages of *C. polylepis* were shown to have different temperature optima, and strains producing alternate cells were less tolerant to high irradiance and high temperatures than strains yielding only authentic cells (Edvardsen & Paasche 1992). In nature, one would therefore expect to find the alternate form mainly at greater depth. The two cell types also differ in toxicity. Strains yielding only authentic cells were much more toxic to larvae of brine shrimp (*Artemia* nauplia) than cultures able to produce alternate cells (Edvardsen 1993). Other *Chrysochromulina* species with an alternate life cycle stage (*C. kappa, C. hirta* and *C. ericina*) have been found to be non-toxic to *Artemia* nauplia (Edvardsen 1993). Differences between the stages in growth optimum and tolerance to environmental factors in these species remain to be examined. No differences in optimal salinity, temperature and irradiance between diploid *P. parvum* f. *parvum* and haploid *P. parvum* f. *patelliferum* strains from western Norway could be detected – nor in toxicity (Larsen & Bryant 1998). The two *P. parvum* forms are thus not known to occupy different niches.

Significance of a haplo-diploid life cycle for bloom dynamics

The maintenance of a haplo-diploid life cycle has been regarded as an adaptation to an environment that is seasonally variable, or that contains two different niches (Valero 1992). Differences in temperature optimum, tolerance to high temperature and irradiance, and toxin-producing ability between the two stages of *C. polylepis* may indicate adapted responses to changing conditions in the water column. *Prymnesium* blooms tend to recur in the same locality (e.g. in Ryfylke fjords, western Norway), and one may speculate that cysts function as an overwintering stage that seeds a new population when the right conditions appear. It is still unresolved whether a *Chrysochromulina* or *Prymnesium* species with a sexual haplo-diploid reproduction has any ecological advantages during bloom formation and development.

On the ecological role of the different life forms of *Phaeocystis*

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Phaeocystis life forms - short description

The eurythermal and euryhaline genus *Phaeocystis* is one of the most widespread marine haptophytes, with species sharing the ability to produce nearly monospecific blooms in many environments. Its unusual heteromorphic life cycle (Fig. 16), which alternates between gelatinous colonies and different types of free-living cells (vegetative non-motile, vegetative flagellate and microzoospore), sets it apart from other members of the class (Rousseau, this volume). Six species have been identified (Zingone *et al.* 1999): *P. globosa, P. pouchetii, P. antarctica, P. scrobiculata, P. cordata* and *P. jahnii*. Genetically, *P. pouchetii* and *P. antarctica* are reported to have evolved from *P. globosa* (Medlin *et al.* 1994).

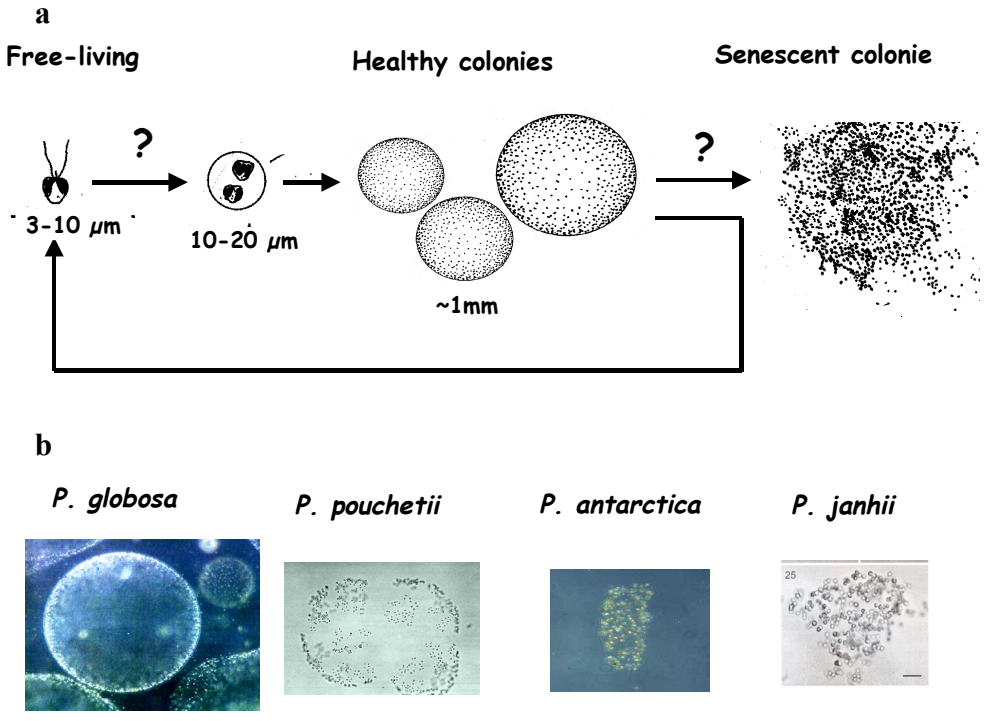


Fig. 16. *Phaeocystis* life forms. (A) Schematic representation of ecologically important forms (free-living cells, colonies, and *Phaeocystis*-derived aggregates) and transition pathways. Question marks indicate lack of knowledge on controlling factors. (B) microphotographs of *P. globosa* (maximum colony diameter: ~8-9 mm), *P. pouchetii* (maximum colony diameter: ~1.5-2 mm), *P. antarctica* (maximum colony diameter: ~1 mm) *and P. jahnii* colonies (reported colony diameter: 150 µm).

The colonies, composed of a few to thousands of cells embedded in a mucilaginous matrix, occasionally reach several mm in diameter. Individual cells, 3-10 µm in diameter, are distributed within the gel matrix of the colonies, which vary in shape and size from little (20 µm) to large homogeneous spheres, and to large ill-formed colonies invaded by bacteria and protists. The latter forms are observed at the decline of the bloom. Colony cells are non-motile and lacking scales. Colony forms were reported for *P. globosa, P. pouchetii, P. antarctica* and *P. jahnii*.

The biochemical composition of the gel matrix has been determined for *P. globosa* and *P. pouchetii*. It is basically polysaccharidic, however with varying monosaccharides and amino groups, depending on environmental conditions (see the review by van Rijssel *et al*. 2000). Colony shape and apparent gel compactness vary between species (Fig. 16B). In both *P. globosa* and *P. antarctica*, individual cells are uniformly distributed around the periphery of the colony, whereas in *P. pouchetii*, the cells are grouped in clusters, usually of four cells, in lobes of the colony. No colonial form or stage has been reported yet for *P. scrobiculata* and *P. cordata*, but this does not necessarily mean that these species are not capable of forming colonies.

Global distribution

Fig. 17 synthesizes current knowledge on the global distribution of *Phaeocystis* species, revised according to criteria described in Baumann *et al.* (1994), Vaulot *et al*. (1994) and Zingone *et al*. (1999). Free-living forms are cosmopolitan in distribution and are an important component of the haptophycean assemblage, which dominates oceanic nanophytoplankton in many areas (e.g. Thomsen *et al*. 1994). Colony blooms are reported in nitrate-rich areas (Lancelot *et al.* 1998), either naturally (Ross Sea, Greenland Sea, Barents Sea) or due to anthropogenic inputs (e.g. Southern Bight of the North Sea, Arabian Gulf, southeast coastal waters of China).

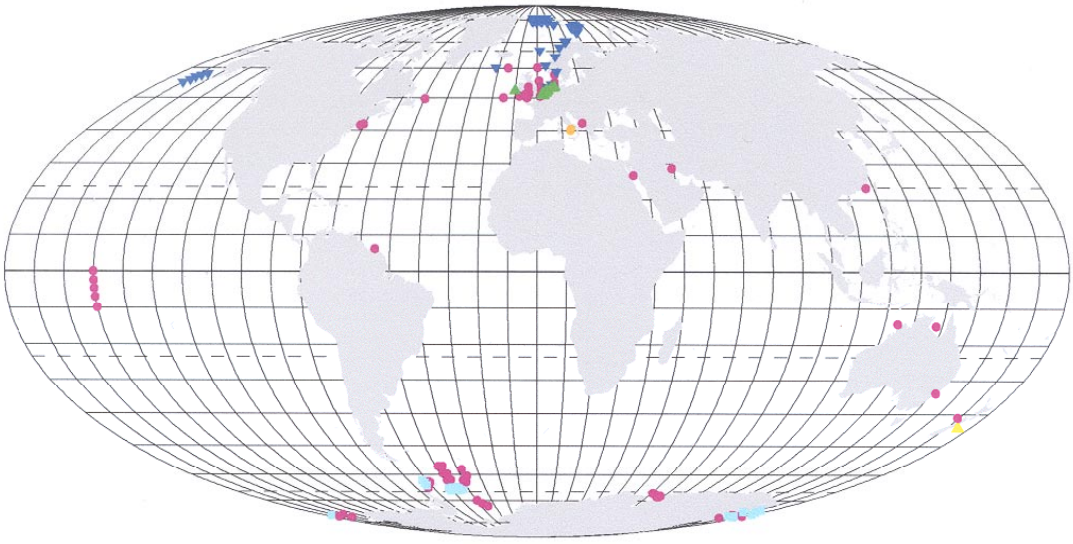


Fig. 17. Geographical distribution of the genus *Phaeocystis* (redrawn from Schoemann 2000). *Phaeocystis pouchetii* is recorded as dark blue triangles; *P. globosa* as green triangles; *P. antarctica* as light blue squares; *P. scrobiculata* as yellow triangles; *P. jahnii* and *P. cordata*, which have the same location, as an orange circle.

Ecology of life forms: free-living cells *versus* colonies

The dominance of one form over the other in natural environments has significant consequence for planktonic and benthic ecosystem structure and functioning (e.g. Hamm 2000) and related biogeochemical cycles (e.g. Wassmann 1994). It can also have severe environmental impacts in coastal areas (e.g. Lancelot 1995). Strain-related morphological and physiological characteristics have been suggested to be of little significance with respect to the autecology and dynamics of *Phaeocystis* blooms (Lancelot *et al.* 1998). The shared ability to alternate between free-living cells and large gelatinous colonies, demonstrated for 4 species, constitutes the key ecological factor. Yet the functioning of ecosystems dominated by *Phaeocystis* colony blooms varies geographically (Hamm 2000). Current knowledge on bottom-up, top-down control of *Phaeocystis* life forms is described below and their impact on biological resources and environment is briefly reported.

Flagellate cells

Maximum specific growth rate is comparable to that of colony cells but free-living cells have been reported as better competitors than colonial forms under ammonium- and phosphate-limited conditions (Riegman *et al.* 1992). Nano-sized free-living cells very rarely form blooms (cell abundance $\leq 10^6$ cells$\cdot L^{-1}$) and are heavily grazed by protozoa, stimulating the development of an active microbial food-web which retains most *Phaeocystis*-derived material in the surface waters (‘regeneration-based food chain’).

Colony forms

It is hypothesized that most of the success and uniqueness of *Phaeocystis* colonies in the environment and their impact on the marine ecosystem and environment is linked to their capacity of forming large gelatinous colonies, and more especially to the structure of the colony matrix. Current knowledge on factors triggering the transition of free-living to colonial forms is discussed by Rousseau (this volume).

Blooming success of *Phaeocystis* colony forms: a better edge to use light and nutrient resources and/or a high resistance to loss?

The colony matrix has a hollow structure composed of heteropolysaccharidic chains (van Rijssel *et al*. 1997; Hamm *et al.* 1999) which form a gel by bridging with Ca^{2+} and Mg^{2+} (van Boekel 1992). Experimental evidence obtained mostly with *P. globosa* suggests that the colony matrix, by acting as an energy and nutrient (Fe, PO_4) reservoir, gives a competitive edge to *Phaeocystis* when resources are scarce and very variable (Veldhuis *et al.* 1991; Schoemann *et al*. 2001). On the other hand, the skin-like structure of *P. globosa* colonies with pore size < 4.4 nm (Hamm *et al.* 1999) has been suggested to prevent pathogen infection (Jacobsen *et al.* 1996) and bacterial colonization. In addition, DMSP produced by *Phaeocystis* cells and accumulating in the colony matrix releases acrylic acid, which, when converted to DMS, would deter grazers. Accordingly, a high acrylate concentration has been measured inside the *P. globosa* matrix (Noordkamp *et al.* 2000). The repellent properties of *Phaeocystis* colonies have never been investigated. The reported resistance of *Phaeocystis* colonies to mesozooplankton grazing is generally attributed to a size mismatch or mechanical hindrance due to increased viscosity. The latter has been suggested but never demonstrated.

Impact on ecosystem structure and functioning

Fig. 18 shows a schematic representation of two *Phaeocystis*-dominated ecosystems (adapted from Hamm 2000): a deep-ocean (Fig. 18A) and a nutrient-enriched shallow coastal (Fig. 18B) system. These diagrams suggest species dependence and geographic variability which could be attributed to differences in the structure of the colony matrix and/or to the presence/absence of large grazers. In the nutrient-enriched coastal system (Fig. 18B), most of the *Phaeocystis* biomass – often dominated by large colonies of *P. globosa* – escapes grazing by indigenous zooplankton. Ungrazed senescent colonies disrupt and/or aggregate and are remineralized by free-living and attached bacteria. Colonial cells released in the ambient after colony disruption are ingested by ubiquitous microzooplankton and are vulnerable to virus infection. In ocean systems (*P. pouchetii* in the Greenland/Barents Sea and *P. antarctica* in the Ross/Weddell Sea), colonies are relatively smaller and grazed by large copepods and euphausids when, present (Fig. 18A). To our knowledge, ungrazed colonies do not disrupt and sink. Senescent colonies aggregate and sink.

Impact on biological resources and the environment

Phaeocystis-dominated ecosystems are invariably associated with commercially important stocks of crustaceans, molluscs, fishes and mammals, but the potential deleterious effect of *Phaeocystis* colonies has not yet been studied. Toxicity of *P. pouchetii* for cod larvae has been shown (Eilertsen & Raa 1995) but never reported for other species. Most adverse effects of *P. globosa* colony blooms on biological resources are reported as mechanical (increased water viscosity; e.g. Pieters *et al*. 1980) or indirect (oxygen deficiency after bacterial degradation of ungrazed *Phaeocystis*-derived material), but have not been seriously studied. Similarly, foam deposits observed every spring on the beaches bordering the eastern Southern Bight of the North Sea are reported as the consequence of huge accumulations of ungrazed gelatinous *P. globosa* colonies (Fig. 18B; Lancelot 1995).

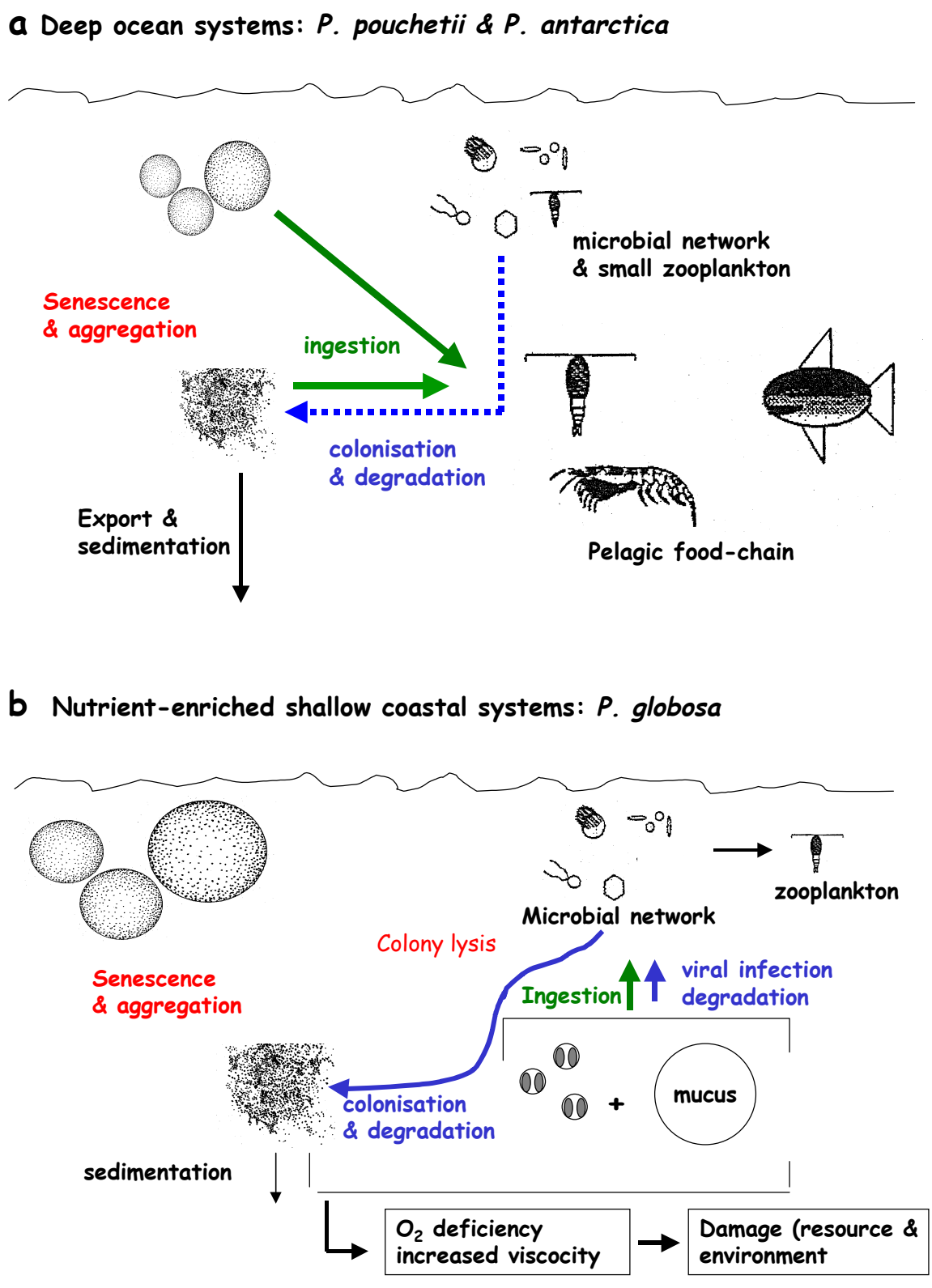


Fig. 18. Diagrammatic representation of the structure of two *Phaeocystis*-dominated ecosystems (adapted from Hamm 2000).

The wax and wane of *Phaeocystis* blooms in relation to life cycle transitions

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Introduction

The wax and wane of blooms formed by the prymnesiophyte *Phaeocystis globosa* negatively affects water quality in the North Sea area by water discolouration, oxygen depletion and foam on nearby shores (Lancelot *et al.* 1987a). In northern parts of the North Sea, *Phaeocystis pouchetii* is thought to produce substances toxic to fish (Aanesen *et al.* 1998). Unidentified *Phaeocystis* species have been recorded on the shelf of the Atlantic Ocean (Kashkin 1963) but these, together with the newly described *Phaeocystis* species in the Mediterranean, *P. cordata* and *P. jahnii* (Zingone *et al.* 1999), do not appear to be harmful. The distribution of *Phaeocystis* species is shown in Fig. 19.

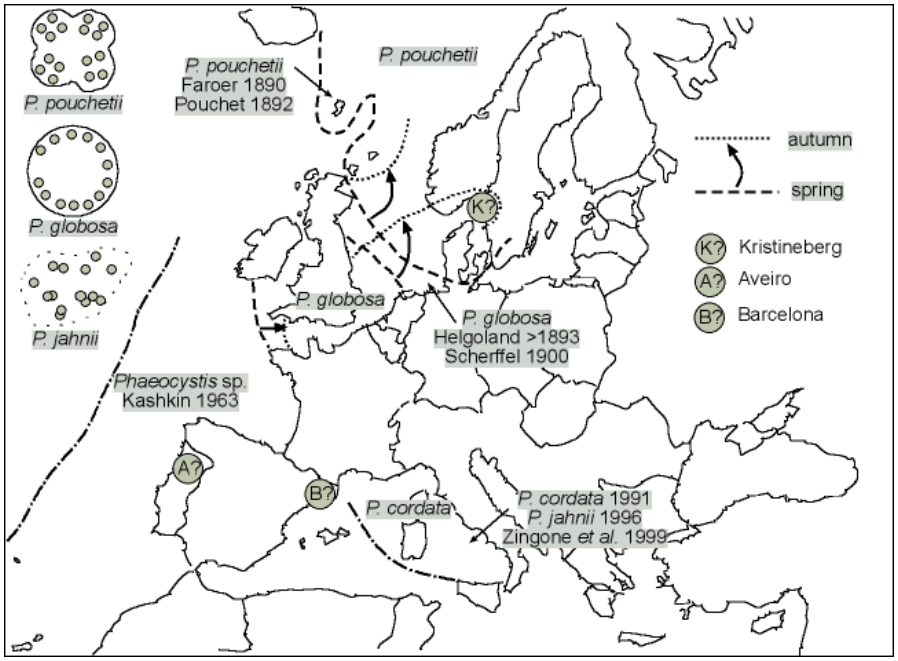


Fig. 19. Distribution of *Phaeocystis* species in Europe. The first microscopic observations of *P. pouchetii* were made by Pouchet (1892), of *P. globosa* by Scherffel (1900) and of *P. cordata* and *P. jahnii* by Zingone *et al.* (1999). The colonial forms of *P. pouchetii, P. globosa* and *P. jahnii* (top left) can be distinguished with the light microscope. The flagellate forms of *Phaeocystis* (including *P. cordata*) can only be identified with electron microscopy. There is an overlap in the spring-autumn distribution of *P. pouchetii* and *P. globosa* in the North Sea (Ostenfeld 1910). The identity of the *Phaeocystis* species occurring on the Atlantic shelf (Kashkin 1963) and in Barcelona, Aveiro and Kristineberg (L. Peperzak, personal observations) are unknown.

Life cycle and blooms

Three different flagellates and a non-flagellate cell type are distinguished in the life cycle of *P. globosa*. The macroflagellate and the non-flagellate cell are diploid, whereas the micro- and mesoflagellates are haploid (Peperzak *et al.* 2000) (Fig. 20). Environmental variables that trigger the transitions are turbulence, nutrient concentrations and daily irradiance. Fig. 20 is constructed from experimental, field and literature data and has not been tested rigorously in culture. Results of sediment incubation experiments and field observations suggest that neither *P. pouchetii* nor *P. globosa* have benthic resting stages (Eilertsen *et al.* 1995a; Peperzak 2002).
The macroflagellate transforms into a non-flagellate cell (the 1st cel of a colony) only when turbulence is low. The surfaces of objects are regions of low turbulence, and in the lab you always find *Phaeocystis* colonies on the glass of your erlenmeyer flasks. In the field young *Phaeocystis* colonies are often observed on diatoms.Blooms of colonial non-flagellate *P. globosa* cells start under nutrient-replete conditions (upwelling, anthropogenic eutrophication), after a daily irradiance threshold has been passed. The upper limit of the bloom maximum, 100 million cells·L^{-1}, is determined by the inorganic carbon (C_i) metabolism of the colony (Peperzak 2002).

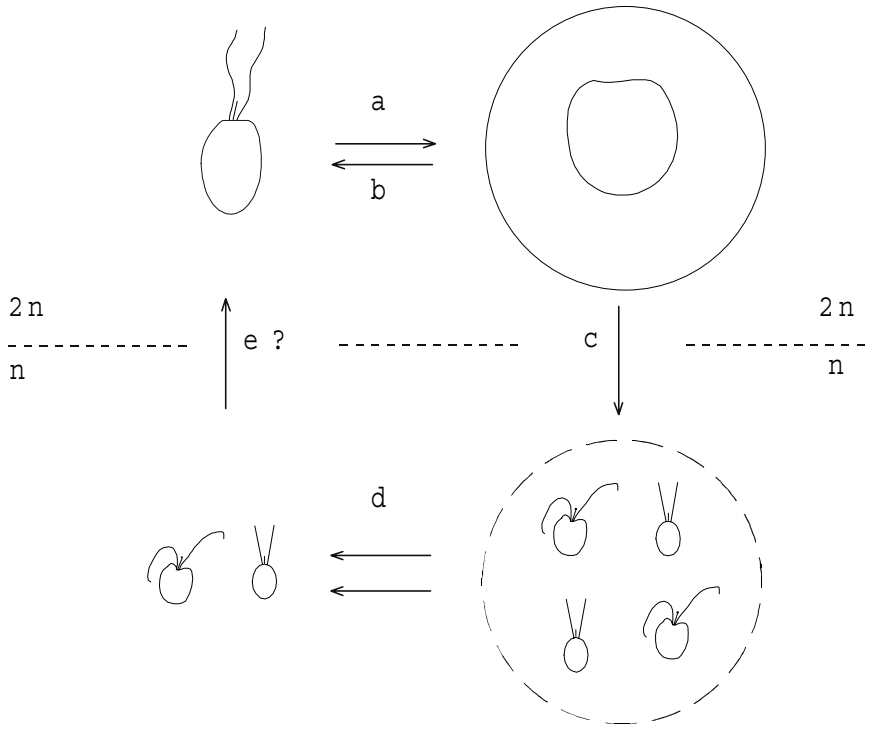


Fig. 20. Life cycle of *Phaeocystis*. n = haploid, 2n = diploid. (A) Colonial non-flagellate cell is formed from a macroflagellate under non-turbulent conditions when nutrients and daily irradiance are sufficient. Normal, vegetative reproduction can lead to a colony consisting of thousands of non-flagellate cells. (B) Diploid macroflagellates from non-flagellate cells are produced physically, e.g. by turbulence. Macroflagellates have a life-time of less than a day. (C) Under nutrient stress and irradiance limitation, haploid micro- and mesoflagellates are formed intracolonially. (D) Meso- and microflagellates escape the colonial matrix. Both are able to reproduce vegetatively. (E) Presumed syngamy of micro- and mesoflagellates leads to the diploid macroflagellate. This step has not yet been observed (Peperzak *et al.* 2000).

The duration of the bloom is set by the second limiting nutrient, which is usually nitrogen or phosphorus.

When nitrogen or phosphorus further limit bloom development, the accumulated *Phaeocystis* biomass (in the form of non-flagellate cells and organic colony matrix) is dissipated via several processes:

1. Colonies settle onto the sea floor and are buried or mineralized (Savage & Hardy 1934; Jennes & Duineveld 1985), or
2. Non-flagellate cells transform into flagellates that escape the colony (Peperzak *et al.* 2000);
3. Microzooplankton invade deteriorating colonies and graze on cells (Peperzak *et al.* 1998);
4. Cells lyse and, together with colony remains, are mineralized pelagically (Brussaard *et al.* 1995; Rousseau *et al.* 2000).

Negative effects of *Phaeocystis* blooms

The negative effects of *Phaeocystis* blooms can be grouped as follows:

- Water discoloration and foam
- Oxygen depletion
- Fish toxins
- Miscellaneous

The most pronounced effect, observed during the wane of *Phaeocystis globosa* blooms, is thought to be the formation of foam (Lancelot *et al.* 1987b). However, there is no formal proof that *Phaeocystis* is indeed the cause of this foam; even the foam composition is unknown. Only recently, a significant correlation between *Phaeocystis* blooms and the frequency of foam was established (Fig. 21). Beer foam is composed of proteins, and the study of beer foam production may therefore be helpful in investigating *Phaeocystis*-foam production.

When large amounts of organic *Phaeocystis* carbon are concentrated near the seabed by sedimentation, oxygen may be depleted (Rogers & Lockwood 1990). In 2001, oxygen depletion was thought to be the cause of a € 20 million mortality of blue mussels in the Oosterschelde, The Netherlands (L. Peperzak, unpublished). The third negative effect could be the production of ichthyotoxins by *P. pouchetii* (Aanesen *et al.* 1998). However, the toxin has not been identified, and it may be possible that the increased lethality of cod larvae (Aanesen *et al.* 1998) was related to *Phaeocystis*-produced DMS. For instance, blooms of *P. globosa* have long been known to affect the migration of herring (Savage 1930).

Miscellaneous Dutch reports of negative bloom effects in 2001 include, for instance, the release of H_2S from sediments after the large *P. globosa* bloom in spring which led to the stop of dredging activities in the outer IJmuiden harbour. In August, skin irritation of swimmers was reported when very high concentrations of colonial *P. globosa* occurred near the mouth of the Rhine.

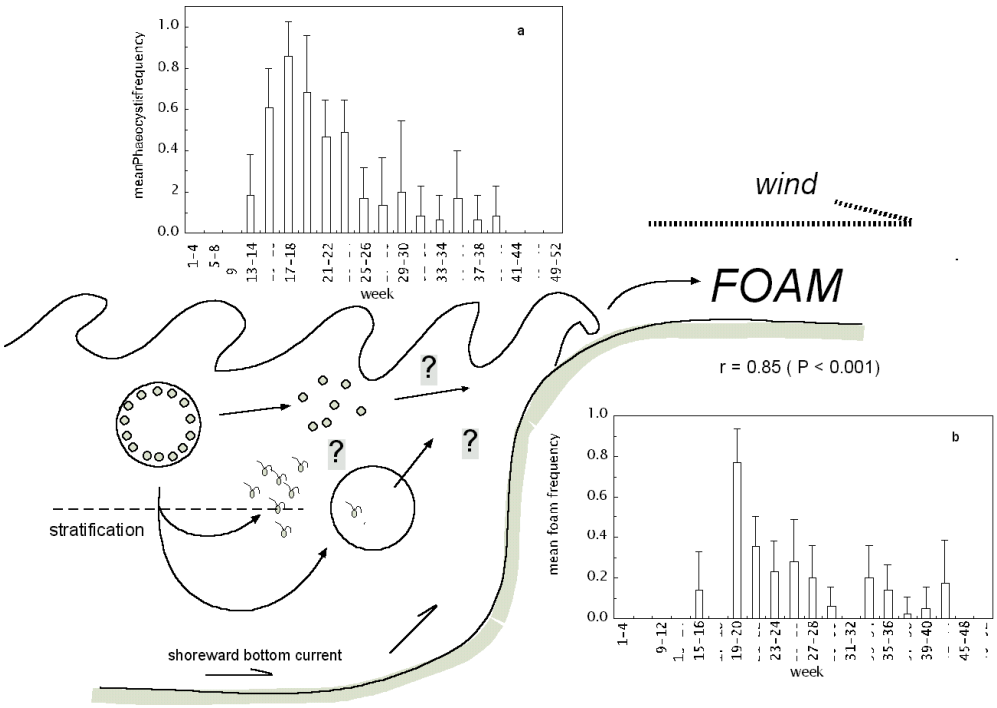


Fig. 21. *Phaeocystis globosa* blooms and foam on the coast. The correlation between the bloom frequency of *P. globosa* in the Dutch coastal zone (panel a) and the frequency of foam observations on the nearby coast of Holland (panel b) was significant (r = 0.85). (L. Peperzak, M. Rademaker & L.P.M.J. Wetsteyn, unpublished). However, the exact mode of production and foam composition are unknown.

Raphidophytes

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Introduction

The raphidophytes are a group of potentially toxic phytoflagellates with benthic cysts (Fig. 22). Prominent genera in marine waters are *Chattonella*, *Fibrocapsa* and *Heterosigma.* In Europe, harmful raphidophytes have been observed recently, mainly in the past two decades (Fig. 22 and references therein). Ichthyotoxic blooms, killing wild and farmed fish, have been recorded in the North Sea west of Denmark and in southern Sweden (Lu & Göbel 2000; Backe-Hansen *et al.* 2001), in the Adriatic Sea (Anonymous 1998), and along the Atlantic coasts of Scotland (Ayres *et al.* 1982), France (Nézan *et al*. 1995; Erard-Le Denn *et al*. accepted), Spain and Portugal (ICES-IOC HAEDAT). In 1989, shellfish were killed in Portugal and in 1991 seafood became toxic in Spain (ICES-IOC HAEDAT).

It has been hypothesized that several raphidophyte species have been introduced recently by shellfish imports (Billard 1992) or by ballast water discharge (Connell 2000). An extension of this recent-introduction hypothesis states that the continuous introduction of formerly isolated raphidophyte populations may lead to ongoing sexual reproduction, and consequently an enhanced capability to form harmful blooms (Kooistra *et al.* accepted).

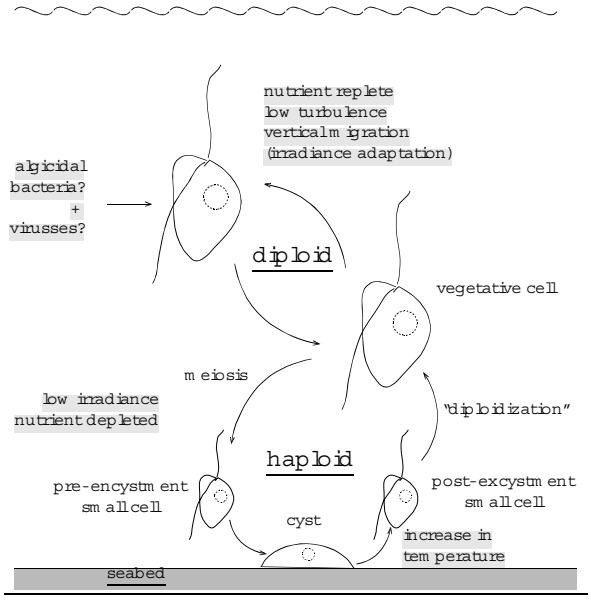


Fig. 22. Schematic haplo-diplontic life cycle of raphidophytes in which diploidization and meiosis have not been observed (adapted from Imai *et al.* 1998) with the environmental factors involved in growth, mortality and life cycle transitions indicated in grey. According to Nakamura *et al.* (1990), however, the haploid 'small pre-encystment cells' fuse to produce diploid 'triangle-shaped' planozygotes that in turn transform into (diploid) cysts (hypnozygotes).

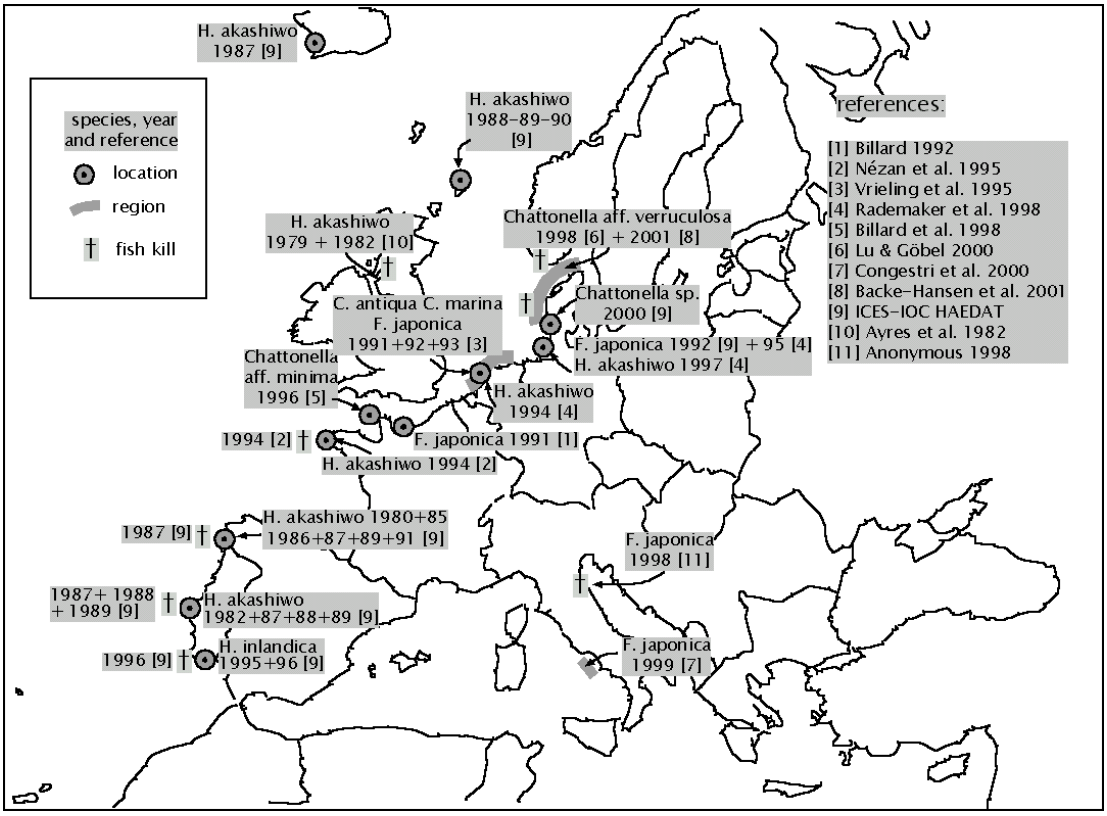


Fig. 23. Observations of potentially harmful raphidophytes and ichthyotoxic events in Europe [references 1-8] were augmented with data from the ICES-IOC HAEDAT (Harmful Event Database, [9]). Flagellate 'X' [10] is *H. akashiwo* according to Li & Smayda (2000).

Knowledge gaps

According to Yamaguchi and Imai (1994), the bloom-forming vegetative flagellate cell type of *Chattonella antiqua* and *C. marina* is large (>20 µm) and diploid, the cyst is haploid and is formed from a smaller haploid flagellate (Fig. 23). On the other hand, Nakamura *et al.* (1990) claim that the cyst of *C. antiqua* is diploid and is formed after the fusion of the small pre-encystment cell to a triangle-shaped planozygote.

The life cycle of *Heterosigma akashiwo* also features non-motile vegetative cells that are agglutinated in large numbers and encapsulated with mucus (Yamochi 1984; Smayda 1998). Apparently, less is known about the life cycle of *Fibrocapsa*, although the presence of cysts was also confirmed (Yoshimatsu 1987). In general, cysts are formed when both nutrient concentrations and irradiance are low. Very little is known about how meiosis and the reformation of the diploid phase take place (Imai *et al.* 1998).

The potentially harmful species identified in European waters are *C. antiqua, C. marina, C.* aff. *verruculosa*, *Fibrocapsa japonica*, *Heterosigma akashiwo* and *H. inlandica* (Billard 1992; Nézan *et al.* 1995; Vrieling *et al.* 1995; Anonymous 1998; Billard *et al*. 1998; Rademaker *et al.* 1998; Congestri *et al.* 2000; Lu & Göbel 2000; Backe-Hansen *et al.* 2001; ICES-IOC HAEDAT). *Chattonella subsalsa* (Mignot 1976),

C. aff. *minima* (Billard *et al*. 1998) and *Olisthodiscus* sp. have been observed in Europe but they are not considered harmful.

So far, harmful effects on fish in Europe have only been described for *H. akashiwo* (Nézan *et al.* 1995; ICES-IOC HAEDAT), *H. inlandica* (ICES-IOC HAEDAT), *F. japonica* (Anonymous 1998) and *Chattonella* aff. v*erruculosa* (Lu & Göbel 2000; Backe-Hansen *et al.* 2001). *Heterosigma inlandica* allegedly caused shellfish mortality in Portugal (ICES-IOC HAEDAT).

Furthermore, with the exception of *F. japonica* (Khan *et al.* 1996) neither the composition of the toxins produced by raphidophytes in Europe, nor the toxin concentrations in raphidophyte blooms have been measured so far. Therefore, more work is needed on the capabilities of toxin production by European raphidophytes and the environmental circumstances that promote toxicity and other potentially harmful effects such as mucus production.

Identification of flagellate raphidophytes in Dutch coastal waters has been performed with light microscopy and with antibodies developed against Japanese strains of *Chattonella* (Vrieling *et al.* 1995). The random amplified polymorphic DNA (RAPD) technique was used to detect differences between species and strains of cultured Japanese *Chattonella* spp. (Murayama-Kayano *et al.* 1998). Whole cell fluorescent *in situ* hybidridization (FISH) and sandwich hybridization assay (SHA) techniques for the identification and quantification of *H. akashiwo* and *F. japonica* have been developed in the USA and New Zealand (Tyrell *et al.* 2001). It can be concluded that the development and application of molecular tools in the identification of raphidophytes has started. However, a complete 'tool box' for all harmful raphidophytes and their life cycle stages is still not available.

A general problem is that the fixation of the delicate raphidophyte cells is difficult, leading to morphological changes and in extreme cases to rapid disintegration and cell loss. The fragility of raphidophyte cells may jeopardize the identification and enumeration of life cycle stages when techniques are employed that are based on some kind of morphological recognition (Tomas 1997; L. Peperzak unpublished results). For the fixation of *F. japonica* a new formaldehyde-Lugol fixative was recommended by Rademaker *et al.* (1998); this fixative should also be tested on other raphidophytes.

Cysts can be sampled and enumerated using standard techniques. The cysts of *C. antiqua* and *C. marina* are, however, morphologically indiscriminable (Imai *et al.* 1998). Raphidophyte cysts may not have been recognized by dinoflagellate-cyst investigators. Furthermore, raphidophyte cysts adhere to solid surfaces such as diatom frustules and sand grains, which may obscure their presence (Imai *et al.* 1998). The presence of cysts in Europe has gone unnoticed and their possible role in bloom formation in coastal regions of Europe has not been studied. It is remarkable though, that blooms in Japan are initiated at temperatures around 20°C that induce cyst germination, whereas in Europe blooms consisting of vegetative cells are usually observed at lower temperatures.

Blooms may be regulated by nutrient concentrations; nutrient depletion probably induces the formation of haploid pre-encystment cells (Imai *et al.* 1998). There are also indications that viruses may be involved in bloom termination, as in the case of *H. akashiwo* (Nagasaki *et al.* 1994), whereas Imai *et al*. (1995) suggested that algicidal marine bacteria killing *C. antiqua* and other raphidophytes also influence their population dynamics.

Approach

Studies should be carried out using field samples (water and sediment) and by collecting environmental data (temperature, salinity, nutrient concentrations, irradiance in the water column). Attention should be paid to small flagellate cells (pre- and possibly post-encystment cells), cysts and possible bloom-affecting species-specific viruses and bacteria.

In addition, experiments should be carried out in the laboratory. Life cycles should be reproduced for each species and the contradiction between the Nakamura *et al.* (1990) and Imai *et al.* (1998) life cycle schemes with respect to cyst formation and cyst ploidy should be resolved (see Fig. 22). The possible toxin production of several potentially harmful species should be established.

Pathways of raphidophyte introductions (shellfish, ballast water) should be identified and possible counter-measures should be investigated.

Methodologies and tools

In general, the techniques for studying life cycles, life cycle strategies and the inducement (triggers) of transitions between the life cycle stages that were discussed for other groups during the LIFEHAB Workshop may be applicable to raphidophytes. The cells' fragility enhances the need to use molecular techniques (e.g. SHA and RAPD) that do not depend on morphological characteristics, and to develop fast and reliable fixation and counting methods. Techniques to identify cysts are needed. Modelling blooms using fuzzy logic tools as, suggested for diatoms, dinoflagellates and haptophytes, will probably also be useful for raphidophytes.

Recommendations

- Investigate the presence of raphidophyte cysts in Europe, and their pathways of introduction
- Extend identification tools with non-morphological (molecular) techniques
- Extend identification tools for all life cycle stages, including the haploid small flagellates and the cyst
- Establish the role of cysts in bloom formation (especially the role of temperature in cyst germination)
- Investigate the role of viruses and bacteria in bloom termination
- Measure toxin production capabilities in European raphidophyte strains

Links with other EuroHAB activities

Raphidophytes are ichthyotoxic and, therefore, studies of their life cycle are linked to investigations of other fish killing groups: haptophytes (*Chrysochromulina, Prymnesium*) and dinoflagellates (e.g. *Karenia mikimotoi*), especially with regard to the identity of the toxins and the mode of toxin action. There is also a link to studies of the introduction of harmful species via ballast water. In BioHAB, the role of viruses and bacteria in bloom termination is studied, which is also of interest to the study of raphidophyte blooms. In HABES, fuzzy logic expert systems (models) are developed for haptophyte, dinoflagellate and diatom HABs.

Priorities

- Reliable identification and enumeration techniques
- Role of cysts in bloom dynamics
- Bloom dynamics: role of nutrients (including trace elements and vitamins), irradiance and temperature

Acknowledgements

Thanks are due to my LIFEHAB colleagues for their comments. Especially the help of Dr. Bente Edvardsen (NIVA Oslo), Prof. Chantal Billard (Université de Caen) and Karin de Boer M.Sc. and Dr. M. van Rijssel (University of Groningen) in the final preparation of the manuscript is gratefully acknowledged. Dr. Monica Lion (IOC-IEO Science Communication Centre on Harmful Algae in Vigo) kindly provided the raphidophyte events filed in HAEDAT (Harmful Alga Event Database).

Life cycles in cyanobacteria

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Introduction

In marine and brackish waters, blooms of cyanobacteria are formed by representatives from several genera of the orders Oscillatoriales (*Trichodesmium* spp., *Lyngbya* spp.) and Nostocales (*Nodularia spumigena, Anabaena* spp., *Aphanizomeno*n sp.). Those orders correspond to Subsections III and IV of the Bergey's Manual of Systematic Bacteriology (Castenholz 2001).

In general, bloom-forming cyanobacteria exhibit simple developmental cycles. They reproduce by cell division perpendicularly to the longitudinal axis of the trichome. Planktonic forms of bloom-forming cyanobacteria are immobile. Vertical movement may, however, appear as a result of sinking and buoyancy, regulated by accumulation of photosynthetic ballast or formation and collapse of gas vesicles which is regulated by light and nutrients (Krompkamp *et al*.1986; Oliver 1994).

Oscillatorialean cyanobacteria from the genera *Trichodesmium* and *Lyngbya* produce only vegetative cells and do not therefore exhibit particular life cycles.

Vegetative cells of Nostocales (genera *Anabaena, Aphanizomenon, Nodularia*) may differentiate into heterocysts or akinetes. Heterocysts are specialized cells where nitrogen fixation takes place. Heterocysts are characterized by thick cell walls, low phycobiliproteins and lack of photosynthesis. They protect the O_2-labile nitrogenase complex from inactivation by oxygen. Heterocyst formation is stimulated in the absence of combined nitrogen (ammonium or nitrate), thus this property provides an important ecological advantage in nitrogen-limited growth environments.

Akinetes are thick-walled cells which are resistant to cold and desiccation. However, akinetes of *Nodularia spumigena* do not resist extremes of dryness or humidity (Pandey & Talpasayi 1981). The factors resulting in akinete differentiation vary from one species to another, and are likely to differ according to growth environment. Akinete differentiation may be triggered by phosphorus limitation (van Dok & Hart 1996) and low temperature (Li *et al*. 1997), and highest akinete formation is often observed during the decline phase after a bloom. Akinete formation of *Nodularia* is sensitive to increasing salinity (Jones *et al*. 1994). Exposure to UV-light delayed cell differentiation *Anabaena aequalis* to akinetes (Blakefield & Harris 1994).

Akinete germination of cyanobacteria requires light. Red light (600-650 nm) is the most active and green light (450-550 nm) the least active part to stimulate akinete germination in *Anabaena* spp. (Pandey & Talpasayi 1981; Reddy & Talpasayi 1981; Yamamoto 1976; Kezhi *et al*. 1985) and *Nodularia spumigena* (Huber 1985). Temperature and pH optima for akinete germination for several species are consistent with their growth optima. Response of germination to nutrients is variable and depends on species. For example, germination of akinetes of *Anabaena circinalis* was suppressed by NH_4, insensitive to NO_3 and required PO_4. (van Dok & Hart 1997), whereas variable results have been obtained for *Nodularia spumigena* (Huber 1985). Trace elements have been found to influence akinete formation and germination in *Nodularia spumigena* (Yumnan & Reddy 1989).

Speculations have been expressed about the role of akines as an overwintering strategy in subarctic and arctic waters. In the Baltic sea, *Aphanizomenon* is present in the water column year round (Niemi 1973) and can be found in sea ice (Laamanen 1996), and therefore akinetes probably do not play a role as a seed population. The situation might be different for *Nodularia spumigena*, because this species requires higher temperatures for growth and has only occasionally been encountered in winter samples. In warm and temperate waters where blooms of Oscillatoriales occur, conditions for vegetative growth are suitable year round and overwintering strategies are not needed.

Knowledge gaps

The role of akinetes as a seeding population for *Anabaena* spp., *Aphanizomenon flos-aquae* and *Nodularia spumigena* in the Baltic Sea is not known. Published information about the factors triggering akinete formation and germination is scarce, and originates from different environments (e.g. rice fields) from where the HABs form. No studies have been undertaken which would clarify the role of akinetes in bloom dynamics in the Baltic Sea, - i.e. in which bloom stage they form, into what kind of basins they concentrate, how they are transported by currents, what triggers their germination, how they enter the euphotic layer, etc.

The relevance of *Gymnodinium catenatum* (Dinophyceae) over-wintering planktonic population *vs*. cysts as seedbeds for the local development of toxic blooms off Western Iberia

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Introduction

Since 1976, *Gymnodinium catenatum* Graham has been frequently recorded in western and southern Iberian waters and has been responsible for the main PSP events reported in this region (Estrada *et al.* 1984; Fraga *et al.* 1990; Moita 1993). Much research effort has been put into understanding its bloom dynamics. However, the origin of the inoculum and the environmental conditions that lead to a sudden and massive bloom are still not clear.

Gymnodinium catenatum identification problems and geographical distribution in Europe

Gymnodinium catenatum Graham is an unarmored chain-forming dinoflagellate with a life cycle involving a vegetative planktonic stage and a benthic hypnozygote (cyst) (Blackburn *et al.* 1989). Most studies of natural samples are based on the species identification in the form of chains or the characteristic microreticulate cyst (Bravo 1986; Anderson *et al.* 1988). Other life-cycle stages, such as single cells, gametes or planomeiocytes are normally overlooked.

Until recently, the taxonomic problems with *G. catenatum* were related to the misidentification of other species with a very similar vegetative stage, e.g. *Gymnodinium impudicum* (Fraga & Bravo) G. Hansen & Moestrup, or a similar resting cyst, e.g. *Gymnodinium nolleri* Ellegaard & Moestrup and *Gymnodinium microreticulatum* Bolch & Hallegraeff (Fraga *et al*. 1995; Bolch *et al.* 1999; Ellegaard & Moestrup 1999; Daugbjerg *et al*. 2000).

Based on the present knowledge, the geographical distribution of *G. catenatum* in European waters seems to be limited to the north by the front of cape Finisterre (NW Spain) (Blanco 1995b; Fraga 1996). The species extends its distribution southwards into the Mediterranean Sea and Moroccan waters (Hallegraeff & Fraga 1998). Previous references to *G. catenatum* in Northern Europe are now recognized as the species *G. nolleri* (Ellegaard & Moestrup 1999).

Where is the inoculum for bloom initiation?

The origin of the bloom population is still not clear, and the two possible hypotheses, a planktonic over-wintering population *versus* benthic cyst beds, are still under debate. There are references in the literature that favour both these hypotheses. According to Fraga *et al*. (1988), *G. catenatum* blooms can form from the accumulation of populations present in shelf waters due to convergence during downwelling conditions. An offshore inoculum of *G. catenatum* associated with oceanic waters along the Iberian coast or with the Azores frontal region was also suggested by Fraga (1996) and Moita *et al.* (1998). On the other hand, Figueiras & Pazos (1991) suggested that cyst germination in the Galician Rías may be the source for the inoculum for *G. catenatum* blooms. In

this situation, according to Fermín *et al.* (1996) and on a short-time scale, the sequence of downwelling-upwelling events may be the mechanism responsible for the local selection and development of *G. catenatum* populations in Ría de Vigo.

Studies on the distribution of cysts in western Iberia have shown that cysts of *G. catenatum* are frequently found on the shelf off NW Portugal and Galicia and have their most northern distribution in Ría de Muros, south of Cape Finisterra (Blanco 1995b; Bravo & Ramilo 1999). Along the Portuguese coast, cysts of *G. catenatum* were ubiquitous, with a distribution center located in the central region of the western coast (Amorim 2001). In most cases, the observed cysts were mainly empty (95% - 100% of total *G. catenatum* cysts, Amorim *et al.* 2001), suggesting both that cysts germinate as soon as the short mandatory dormancy period is completed (< 12 days), and that extensive cyst beds do not accumulate in the area. The few *G. catenatum* cysts with cell contents may represent cysts that lost their viability and will never excyst, or that have been reworked from deeper sediments and may still germinate, or they may represent cysts that are being produced in the overlying waters. If the later hypothesis is true, data on cysts with cell contents seem to indicate that cysts are being produced either in mid summer or late autumn-winter (Amorim *et al.* 2001). Cysts may therefore be continuously providing an inoculum to the water column, but it seems less plausible that blooms have their origin from massive cyst germination. However, as observed in the Ría the Vigo, we cannot neglect the possible importance of cyst germination in the development of local *G. catenatum* blooms (Figueiras & Pazos 1991; Fermín *et al.* 1996).

Are there particular areas where physical conditions may favour the development of *G. catenatum*?

On the central coast of Portugal, the highest relative abundance values of *G. catenatum* cysts were observed south of recurrent plumes associated either with major capes or bottom topography (Amorim 2001). Accordingly, during summers of 1985 and 1994, previous to the occurrence of extensive and massive autumn blooms of *G. catenatum* along western Iberia, the motile stage was also restricted to central Portugal, in the region of Lisbon (Fig. 24) (Moita 1993; Moita *et al.* 1998). As exemplified in Fig. 24, the occurrence of this species was favoured along the eastward side of an upwelling plume extending southwards, within warmer waters (Moita *et al*. submitted). A similar distribution pattern was observed in summer 1999, but it was not followed by the development of blooms. These results seem to indicate that along the eastward edge of plumes that extend southwards, there is likely to be a weaker possibly northward flow where phytoplankton populations such as chain-forming dinoflagellates may accumulate close to the core of the upwelling plume without being advected away. A similar situation was observed to occur along the leeward side (south) of plumes extending westwards where *G. catenatum* cells were observed to accumulate near the coast (Moita *et al*. submitted). These results suggest that flow fields associated with upwelling plumes must play an important role on the dynamics of *G. catenatum* populations on the central coast of western Iberia. Even when this species was rare along the coast, *G. catenatum* was recorded where it was apparently being concentrated by physical accumulation (Moita *et al.* submitted). The retention area described here is probably a general phenomenon that helps to explain patches of *G. catenatum* elsewhere on the Iberian coast.

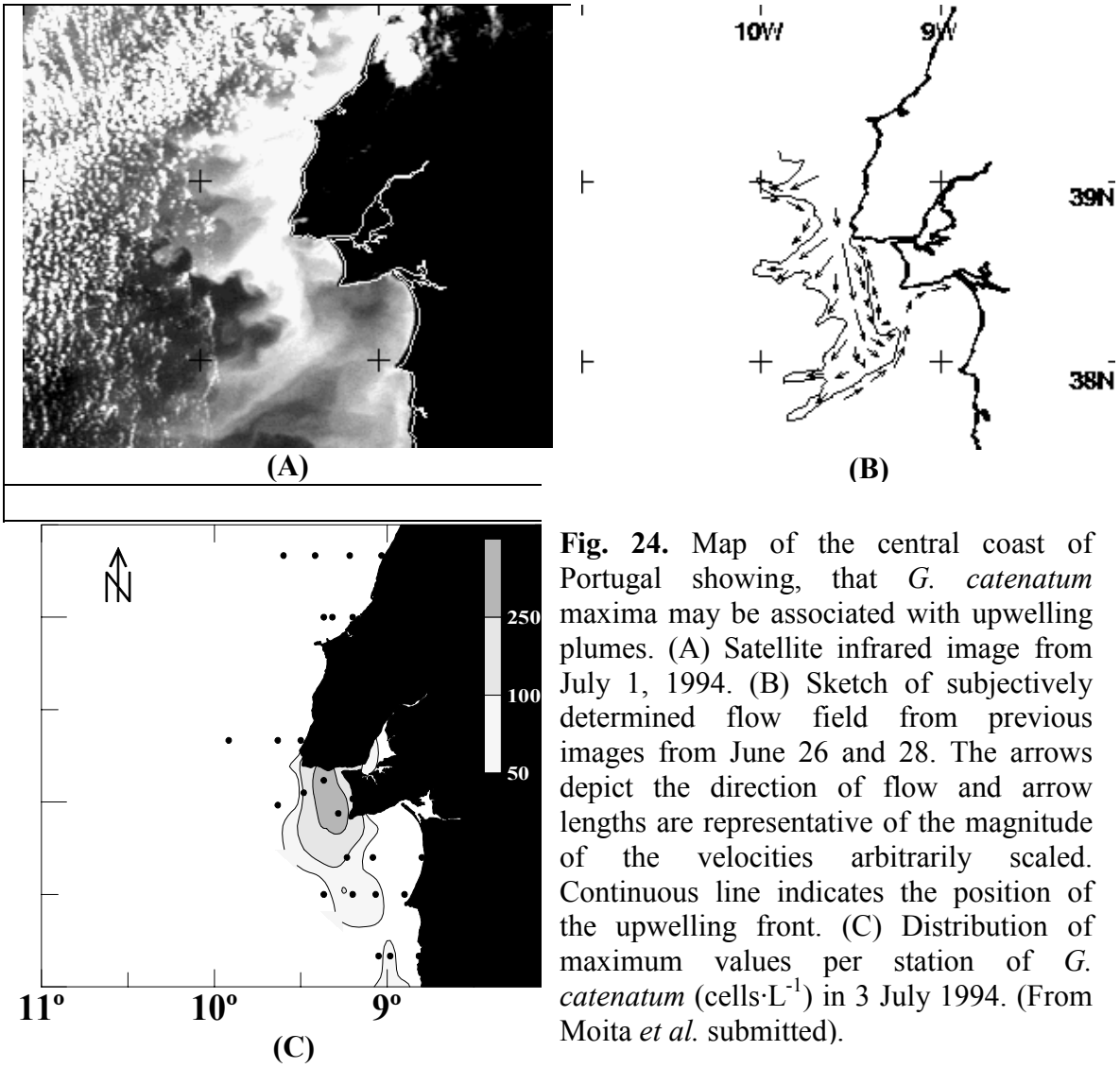


Fig. 24. Map of the central coast of Portugal showing, that *G. catenatum* maxima may be associated with upwelling plumes. (A) Satellite infrared image from July 1, 1994. (B) Sketch of subjectively determined flow field from previous images from June 26 and 28. The arrows depict the direction of flow and arrow lengths are representative of the magnitude of the velocities arbitrarily scaled. Continuous line indicates the position of the upwelling front. (C) Distribution of maximum values per station of *G. catenatum* (cells·L^{-1}) in 3 July 1994. (From Moita *et al.* submitted).

Bloom initiation may depend on a minimal concentration of cells in the water column (~100 cells·L^{-1}, Slobodkin 1953 in Wyatt, this volume). These concentration values may be favoured in areas of retention where the species is dividing asexually without being dispersed, or in areas of physical accumulation of cells. Under favourable conditions, results presented above suggest that these areas could represent the source inoculum for the autumn blooms along the Iberian coast, and support the hypothesis that blooms can be originated from an over-wintering planktonic population. Furthermore, these concentration areas may favour sexual reproduction by increasing the probability of gamete encounter, eventually leading to cyst production. In this case, cysts could be produced even during the absence of recorded blooms.

In order to further investigate if there are persistent residual populations of vegetative cells, even when blooms do not occur, future field investigations should include sampling strategies with higher spatial and temporal resolution, particularly focused on potential areas of retention or accumulation, such as the south of major capes.

Harmful algal events in Irish waters. The importance of life cycles from an ecologist's perspective

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Introduction

Within the Irish coastal zone, research on harmful algal events is directed towards the elucidation of the origin of azaspiracid, the distribution of *Pseudo-nitzschia* spp. and production of domoic acid, and the dynamics of harmful algal events and their interactions with the physical environment. Culture of *Protoperidinium* and *Pseudo-nitzschia* is still at an early stage, but other efforts are addressing the questions pertaining to the sources and sinks of harmful populations. Recent evidence has shown:

- how far high density populations of *Karenia mikimotoi* can be advected;
- where *Dinophysis* may encyst (and excyst);
- new locations where *Alexandrium* may overwinter.

Karenia mikimotoi

In a recent series of papers (Hill *et al*. 1996; Hill *et al*. 1997a; Hill *et al*. 1997b; Brown *et al*. 1999; Horsburgh *et al*. 2000), it has been demonstrated that associated with tidal fronts are fast (> 0.2 $m \cdot s^{-1}$), narrow (10-20 km wide) baroclinic jets which bound cold (or salty), dense pools of bottom water which can remain trapped in deep basins on the continental shelf during the summer months. One of the key insights of this work is that the bottom density front induces significant along front transport of seston, including phytoplankton. The pool of cold dense winter water, isolated below the thermocline due to the onset of stratification, is also separated from warm tidally mixed waters close to the coast by horizontal bottom density fronts. Dynamically, it would be expected that narrow jet-like circulations would also be associated with these fronts (Garret & Loder 1981) and has been demonstrated to occur along the northeastern English coast (Brown *et al*. 2001) and elsewhere.

More specifically, a summertime survey during 1998 (Brown *et al*. 2001) revealed that the thermally stratified Celtic Sea is dominated by strong and well-organised jet-like flows. Here, a broad comparatively slow northward flow passes along the Cornish coast. In St. George's Channel the flow narrows and intensifies, crossing the channel and returning south, either into the centre of the Celtic Sea or as a near coastal westward flow. Exchange between the Celtic and Irish Sea is comparatively weak. Data presented by Raine & McMahon (1998) indicate that flow continues around the south-western tip of Ireland. Recently collected and as yet unpublished data provide good grounds to believe that the flow extends northwards along the entire western coast of Ireland.

Karenia mikimotoi is known to occur in high densities all around the southern, eastern and northern perimeter of the Celtic Sea. For example, blooms have occurred in the vicinity of the Scilly Isles, associated with the Celtic Sea Front, and off the south and southwest coasts of Ireland. The inference is therefore that what we are studying is a coherent population transported anti-clockwise around the perimeter of the Celtic Sea. This occurs as an intense sub-surface maximum associated with the pycnocline,

manifesting itself at the surface at tidal or weak coastal fronts. Yet we know very little about survival strategies of this organism, particularly those such as the production of exotoxins (Gentien 1998), which are related to local small scale turbulence, and are very likely to be significant during the weeks required to transport high density populations along the path outlined.

***Dinophysis* spp.**

The bays of southwestern Ireland support approximately 80% of the national rope cultured mussel (*Mytilus edulis*) production. Contamination with DSP toxin is a regular problem in the region, and is caused by advection of populations of *Dinophysis* spp. (*D. acuta* and *D. acuminata*) into the bays (Raine *et al*. 1993; McMahon *et al*. 1998). These populations are known to accumulate in a region of slack residual circulation off the south coast of Ireland. High density sub-surface layers have been observed here, with concentrations of up to 125 cells·mL^{-1} (*D. acuminata*). This area, which in summer becomes depleted in oxygen (<70% saturation), is relatively high in remineralized nutrient and is thought to support a resident population of both species of *Dinophysis*. If this is the case, then elucidation of the timing of excystment, and the potential of the organism to form high density thin layers in its life cycle becomes of vital importance in prediction of these events.

Alexandrium

There is a resident population of *Alexandrium tamarens*e within Cork Harbour on the south coast of Ireland (McMahon & Silke 1997). The population regularly appears in summer, and the location of a seed bed for cysts has been identified. Occurrences of *Alexandrium* elsewhere around Ireland have been very infrequent and in low cell density.

Field investigations in the summer of 2001 revealed that the salinity of coastal waters along the west coast of Ireland was noticeably reduced out to the Irish Shelf Front. This front is a surface to bottom front separating Irish coastal water from water of more oceanic characteristics (S >35.2). It runs roughly parallel to the western Irish coastline up to 150 km offshore. Salinities of 34.9-35.1 observed prior to 2001 inside this front were reduced by approximately 0.3-0.4, after the wettest autumn on record. An extended *Alexandrium* cf. *minutum* bloom was evident near the coast in the northwest. Surface concentrations of 30-50 cells·mL^{-1} were recorded in the vicinity of Erris Head, Co. Mayo, and examination of water samples showed its presence in a wide area over the shelf. Although toxicity results suggest that the strain was not toxic, this is the first recorded instance of such an extended bloom in the region. A key question as regards its life history is what is the influence of increased run-off in promulgating the bloom, which most likely had an origin in one of the many coastal inlets along the western Irish seaboard.

The role of alternate life stages in the timing and succession of phytoplankton blooms

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Introduction

Harmful algal blooms are often regarded as "exceptional" events, "in that they are 'noticeable', particularly to the general public, directly or indirectly through their effects, e.g. visible discoloration of the water, foam production, fish or invertebrate mortality or toxicity to humans" (ICES 1984). A somewhat different concept is that of "unusual" or "episodic", which implies that a new event occurs, either caused by the presence of a species not recorded before, or due to unprecedented abundance, or to harmful effects unexpectedly brought about by some otherwise common species. From an ecological point of view, most harmful algae are "normal" components of plankton communities, which become noticeable because their presence interferes with human activities. In this context, the main question to answer in order to gain predictability of harmful events concerns the mechanisms driving the seasonal pattern of occurrence and recurrence of algal species in general.

Following the physico-chemical paradigm, which has dominated ecological theories on plankton over the last decades, species occurrence and abundance are driven by a set of optimal or adequate environmental factors. Several different models have been suggested (Margalef 1978; Reynolds 1988; Reynolds & Smayda 1998) whereby selected groups of species occur regularly in association with a set of physical and chemical conditions. On the other hand, only limited consideration has been given to the role of endogenous regulation in species timing (Hensen 1887; Garrison 1981; Eilertsen & Wyatt 2000).

In this paper, I will deal with the occurrence of harmful phytoplankton species, with two main questions proposed for discussion:

- how do harmful/non-harmful species succeed in being present at a given time of the year?
- to what extent do heteromorphic life cycles affect the regularity of seasonal patterns of occurrence of harmful/non harmful species?

The timing of species occurrence

The concept of annual recurrence in the occurrence and peaks of phytoplankton species underlies the modern paradigms of phytoplankton species succession and is widely accepted since the first multi-annual investigations, e.g. in Kiel Bight (Germany):

> "One form appears, grows and vanishes yet again from the surface waters...and this play repeats itself year after year with the same regularity as every spring the trees turn green and in autumn lose their leaves; **with just such absolute certainty as the cherries bloom before the sunflowers, so *Skeletonema* arrive at their yearly peak earlier than the *Ceratium*"** (Schutt 1892, translated by Mills 1989)

Here the parallel with the terrestrial vegetation is explicit. In fact, there are two different analogies, one referring to the seasonal cycle of the caduceus leaves and flowering trees, the other to the annual timing of seed flowers. Both are relevant to the issue of phytoplankton timing. Microalgal species that form resting/dormant stages have been compared to seed plants (Wyatt & Jenkinson 1997) which allocate energy in protecting their gametes and ensuring an optimal anchoring and/or dispersal mechanism. On the other hand, species that do not form resting cysts/spores presumably persist through the unfavourable season at minimum physiological levels, comparably to leafless trees. In this context, literature data can be explored to ascertain whether a difference in timing pattern is found between species adopting one or the other of the two life-strategies mentioned above.

Annual timing in cyst- and spore-forming species

There are not so many studies that compare cyst and vegetative stage occurrence in the marine environment (Wall & Dale 1968). However, it is clear that a rather high heterogeneity of life-strategies exists even within the apparently homogeneous category of organisms that are able to regulate their presence in the water column through excystment/encystment dynamics.

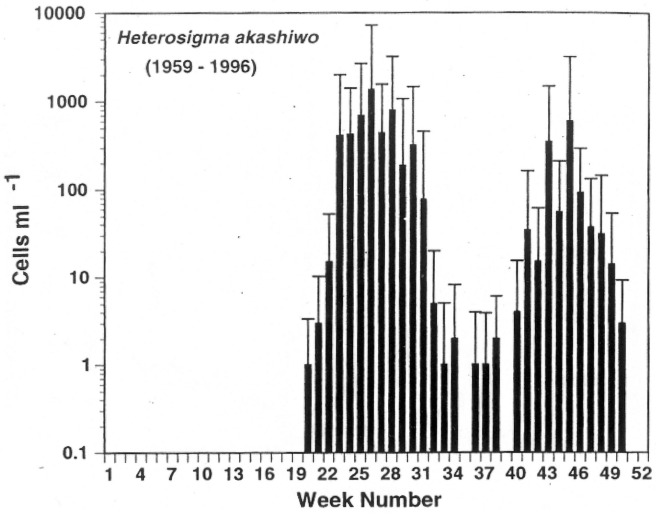


Fig. 25. The analysis of a long-term data series shows that the cyst-forming raphidophyte *Heterosigma akashiwo* rarely occurs in Narragansett Bay before the 19th week of the year (Li & Smayda 2000).

Within the same community of cyst- forming species, three kinds of germination-time modes were distinguished in Onegawa Bay, Japan (Ishikawa & Taniguchi 1997): species whose cysts germinated a) incessantly (*Scrippsiella* spp.), b) sporadically (*Protoperidinium conicum*) and c) seasonally (e.g. *Protoperidinium conicoides*, *Ensiculifera carinata*). Only the latter case would result in a rather synchronous contribution to plankton, whereas the other strategies apparently reflect a more opportunistic behaviour.

In the case of *Scrippsiella hangoei* and *Peridiniella catenata* (Kremp 2000a; Kremp 2000b), a six-month mandatory dormancy period allows the species to

withstand unfavourable conditions and be ready to begin its growth season, preceding the fast-growing diatoms at the early stage of the annual succession. Despite the similar occurrence pattern, notable life-strategy differences were found between the two species: *S. hangoei* produces many cysts at the end of the bloom but their germination rate is low, whereas *P. catenata* produces a lower number of cysts with a high excystment success.

Synchronous diatom spore germination triggered by the 12:12 photoperiod at the spring equinox was hypothesized to be the start of the early spring bloom at several sites from the Norwegian coast (Eilertsen *et al.* 1995).

Other cases of recurrent occurrence of cyst-forming species are known. These include *Heterosigma akashiwo* (Fig. 25) (Imai & Itakura 1999; Li & Smayda 2000) and *Alexandrium tamarense* (Wyatt & Jenkinson 1997). Both endogenous and exogenous triggering factors were invoked to explain regularity in the occurrence of these species. For *H. akashiwo*, the occurrence timing was associated with temperature, whereas for *A. tamarense* the lunar cycle (Wyatt & Jenkinson 1997), or an internal clock (Anderson & Keafer 1987) were suggested.

Annual timing in species without heteromorphic overwintering stages

The hypothesis that benthic stages initiate the regular occurrence of plankton species was put forward by Hensen (1887), based on his observation in the Kiel Bight. At the same site, however, the most predictable species in the annual cycles are non-cyst forming species, such as *Ceratium*, whereas species with benthic resting stages show a high interannual variability (Smetacek 1985). A similar situation is recorded in the Gulf of Naples, where non-cyst forming species like *Pseudo-nitzschia* spp., *Prorocentrum triestinum* and *Calciopappus caudatus* show a higher regularity as compared to the cyst-forming *Scrippsiella* spp. and *Chaetoceros socialis*. It appears that opportunistic or "timely occurrence" strategies are equally spread among cyst and non-cyst forming species.

What can be the trigger for a seasonally recurrent pattern in cyst and non-cyst forming species? The physico-chemical paradigm could be invoked, yet the regularity of occurrence is higher for the biological than for the physical signal (see e.g. Li & Smayda 2000). This suggests a possible role of internal clocks and/or of more reliable environmental variables such as the photoperiod.

Conclusions

To be present when favourable conditions exist is one of the main keys to the success of a species. Especially if the favourable condition includes not only physical and chemical factors but also compatible biological interactions (e.g. reduced grazing pressure, limited competition, etc.), a species will achieve the biomass needed for sexual reproduction, cyst production, increase of genetic variability and for other processes that promote its persistence. Literature suggests that both species that produce heteromorphic resting stages and species that apparently do not can display a remarkable punctuality in their annual occurrence. A wide range of mechanisms could favour this punctuality. These include circa-annual clocks of the growth response, reaction to changing photoperiod, mandatory dormancy for resting cysts. These mechanisms highlight the importance of biological regulation in plankton succession, and suggest the need for investigations that aim at clarifying species-specific life-strategies.

Application of ploidy analysis and DNA sequencing to examine life cycles in harmful microalgal species

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Introduction

Molecular biological tools have improved our ability to clarify life cycles in microalgal species in several ways. In order to identify the organisms in general and more specifically to identify all life cycle stages in cultures and field samples, methods such as DNA sequencing and molecular probes (oligonucleotide probes, antibodies, specific primers) can be used. Flow cytometry and microfluorometry are valuable tools to determine ploidy level in cell types to indicate their role within a life cycle. Expression of genes regulating cellular processes connected to the life cycle may be measured by e.g. real-time PCR, expressed sequence tag, and differential display of mRNA. In order to determine the significance of a life cycle strategy for bloom dynamics of a harmful algal species (HAS), spatial and temporal genetic diversity as well as gene flow should be estimated using DNA fingerprinting, e.g. microsatellites (Molecular techniques, this volume)

Application of molecular methods to phytoplankton biology and ecology is a large and rapidly expanding field. I will restrict this report to briefly describe the use of DNA sequencing and ploidy analyses in life cycle studies of HAS, and present examples on how these methods have been used within the genus *Chrysochromulina* to obtain information about the life cycle of these algae.

DNA-sequencing

The nuclear ribosomal operon (rRNA genes and spacers) has been extensively used to genetically characterize microorganisms and study their phylogenetic relationships. There is now a huge database available for the small and large subunit rRNA genes (SSU and LSU rDNA), with more than 3000 SSU sequences for eukaryotic organisms in the Ribosomal Database Project (Van de Peer 2000). When the coding rRNA genes do not provide sufficient resolution, non-coding regions such as internal transcribed spacers ITS 1 and ITS 2 rDNA may prove to be valuable markers. These spacers are expected to have higher variability than the coding SSU and LSU regions and are usually suited for studies at the species or population level.

Identification of an organism (vegetative cells, zoospores, cysts, etc.) by DNA sequencing includes the following steps: 1) isolate cells from natural material or culture; 2) extract DNA (not obligate); 3) amplify DNA region by PCR; 4) sequence PCR-products; and 5) analyze data and compare (align) with existing sequences in databases. Information about the genetic variability within the taxon and related taxa is necessary to decide on the identity of a cell type. At the species level, it is thus necessary to determine the nucleotide sequences of several species within a genus and several strains within a species. Once the DNA sequence of a taxon and related taxa has been established, one can also design oligonucleotide probes that recognize only certain taxa (see Guillou, this volume). The detection method can be by e.g. specific PCR, fluorescence *in situ* hybridization, real-time PCR assay (Bowers *et al.* 2000) or heteroduplex mobility assay (Oldach *et al.* 2000).

Ploidy analyses

There are several methods available to determine ploidy levels in microalgae. The most direct way is to count the chromosome number in stained cells with condensed chromosomes under the microscope. This has been done in e.g. dinoflagellates, which have a large and continually condensed nucleus (e.g. Trench & Blank 1987). When the nucleus and chromosomes are small, such as in haptophytes, measurement of the total amount of DNA in the nucleus is more convenient. Cells are then stained with a fluorochrome binding specifically and quantitatively to DNA. The intensity of the emitted light (fluorescence) from each cell is then measured by a microfluorometry system connected to an epifluorescence microscope or by a flow cytometer. In a microfluorometry system one cell is analyzed at a time, whereas with flow cytometer thousands of cells can be analyzed within a few minutes. DNA microfluorometry was applied to determine ploidy levels in e.g. *Chattonella* spp. (Yamaguchi & Imai 1994) and flow cytometry for life cycle studies in e.g. *Phaeocystis* spp. (Vaulot *et al.* 1994).

Chrysochromulina polylepis

In the following, I will describe how the above methods have elucidated the life cycle of *C. polylepis* and related species. As previously described (Edvardsen, this volume), *C. polylepis* was found to have two morphologically distinct, motile cell types (authentic and alternate) that first were thought to be separate species. However, repeated examinations in transmission electron microscopy (TEM) of six alternate clone cultures (cultures originating from a single alternate cell) during more than a year showed that authentic cells appeared in all cultures after about 3-13 months.

The nucleotide sequence of the SSU rRNA gene was determined in alternate *C. polylepis*, in part of the gene of authentic *C. polylepis,* and in several other *Chrysochromulina* species (Edvardsen & Medlin 1998). The two *C. polylepis* forms were identical in the analysed region, whereas the other *Chrysochromulina* species differed in 22-110 bp out of 1802 bp in the gene. The first internal transcribed spacer (ITS 1) was then analyzed in three alternate and three authentic *C. polylepis* strains from Norway, one authentic *C. polylepis* strain from Sweden, one *C.* aff. *polylepis* strain from Great Britain and four strains of other *Chrysochromulina* species. Strains of both cell types isolated from Norway and Sweden were identical in this region, but differed from that in the British strain and other *Chrysochromulina* species. Interspecific variation in the ITS 1 regions was very high, with about 50 per cent divergence between closely related species. Interspecific length variation in ITS 1 was so great that it prevented reliable sequence alignment (Fig. 26).

DNA sequencing of *C. polylepis* and related taxa have shown that authentic and alternate cells are one and the same species.

Flow cytometric (FC) ploidy analyses were performed to investigate whether the two forms were joined in a sexual life cycle (Edvardsen & Vaulot 1996). Authentic cultures contained cells with the lowest level of DNA and these were termed haploid (Fig. 27A). In two pure alternate cultures the cells contained twice as much DNA as authentic cells and were termed diploid (Fig 27B). Other apparently pure alternate cultures contained both haploid and diploid cells or only haploid cells. The life cycle of *C. polylepis* apparently embraces at least three cell types: authentic haploid, alternate haploid and alternate diploid cells. The ploidy level of benthic stages has not yet been determined.

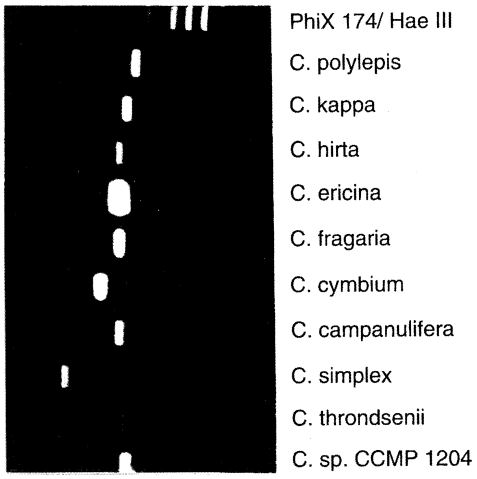


Fig. 26. Agarose gel with ITS 1 rDNA fragments from *Chrysochromulina* species obtained by PCR. The fragments of *C. polylepis* consisted of 436 bp. (From Medlin *et al*. 2000b).

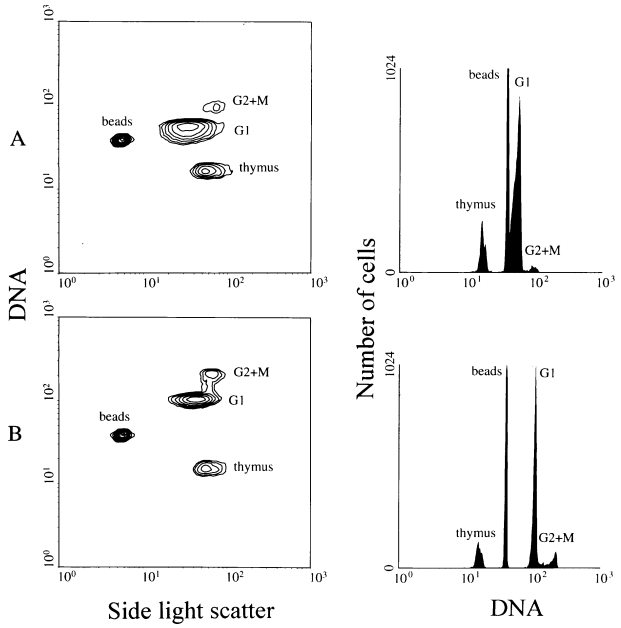


Fig. 27. Flow cytometric signatures of authentic (A) and alternate (B) *Chrysochromulina polylepis* cultures growing exponentially in continuous light. Left: Distributions of DNA content per cell (log green-orange fluorescence) versus side light scatter (related to cell size, arbitrary units, AU). Right: Distributions of DNA content per cell (log green-orange fluorescence, AU). G1, G2 and M refer to phase in the cell cycle. Nuclei of calf thymocytes served as internal standard for DNA fluorescence and beads (0.95 μm) for light scatter. (Modified from Edvardsen & Vaulot 1996).

Chrysochromulina spp.

Since three motile cell types are included in the life cycle of *C. polylepis*, other *Chrysochromulina* species could also be joined in a common life cycle. The nucleotide sequence of SSU rDNA of 28 *Chrysochromulina* strains representing 22 species were determined (Edvardsen *et al*. 2000; Eikrem *et al.* unpubl. data). All species were different in this gene and as a rule identical within a species, indicating that they were all separate species and not joined in a common life cycle with a different species.

Different strains of a species were compared morphologically (TEM), genetically (ITS 1) and in genome size (FC) to examine whether more species possess alternate stages.

Two ploidy levels were found in four *Chrysochromulina* species: *C. polylepis*, *C. ericina*, *C. hirta* and *C. kappa* (Edvardsen 1998). The ITS 1-region was identical within a species. It could well be that other species also have two ploidy levels, but that not both were represented in our cultures.

Conclusion

In conclusion, both SSU and ITS 1 rDNA have proven valuable molecular markers in identifying a link between morphologically different, alternating forms of *Chrysochromulina*. FC analyses of DNA content per cell provided indirect evidence for a sexual haplo-diploid life cycle in several *Chrysochromulina* species.

Different molecular techniques to examine life cycles of phytoplankton or HAB species

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Introduction

Life history stages play a key role in harmful algal bloom (HAB) dynamics. Approximately 10% of the 2000 marine species of dinoflagellates are known to produce resting cysts (Dale 2001). However, the presence of as yet unknown resting cysts is suspected to occur in numerous other species (e.g. *Karenia mikimotoi* and *Dinophysis acuminata*). Furthermore, extensive ecological studies are hampered by difficulties in identifying the cysts, as this usually requires germination of the cysts. In addition, it is difficult to detect organisms at low concentrations in mixed natural assemblages. The advantage of molecular techniques is that they may allow, without prior cultivation, a clear identification of the different stages of HAB life cycles. When applied to field samples these techniques may also provide the precise detection of both vegetative cells and resistant cysts, which are either free living in the water column or associated with sediment. In the future, molecular tools will probably permit the study of genetic regulation of the encystment-excystment processes in HAB species.

Detection of taxa using destructive-PCR methods

During ecological studies, it is important to detect and rapidly characterize HAB stages both in culture and natural samples. In this context, destructive-PCR assays have been the most employed to date. They have been successfully employed to detect various toxic dinoflagellates in natural samples (see Table 5). A target gene is amplified using general or specific primers and the polymorphism of the PCR products is assessed by different fingerprinting methods.

Limitations of the destructive-PCR methods: Destructive methods provide only a semi-quantitative assay (Penna & Magnani 1999; Bowers *et al.* 2000; Coyne *et al.* 2001), as gene copy number and amount of DNA are variable between species and strains. Furthermore, information on the cell morphology is lost. For example, destructive methods do not allow discrimination between the life stages (resting cysts or vegetative cells), or even free DNA.

Table 5. Examples of different PCR-based assays applied to toxic dinoflagellates.

PCR-based essay	Confirmation of the PCR products	Target species	Reference
Direct PCR	RFLP	*Karenia mikimotoi* *Alexandrium minutum*	Godhe *et al.* 2001
Direct PCR	Cloning and sequencing	*Gonyaulax polyedra*	Rollo *et al.* 1995
Semi-nested PCR	Direct sequencing	*Karenia mikimotoi* *Alexandrium* spp. *Dinophysis acuminata*	Guillou *et al.* submitted
Direct PCR	HMA/SSCP	*Pfiesteria piscicida*	Oldach *et al*. 2000
Direct PCR	DGGE	*Pfiesteria piscicida*	Coyne *et al.* 2001

Detection and quantification of taxa using non-destructive methods

In order to be able to connect two different morphological cell types within the same life cycle, we often need to work using non-destructive methods. **Whole and single-cell PCR approaches** allow us to characterize life cycle stages, by placing entire cells directly in the PCR tube. Several authors (Galgani *et al.* 1994; Bolch 2001; Edvardsen *et al.* in prep; Guillou *et al.* submitted) have demonstrated that this method can be applied to intact, living and fixed algal cells or to resistant cysts. Non-destructive methods such as **whole-cell hybridization** (Adachi *et al.* 1996a; Anderson *et al.* 1999), are the most promising methods to quantify accurately target cells in natural samples. Field samples are collected and after being fixed and dehydrated, cells are hybridized with class, genus, or species specific oligonucleotide probes which are labelled with fluorochromes. Hybridized cells are then enumerated under a fluorescence microscope or by flow cytometry. Within the last 10 years, fluorescent *in situ* hybridization (FISH) has been widely used to identify free-living or substrate-associated bacteria in aquatic environments (Amann *et al.* 1995). With a confocal microscope, discrimination between cysts and vegetative cells is possible (see Fig. 28).

Genetic regulation of transitions between different life cycle stages

So far, encystment and excystment pathways and sexual reproduction determinisms in HAB species have been studied exclusively using morphological or chemical methods. Identification of the genes involved in these processes and how they are regulated will lead to a better understanding of triggering factors. Since very little is known about the genetic regulation of these processes, the first step must be to compare the genetic expression in the two phases.

There are different strategies to analyze expressed DNA or mRNAs (converted in cDNAs), such as cDNA subtraction, EST method (Expressed Sequence Tag), and differential display. cDNA subtraction consists in the hybridization of one set of cDNAs with the other one, in order to detect unique or differentially regulated expressed genes.

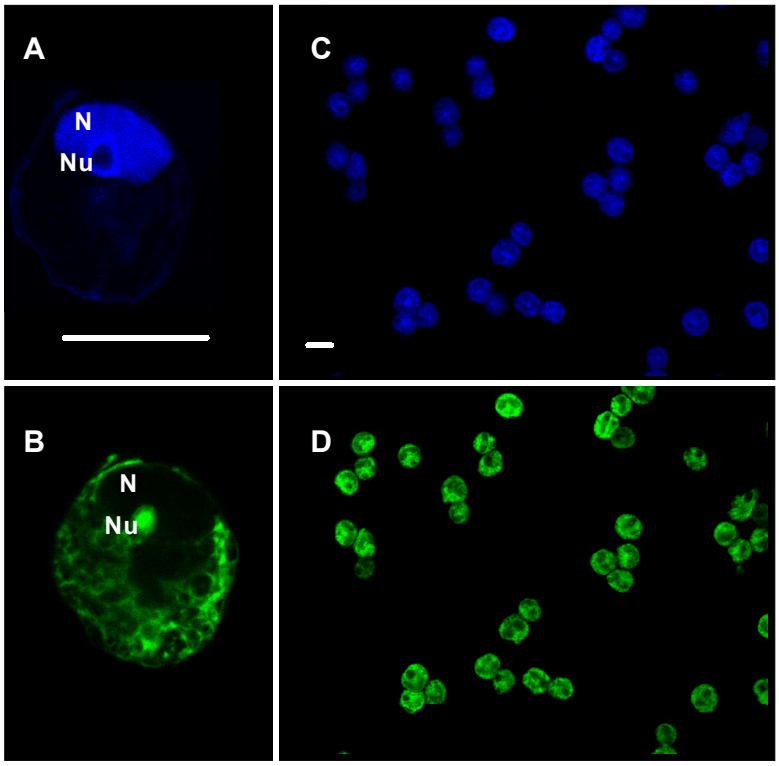


Fig. 28. Cysts (A, B) and vegetative cells (C, D) of *Alexandrium fundyense* detected by TSA-FISH (tyramide signal amplification of fluorescent *in situ* hybridization) and confocal microscopy. (A) Condensed nucleus, stained with DAPI surrounding an unstained nucleolus (blue, excitation 380 nm). (B) The same section as (A) but excited at 488 nm (green), showing cytoplasmic and nuclear (nucleolus) 18S rRNA hybridized with a general eukaryotic probe (EUK1209R). (C) Vegetative cells stained with DAPI (blue, 380 nm excitation). (D) The same image as (C), but excited at 488 nm (green), showing 100% of the cells hybridized with the general eukaryotic probe. N = nucleus; Nu = nucleolus. Scale bars = 20 μm. (After Biegala *et al.* accepted).

This method was recently applied to identify the gene family expressed during the sexual reproduction of the diatom *Thalassiosira weissflogii* (Armbrust 1999). EST analysis consists of systematic sequencing of extremities of randomly picked cDNA clones. In general, 40% of the putative functions of partial coding regions can be assessed by sequence comparison. Recently, it was also applied to compare the microscopic gametophyte and macroscopic sporophyte of the brown algae *Laminaria digitata* (Crépineau *et al.* 2000). The differential display allows the detection of more rare mRNAs. Two or more sets of cDNAs are amplified by PCR using a combination of randomly selected primers and then compared on agarose or polyacrylamide gel electrophoresis. Differentially amplified cDNAs can then be excised, cloned and sequenced. This method is rapid and sensitive. It was recently applied to assess the cell cycle-regulated genes in the toxic dinoflagellate *Alexandrium fundyense* (Taroncher-Oldenburg & Anderson 2000) and the excystment-regulated genes in the Ciliophora *Sterkiella histriomuscorum* (Villalobo *et al.* 2001).

Models of bloom dynamics: what is needed to incorporate life cycles and life stages?

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Introduction

The study of harmful algae is a subject of growing interest which requires a detailed scientific knowledge of an heterogeneous group of organisms exhibiting very complex life cycles, toxic or noxious effects being associated to one specific life form and to a density threshold. Numerical experimentation is valuable, to test the current knowledge of HAB dynamics and to explore different scenarios of life stage transitions and success.

Modelling is a stepwise process that includes three complementary objectives: 1) understand laboratory-controlled process studies and estimate relative importance of different processes studied *in vitro*; 2) *in situ* validation of a set of hypotheses derived from *in vitro* experiments; and 3) extrapolate and simulate different scenarios. Such an iterative process is necessary for the integration of all the knowledge into a general scheme.

There are different types of HAB models, which mostly depend on the occurring "harmful event".

Modelling only the biomass (so-called biogeochemical models) implies that an average population is considered and is not species-specific. This hypothesis is valid if one exploits the results only in terms of biomass production and does not extend interpretations to species in particular. In the context of this conceptual averaging procedure, all phytoplanktonic diversity is reduced to few biological reactors (diatoms, non-diatoms, …) transforming chemical energy and light into biomass with averaged kinetic parameters. The driving forces are traditionally inorganic nutrients, light and temperature. This approach has some advantages depending on the objectives pursued, such as eutrophication and climate change. However, simplifications made do not allow any prediction for a single species and they are not suitable for HABs because HABs are species-specific.

Modelling a species of interest requires specific knowledge of a whole suite of processes among which are the following: inoculation scheme (geographical and timing), vegetative cell growth and loss factors (specific grazing, bacterial and viral infection) and sinking. It is essential to realize that nutrient depletion may not be the sole cause for bloom decay. Knowing what influences a species is not really sufficient and the interactions within the community are often essential, which demonstrates the need for embedding the species-of-interest into a general biomass model. As an example, the *Phaeocystis* model developed by Lancelot (Lancelot *et al*. 1997) has 3 phytoplankton groups (diatoms, nanophytoplankton and *Phaeocystis*), each with its specific light, temperature, nutrient requirements, grazing control, biodegradability and sinking rate.

Modelling a toxic event imposes additional constraints which are related to the physiology of toxin production (depending on the life stage and physiological

conditions) and cell density (i.e. which threshold is required to induce toxic or noxious effects). Spatial heterogeneity is commonly observed, the "bloom" usually does not involve large biomass at the scale considered for a spring bloom. For this reason, vertical averaging of a population is not generally suitable. This raises the more general question of the definition of spatial scaling for the population which will be discussed later.

At this stage, there is no model reproducing a full life cycle of one species. Even one of the most advanced modelling exercise on *Phaeocystis* (Lancelot *et al.* 1997) considers two semi-independent state variables because the factors triggering the colony form are not known.

The framework for population dynamics

The robust equation for the local number of organisms in one life stage in per unit volume can be written in the following form:

$$\frac{\partial n_i}{\partial t} = \mu n_i - m n_i - \nabla.(n_i \bar{v}) - \nabla.(n_i \bar{u})$$

with μn_i = growth; m n_i = mortality (grazing, mechanical damage, death); $\nabla.(n_i \bar{v})$ = 3-D, time variable transport; and $\nabla.(n_i \bar{u})$ = motion of organisms relative to water.

A major challenge is to understand the processes to a level of detail that allows a simplified mathematical formulation (parameterization) and that still reproduces the salient features of one HAB population dynamics.

For each of the life stages, a specific simplification will have to be made because it is likely that within any life stage, the most important processes will differ. A precise knowledge of the transitions between stages (timing and yields) will also be critical to the success of the modelling exercise.

In terms of modelling, these life cycle transitions induce switches between systems which involve either a different parameterization or a different set of process formulations. It is therefore understandable that not only the primary processes regulating one phase must be properly defined, but also that the correct timing (or triggering factors) have to be applied for adequate simulation of the population history (Fig. 29). Another difficulty resides in the sometimes large differences in time scales, like the transition from vegetative to dormant phases.

Knowing the large diversity in harmful algal species, it is obvious that generalizations for this heterogeneous group of species responsible for harmful algal events would probably be inadequate. On the basis of present knowledge, it is my intention to present some general ideas, but no specific recipe. Interactions between meso-scale physics and behaviour, although important, will not be discussed in this presentation.

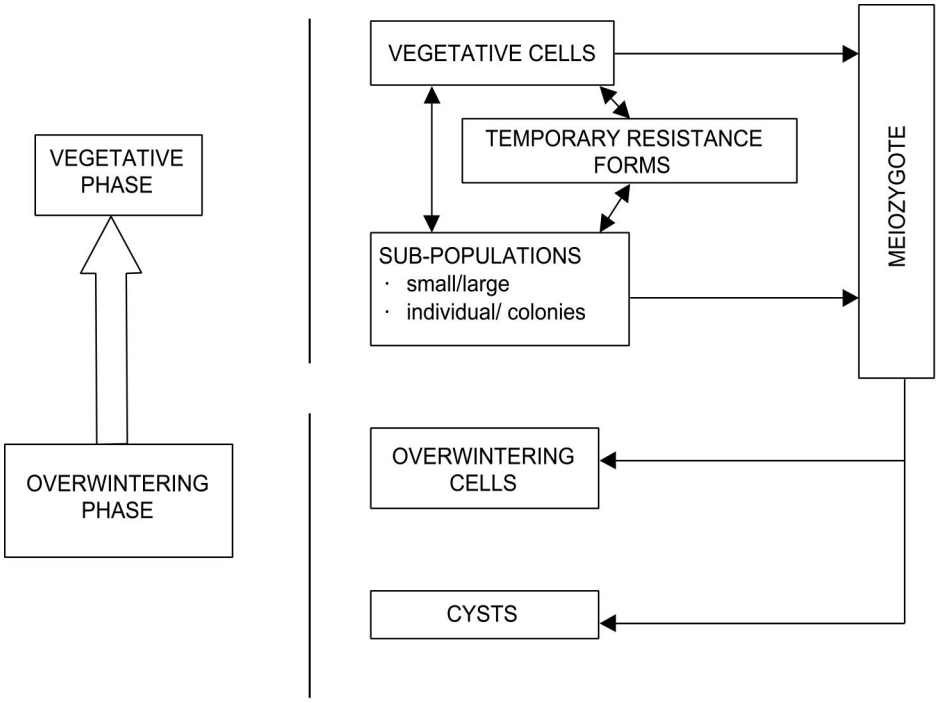


Fig. 29. Schematic flow diagram between the different state variables. Case specific studies may not require this degree of complexity. Each transition (arrows in the diagram) requires definition of triggering factors and fluxes between the two stages.

The vegetative phase

Although usually simulated, the exponential growth phase is not known in sufficient detail for many species of interest. It is rather common in HAB species that all cells do not divide at the same rate. Some may enter temporary dormancy, differentiate between large and small cells, produce colonies and individual cells; each of these stages having consequences on the overall growth rate of the population.

Some species present cell differentiation in size while in the vegetative phase. It has been described for *Karenia mikimotoi* both in the field and *in vitro* (Gentien 1998). Cell size tended to be smaller towards the end of the bloom but both sizes were present during the bloom. Partensky & Vaulot (1989) separated small and large cells of *K. mikimotoi* having the same taxonomic characters and DNA content. They reported that small cells could produce large cells by simple enlargement of cells during the stationary phase but that, in contrast, production of small cells by budding of the left epicone took a variable number of generations ranging from 1 to 50. This indicates that the formation of small cells is probably not continuous, but appears sporadically. Parameters inducing this differentiation are not yet defined. However, these authors concluded that small cells preferentially used nitrate, whereas large cells could use both nitrate and ammonium. The reduced size of the small cell sub-population allows an optimization of photon capture and nutrient uptake (Raven 1986). Partensky & Vaulot (1989) reported a division rate of 1 d^{-1} for small cells, whereas large ones divided at 0.6

d^{-1}. Such a large difference in exponential growth deserves further studies on the induction of small cells budding. Cell size differentiation has been noted by different authors for different species of *Alexandrium* and appears rather common, including possibly *Dinophysis* (Reguera, this volume) with unexplored consequences on growth rate.

Phaeocystis is a good example of differentiation between the colonial form and single cells (Lancelot & Rousseau, this volume).

When environmental conditions become unfavourable, many species create temporary cysts. *Alexandrium* cells submitted to mechanical stress may produced temporary cysts (Garcés, this volume), with a behaviour differing from vegetative cells in terms of motility. They still appear to produce. This may confer to the population some advantages in terms of limiting dispersal.

Another type of temporary cyst phase without ecdysis has been observed in the case of *Alexandrium tamarense* and *A. minutum*. Laabir & Gentien (1999) have reported this quiescent stage, following cell transit in the tractus of *Crassostrea gigas*. This stage had a duration of up to 4 hours. Up to 25% of the cells were constantly in this stage during a 24 h hourly sampling scheme, which induced a bias in cell cycle estimation. It is not known if this occurs in the pelagic phase and what consequences this might have on the gross and net growth rate.

Factors inducing these transient stages are not defined and their consequences on population net growth are unknown, but are likely.

Overwintering encystment

This section deals specifically with species producing cysts, an important group of species in terms of toxicity.

A population producing overwintering stages can only maintain itself in an estuary or a geographical area for years if the ratio

$$\frac{cyst_{n+1}}{cyst_n} \geq 1$$

This ratio represents the yield in cysts with an excystment potential based on viability and availability of cysts in a geographical unit from the year 1 to the year 2 at the same period. Wyatt & Jenkinson (1997) in a comprehensive paper, present some of the critical elements ruling the population dynamics and base their discussion on the seed desert plants. The major problem is the integration contour inside which the total number of cysts is estimated. Resolution of this phase and understanding the recurrence of blooms from one year to the next requires mapping of seedbeds and modelling of sediment transport during at least annual cycles. Such global sedimentary models are now within reach, however annual transport modelling of one single particle type (such as the cyst of one species) is not yet available.

Alexandrium toxic events are often confined to estuaries and do not spread into neighbouring estuaries. In this case, the cyst distribution is bounded by a threshold in mating success. Populations expelled to the more open sea at low tide are presumably diluted to a point at which gametes when produced have negligible encounter probability.

Encounter rate of particles is described by a collision kernel (after Pruppacher & Klett 1980), which depends on different components (isotropic particles motion, differential sedimentation and shear). Gametogenesis is probably, due to cell membrane modifications, favouring encounter. Probert (this volume) has observed flagellar

attachment. Understanding the changes in membrane properties and in behaviour during the gametogenesis could shed some light on the following. The success rate of mating has been reported to be 20-40% (Anderson *et al.* 1984), whereas Wyatt & Jenkinson (1997) had to invoke pheromones from theoretical considerations. A possible change in cell effective diameter could similarly increase the encounter rate. All contributions to the collision kernel depend on the radius (r) of the particles to different powers of r. In the case of emission of a chemical signal, the radius to be considered would the radius of a sphere due to radial diffusion from the cell surface (this radius depending on the outflux from the cell and the decay rate of the signalling agent). An increase in the efficient cross-section will lead to an increased collision rate.

Some studies have produced evidence of increased polysaccharide cell excretion under N-deprivation (Engel 2000). It is known that gametogenesis is induced when cells are nutritionally unbalanced (Probert, this volume). A subsequent increase by 3 of the effective diameter (i.e. cell plus glycocalix or the chemical sphere radius) could increase by 27 the encounter rate for a given shear and a given cell concentration. The same rate would be achieved by a factor 5 cell density increase. In the real viscous world in which the cells evolve, this would only be approaching realty if transparent exopolymer particles (TEP) produced stays close to the cell; otherwise, it can be shown that TEP production would decrease the encounter rate, due to changes in viscosity.

As discussed above, to understand, simulate and predict the gamete mating success which determines the spatial distribution of the inoculum, a detailed study of fine processes integrating cell physiology and shear transmission through the viscous domain is needed.

Excystment and/or inoculum

Sexual reproduction, forming cysts, has not been observed for all species. Vegetative cells may overwinter as in *Karenia mikimotoi* which was detected at the level of 2 to 5 cells·L^{-1} in the Bay of Brest. This cell density is sufficient for the summer bloom build-up. However, it does not imply that the source cells are homogeneously distributed in a large area, and the location of the inoculum should be determined. It is obvious that the Gulf of Mexico is the source for *Karenia brevis*, just as Western Brittany seems to be the inoculum source for *K. mikimotoi* (Gentien *et al.* 1998).

For many species presenting a cyst phase, the relative contributions of cysts and vegetative stock have not been quantified. Conclusions reached by Wyatt (this volume) that the knowledge of the seed bed locations extended to overwintering cells has to be stressed, is contrary to what is required for a biomass model for which the inoculum does not appear critical.

When triggering conditions for excystment become favourable, the newly produced environmental conditions should be fit for growth and development of the population. That the relatively small number of factors triggering excystment (temperature threshold, bottom stress threshold, biological clock) are met does not necessarily imply that growth conditions are completely fulfilled (nutrient status, light, etc.). In those cases, the population starts to develop and does not reach the bloom stage and re-encysts or is dispersed. Latency time quantification and its possible variations with environmental factors appears to be essential.

For species relying on a seed bed, a certain degree of synchrony in excystment is also necessary for the build-up and maintenance of the population in a given area. Population must achieve a sufficient density to produce cysts at the end of the growing

period. If the vegetative cell flux from the sediment is too low, vegetative cells will be dispersed without any future chance to gather and mate. As the critical size for maintenance of a phytoplankton patch depends on the ratio of dispersive capacity to the growth rate, the limit flux of cells will be directly related to the intensity of diffusion which depends on the local hydrodynamics.

Conclusion

Species-specific models have to be designed before one may address simulation of harmful events. These models quite often require embedding into a general biomass model.

Each life stage requires a different set of equations covering the most essential processes. Transitions to each life stage have to be identified in terms of triggering factors (temperature, nutrients, light, biological clock) and yields. Special attention should be paid to transitions from slow to rapid systems since these switches may artificially condition the general dynamical behaviour of the model.

Confronting the Complexity of HAB Dynamics

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Originally, the modelling of HABs was viewed as a branch of physical oceanography, and the resultant numerical simulation models were based on the concept that plankton are passive elements in a complex fluid environment. Increased participation by biologists in HAB modelling has led to more realistic models, although simply adding information on the dynamics of algal life stages sometimes makes these models more complex without necessarily adding to the performance and reliability of the models. We should consider complementary approaches that are more appropriate for complex systems. Given the scarce and sometimes unreliable data contained in short time series for HABs, rule-based models may provide a good starting point for understanding and modelling these systems.

HAB dynamics are complex. Physical, chemical and biological factors interact in ways that are often erratic and unpredictable. Cysts are transported between the sediments and the euphotic zone, some organisms have alternate reproductive strategies and many different life stages, and undetectable traces of rare elements may play a critical role in triggering a bloom.

There are several different ways to approach the modelling of complex systems (Silvert 1981; 2001a). The most common and widely accepted method is to build the model from the ground up. This approach is highly reductionistic and is based on the idea that a model is an assemblage of submodels that represent all the various processes going on in the system. There is, however, a major flaw with these massive reductionistic models: they often do not work. It is an unfortunate fact that most really big models do not produce realistic results (Lee 1973). Of course the usual explanation is, "We must have left something out", but numerous studies have indicated that as models of this sort get bigger and bigger with more submodels and other details, they do not generally converge on an accurate solution. There are many reasons for this, one of which is that all modellers start from a personal background with a point of view determined by their perception of the system being modelled. If they do not understand all components of what they are modelling, they may make a real mess of it, or, at best, leave out something that is important. And although it seems intuitively clear that complex systems require complex models, in practice this is not always the case. In fact, the more details a model contains, the more likely it seems to be that the results of the model will turn out to be affected by details that were omitted, and the performance record of complex multi-disciplinary models is not good.

Originally most modellers in the HAB field were physical oceanographers, and while the physical aspects of their models were generally good, the chemical and biological components tended to be oversimplified. One common aspect of such models is that they treat plankton as passive tracers that are transported by water masses like dye patches, when in fact there are several processes that can cause plankton to move relative to the water. However, it is fortunate that HAB modelling has moved out of the grip of the physical oceanographers and increasingly involves biologists (cf. Lancelot *et al*. 1997; Verity 2000; Hannon *et al*. 2001).

Even so, it would be unfair to single out physical oceanographers as the only culprits committing crimes of omission in this matter of including all relevant factors. Biologists also tend to ignore important factors – for example, phytoplanktologists often seem oblivious to the effects of grazing and nutrient recycling by zooplankton, and the possibility that at different times and different conditions one might have both top-down (i.e. grazing) and bottom-up (i.e. nutrients) control of primary production is rarely countenanced by biological specialists. Thus, increased participation of biologists in HAB modelling has certainly improved the biological content of these models, but there is always a risk that not all biological factors are dealt with adequately, and of course there is also the risk that the quality of the physical oceanography may suffer if physical oceanographers do not remain fully involved. The unfortunate fact is that we all have our blind spots, and we must take this human failing into account when we design our models.

An alternative to the ground-up development of models based on what we know (or assume) about the dynamical structure of complex systems is to start from the top down and build models that behave in the way that is actually observed, rather than first building the model and then hoping that it generates reasonable behaviour. I originally referred to these models as “top-down”, but because of the confusion this caused with models of top-down control of ecosystems, I now prefer the term “data-driven”.

An important distinction should be drawn between data-driven modelling and statistical curve-fitting, although the two sound very similar. The goal of the statistician is just to fit the data, without concern for the structure of the model – that is why polynomial models are often used, even though nothing in nature generates polynomials. Data-driven modelling seeks to uncover the structure of the system by tracing its behaviour back to the processes that generate that behaviour. This distinction is particularly important in the study of HAB dynamics, since statistical models are generally only useful for interpolation – they can describe events that have happened before, or that resemble ones that have occurred in the past, but once you go beyond the historical record you have no way of knowing what will happen. Many HAB occurrences are extreme events, going well beyond what has been recorded in the past, and we need to understand the underlying dynamics in order to understand these processes. Furthermore, there are seldom sufficient data to fit a statistical model, given the multivariate complexity of algal blooms and the relatively short and incomplete time series that are usually available.

Rule-based modelling offers a very different approach, and although it can lead to substantially different types of models, it has proved very effective in developing understanding of complex systems. It has a charming simplicity that belies its power, and because it avoids many of the hidden assumptions of statistical and other more explicitly quantitative techniques, it actually has far greater flexibility.

The major problem with rule-based systems, and the probable reason why they have made minimal inroads in ecological modelling, is that it is difficult to state the rules in the precise way that scientists expect. The earliest successful applications of rule-based models were to discrete systems, where a crisply defined rule led to clearly defined outcomes. One of the earliest uses was in the design of computer systems, where the configuration choices could be determined by clear rules, but a better example of how a rule-based model can be developed, and one with obvious

application to modelling HAB dynamics, is in the area of medical diagnostics. Physicians also deal with complex and poorly understood systems, they almost never have adequate data with long time series on the objects they study, and yet they have to interpret a small set of observations to make life and death decisions. To deal with imprecise concepts such as "fever" and "pale", they use fuzzy set theory, a well-known but much deprecated branch of mathematics that has been around for about 35 years (Silvert 1997; 2000; 2001a,b). The strength of fuzzy set theory is that it brings mathematical reasoning into correspondence with common sense, whereas its weakness is that it sounds too much like common sense and not enough like mathematics. It is in fact very simple, so simple that we tend to say “it is too simple to be right” rather than “oh, the right answer is simpler than I thought”.

In the long run, our objective should be to use the results of rule-based models to develop the best possible models, and they can help by identifying patterns which should appear in the output of these models. Our understanding of how HABs develop should not stop with identification of simple patterns, such as a correlation with warm spring temperatures – we need to ask why these patterns arise. Is it stratification? Is it the chemical change caused by a non-harmful spring bloom? Does it have anything to do with grazing? The important aspect of any type of modelling is that we use all the information that we have, and use it effectively, which means that we should be prepared to apply any analytical tools that might lead to better understanding. Whether the resulting model is rule-based, or whether it is a numerical simulation model whose structure is developed in the context of identifiable rules, is not the major issue – what really matters is that we formulate the problem correctly, and not how we implement the representation of the system.

This abstract describes just a few of the tools for doing this, rule-based and fuzzy-rule-based models, but there are many approaches to the problem of organising subjective and even intuitive insights into a useful understanding of how complex systems work. In fact, a whole field of “knowledge engineering” has grown up around this type of analysis, using tools such as Delphi analysis to “extract” information from the minds of experts who might not be prepared to put their ideas into equation form, and to translate this information into models known as “expert systems”. Clearly one of the advantages of this type of approach is that it bridges the gap between people with understanding and people with mathematical expertise, a gap which is often manifest at meetings devoted to HABs and other complex phenomena.

It is not my intention to suggest that only rule-based systems can be used to develop models of HAB phenomena, and certainly there is no question that detailed simulations based on reductionistic fine-scale models of physical phenomena, going back at least as far as the early work of Riley (1946), have told us a great deal about primary production and the development of HABs in particular. The key issue is not whether any particular modelling approach has merit, but whether there is an approach so powerful and general that it is the only one we need – and the answer I suggest is clearly “NO”.

How can we combine the population dynamics of life history stages?

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Introduction

Dormant cells are well known stages of the life cycles of a variety of phytoplankton species from different groups, and probably obligatory in many of them. The duration of the dormant phase is often much greater than that of the multiplicative phase, the dormant cells are non-motile, and except in unusual circumstances are likely to remain in the same place until they hatch. Furthermore, their survival rates following settlement are likely to play an important part in population levels achieved in subsequent seasons. They would therefore seem to present more accessible targets for any control measures which might be applied in cases where a particular species becomes a pest. Yet in the past there sometimes seems to have been a certain reluctance, at least amongst plankton ecologists, to accept that these dormant stages do have a significant impact on the population dynamics of the planktonic phases, and in general it is probably fair to say that their importance has often been underestimated. The sources of this reluctance can perhaps be traced to an exclusive concern with events in the water column, to the planktonic perspective. This perspective is contrasted here with a view from the seed bank, and some ways are suggested in which both perspectives can be broadened to achieve a more integrated view of the population dynamics and life cycles of phytoplankters.

The planktonic perspective

The planktonic perspective is concerned with the dynamics of vegetative growth; the seeds are one source of inoculum, though not necessarily essential since low concentrations of vegetative cells conceivably form the latent phase which precedes population growth, or a bloom might be initiated by advection from elsewhere. The pre-bloom location and source of the inoculum are not normally primary concerns, but the numbers are. Simulation experiments of microplankton dynamics indicate that a minimal inoculum of the order of 10^{2} cells$\cdot L^{-1}$ is necessary to generate blooms on the observed time scales (Slobodkin 1953). So if concentrations of residual or advected cells are much lower than this, germination of resting stages begins to look essential. Similarly, seed formation may often be responsible for ending a bloom (e.g. Sgrosso *et al.* 2001), but again several other mechanisms are available to reduce population numbers, any or all of which might intervene before seed formation can take place (grazing, parasitism, dispersal), and it is with these other alternatives that most studies have been concerned.

If the disappearance of a bloom is a very sudden event, then the quasi-simultaneous formation of resting stages is a likely cause. One of its most important characteristics would then be its timing. Information on the yield of seeds, i.e. the ratio of seeds produced at the end of the growth season relative to the size of the inoculum at the beginning, is a necessary input variable to seed bank dynamics. In either case, the origin and fate of the seeds are not normally thought of as essential elements in accounts of bloom dynamics. But if life cycles are the focus of interest, or if it is established that seeds are a necessary prerequisite for the development of blooms, then their fate following a bloom needs to be taken into account.

Here the problems of describing the fate of resting stages following a bloom are examined. The area in which seeds settle is generally known as the seed shadow or seed dispersal kernel. It is assumed initially that the seeds behave as passive particles. Phenomenological models of the seed shadow are usually based either on the frequency distribution of the distance moved from the source, or on the density of seeds relative to the distance from the source, and can thus be described by probability density functions (PDF), with radial integration around the source. Observations of seed shadows in terrestrial environments indicate that these distributions are generally leptokurtic with long tails. This is presumably because local and long distance dispersal are qualitatively different since different processes can operate over different spatial scales. We must also remember that secondary dispersal following an initial settlement may be important as in many invertebrate larvae. A practical problem is that simple PDFs (gaussian, negative exponential,…) fail to provide sufficiently fat tails whilst functions which do so overestimate dispersal near the source. These considerations have suggested the use of hybrid models (Clark *et al*. 1999).

It is usually assumed that seeds disperse from a "point source", but it is obvious that the vertical and horizontal distributions at the time of seed production need to be taken into account. Except in static unstratified water columns, even for passively sinking particles, the form of the seed shadow will be determined by interactions between the oceanographic conditions and the distribution of the source. We can consider three extreme cases of vertical distribution of the vegetative population. It may be evenly dispersed throughout the mixed layer, or it may form a thin layer at the surface or in the pycnocline. These distinct vertical distributions will generate quite different seed shadows since the sinking particles will experience distinct flow patterns between their site of origin and the sediments. Perhaps an analogy can be drawn with the effects of the form of tree canopies on the fall of seeds in forests modelled by Cousens & Rawlinson (2001).

Even if seeds disperse passively, the distance and direction travelled are controlled by interactions between their density and form, species characteristics, and the density, viscosity and turbulent characteristics of the water (e.g. McNair *et al.* 1997). But many marine propagules are motile, can control buoyancy (cf. aerial spiders and clam larvae), the timing of seed production in relation to tidal and other cycles can be controlled, and the spatial location by e.g. vertical migration, and they may vary greatly in competency. For these reasons, mechanistic models of seed shadows which take these processes into account are likely to provide insights into seed bank dynamics additional to those provided by phenomenological models, and will be essential if the processes leading to seed production are to be incorporated into dynamical models of life cycles.

The seed bank perspective

We normally think of dormancy strategies that are effective on annual time scales (overwintering, aestivation), but shorter time scales are also important in many inshore environments, and free-swimming and quiescent stages are known to alternate with diurnal and lunar rhythms on e.g. intertidal mudflats and in tide pools. In insects, Mansingh (1971) recognized three categories of dormancy: quiescence, oligopause, and diapause. The ecdysal cysts of *Alexandrium* can be equated with Mansingh's quiescence, formed in response to non-cyclic deviations from normal environmental

conditions of short duration such as increased turbulence, and the zygotic cysts with the more highly evolved diapause in which cyclic unfavourable conditions for vegetative growth are anticipated and programmed for, and the return of favourable conditions does not necessarily break dormancy (Wyatt & Jenkinson 1997). In some species, there is a mandatory dormancy period; perhaps in those species in which this is not so, we might recognize a parallel to oligopause. Here the main emphasis is on diapause as defined by Mansingh.

Prakash (1967) speculated that *Alexandrium* cysts might overwinter and re-inoculate the same waters where they had been formed, and direct evidence of this was later obtained by Anderson & Wall (1978). In the context of dinoflagellates generally, Wall (1975) listed five functions of the cyst stage. Three of these are directly linked to the population dynamics of the planktonic phase, to re-seed the same area where the cysts were produced, to control the time at which the surface waters are repopulated, and to aid dispersal. The genetic role of cysts was also listed. Wall's last function was that of surviving unfavourable conditions, which is sometimes the only function recognized. From the perspective of the seed bank, the essential need for long-term survival is that it replenish itself. This demand is achieved by sending colonists into the water column which if they are successful will eventually provide new recruits to the sediments. There may also be midwater alternatives to the sediments, as has been suggested by Misha-Irina (Shirshov Institute) for the deep Mediterranean (unpublished).

In terrestrial biology, the evolution of dormancy is thought to be favoured in temporally unvarying environments where there is spatial variation in establishment conditions. This presupposes that the dormant stage can detect environmental quality. If this is not so, then dormancy strategies should indicate a temporally variable environment. It is also thought that life history attributes which reduce the impact of environmental variation on fitness show patterns of negative covariance (e.g. Ellner *et al.* 1998). Can we learn anything by transferring these intuitions to the planktonic environment? For example, is high variability in the duration of the season suitable for growth associated with early production of resting stages? The same trend might be associated with density dependence of the growth stage since reproductive potential would then be reduced as the season progressed (Taylor 1980). Or alternatively, is delayed germination associated with growth season variability? This strategy reduces the risk that all resting stages will be lost to an unfavourable year, and raises the long-term probability of persistence; but the advantage is offset by a reduction of short-term growth potential when conditions are favourable since the inoculum size is reduced. These questions have not been addressed for phytoplankton species, but there do not seem to be any reasons why they cannot be answered given appropriate sampling. From the point of view of the question posed in the title, the important information needed centres on the contribution the seed bank makes to the inoculum following germination, and how this contribution is distributed in time.

Combining the two perspectives

The key life history variables which emerge from these considerations are the time at which the vegetative phase produces resting stages, which in the absence of other causes dictates the end of the planktonic phase, and the proportion of resting stages which germinate, which, if it is the only source of inoculum, determines the initial growth rate. These two links connect the planktonic phase with the seed bed and

complete those life cycles in which there is a simple alternation between reproductive and mandatory resting stages. Only quite recently have attempts been made to model the impacts of seed inputs and outputs on the initiation and termination of phytoplankton blooms (Eilertsen & Wyatt 1998; 2000); this model provided inocula to the water column from resting stages, and caused the vegetative cells to undergo gametogenesis and syngamy to form resting stages at concentrations much lower than those dictated by the carrying capacity. It thus addressed two of the functions listed by Wall (1975), that of controlling the timing of the vegetative phase, both its beginning and its end, and survival through the unfavourable season. But the spatial problem raised by Prakash & Wall (1978) remains unresolved. How are the movements from the planktonic phase to the seed bank and back again controlled so that spatial coherence or closure is maintained? Circulation patterns can clearly aid this requirement; Pitcher (1990) describes a mechanism that achieves closure for diatoms in an upwelling system off South Africa, and we can suppose that a combination of depth regulation (by buoyancy control or vertical migration) and partially closed horizontal and vertical circulation patterns must form the general scheme for achieving closure.

Not all life cycles consist of simple alternations between vegetative and resting stages. The role of motile planozygotes is particularly intriguing, and may indicate that the settlement process is under some biological control. Some studies of benthic invertebrates have recognized a process of “directed dispersal” in which settlement is dependent not only on the quality of the sediment but on social constraints, and there is a disproportionate arrival of recruits to more favourable sites. Are such tactics possible in some species by virtue of motile planozygotes? Excystment too may be more than a simple one to one production of vegetative cells by planomeiocytes. We need more information on the functions of these “intermediate” stages of life cycles to reach a fuller understanding of the population dynamics of life histories.

GAPS IN KNOWLEDGE

LIFE CYCLES IN DIATOMS. D.G. MANN AND S.S. BATES

Diatoms make up only a small proportion of HAB species (APPENDIX 1). One genus in particular (*Pseudo-nitzschia*) is causing increasing problems in European waters. Other genera are problematic elsewhere in the world, but have the potential to cause problems in Europe, either if there were to be changes in the physical or chemical environment (e.g. through eutrophication or climate change), assuming that they are already present in small numbers or as hidden ("cryptic") species, or if they are introduced (e.g. via ballast water). Because many of these diatoms have not been known to cause problems until recently, little is known about their life cycles, especially in relation to bloom initiation. The Discussion Group concluded, however, that we need a better knowledge of the physiological ecology and life cycles of diatoms in general, not just HAB species.

Cell size

What is known about diatom life cycles is that the cells must decrease to a certain size threshold before they are capable of reproducing sexually, and that for most diatom species sexual reproduction regenerates the original large size of the cell via an "auxospore" (Mann, this volume; Bates & Davidovich, this volume). The difficulty is in determining when this actually occurs in the field because: (1) only a low percentage of the population may reproduce sexually at one time; (2) the sexual stages (gametangia, gametes, zygotes, and auxospores) may be difficult to identify; and (3) some of these sexual stages may be fragile and therefore not preserved during sampling. There is a wide size window for sexual reproduction and so synchronicity of induction in natural populations is unnecessary and indeed does not occur. Both the percentage of the population undergoing sexual reproduction, and its timing during the year, are unknown for the toxic and harmful diatoms of concern. Such information would help us understand the magnitude and timing of diatom HABs.

The Group discussed two approaches for determining when sexual stages are produced: (1) measurement of the size-frequency distribution of diatom cells over several growth seasons, but at short time intervals within each growing season; and (2) development of molecular probes for identifying both sexually induced cells and the different sexual stages.

It is important to know the cell size distribution of diatom populations, not only because size is a trigger for diatom sexualization, but also because of size-selective grazing and susceptibility to parasites and pathogens. Ideally, one would look for a gradual shift in cell sizes to smaller cells, then for the appearance of large cells to indicate when sexual reproduction took place. In reality, this has been observed in only a few diatom species, almost none of them marine phytoplankton. Also, factors other than vegetative cell division may be responsible for changes in cell size, e.g. parasitism, unfavourable conditions, or "accidents" during cell division; some of these could result in abrupt size changes. Large cells of a given species are rare in natural populations,

primarily because of the short-term penalty of sexual reproduction and auxosporulation and often because of the low percentage of cells reproducing sexually, and perhaps also because of selective loss of large cells due to grazing and/or sinking. Many cells would therefore have to be measured (500+) in order to detect the rarer large ones. This is tedious and time consuming and therefore automated methods (e.g. flow cytometry, image analysis) must be developed if size spectra are to be monitored routinely. One approach discussed was to model changes in the size spectrum, after making assumptions about the shape of the initial size spectrum, the rate of size decrease, and the percent of the population undergoing sexual reproduction to regenerate large cells.

An additional approach is to develop molecular probes against any or stage specific mRNAs or proteins and to apply these to field populations; molecular probes for particular DNA sequences have already been used to discriminate among different species of *Pseudo-nitzschia* or the dinoflagellate *Alexandrium*. This would let us determine when sexualization occurs, and the percentage of the population undergoing sexualization. Probes (for sex determining genes or monoclonal antibodies against cell surface antigens) could be used to distinguish "male" from "female" cells, in the case of dioecious pennate species (e.g. in *Pseudo-nitzschia*, *Nitzschia*). With this tool, one could determine the ratio of cells of opposite sex in natural populations (Davidovich & Bates, this volume). A current PhD study is underway to identify which genes are being up- and down-regulated during the growth of *Pseudo-nitzschia*, by isolating mRNA and applying the DNA microarray approach (Katie Rose Boissonneault-Cellineri, MIT/WHOI Joint Graduate Program in Oceanography, Cambridge, MA, USA). This approach could be used to develop specific sexualization probes, through comparisons of sexualized vs vegetatively growing cells. Laboratory studies are thus first required in order to be able to induce and maintain synchronous sexualization, so that mRNA profiles can be identified for each stage in sexual reproduction and auxospore formation for a given harmful or toxic diatom species, so that molecular probes can be designed. This will require new studies since synchronous sexualized cultures have not yet been obtained for any HAB diatom, and indeed, sexual reproduction of *Pseudo-nitzschia* has only been studied in culture in two species.

Resting stages

Remarkably little is known about the resting stages (morphologically distinct resting stages = resting spores; not part of the sexual cycle) of most diatoms, and virtually nothing about the formation and role of resting stages in harmful or toxic diatoms in particular. There seems to have been a decline in research in this area since the 1970s. One problem is that in some species there is little visible difference between resting stages and vegetatively dividing cells. Some centric resting stages are characterized by slight or major differences in valve morphology and content of storage products, and the valves are sometimes more silicified; the most highly differentiated resting spores are found in e.g. *Chaetoceros* and *Rhizosolenia*. Very few pennate diatoms are known to produce resting stages.

The role of diatom resting stages is not clear. One would think they would have a role analogous to that in dinoflagellates, i.e. as a mechanism to withstand adverse conditions and to act as a "seed bed" to inoculate the water column under more favourable conditions. However, resting stages do not always survive anoxic conditions in sediments and are therefore unlikely to act as a sedimentary inoculum for future

planktonic blooms. It has been hypothesized that resting stages may promote rapid sinking of the cells at the end of a bloom, or act as predation-resistant stage.

One must still explain the disappearance of diatom cells at certain times of the year, e.g. during harsh winter conditions, and their re-appearance later, under more favourable conditions. In the case of *Pseudo-nitzschia multiseries* in embayments of eastern Canada, blooms occur only in the fall; few, if any, cells are seen in the water column at other times. Only a small effort has been made thus far to look for cells (resting or vegetative) in the sediments during the non-bloom periods of the year, but nevertheless it is striking that no such cells have been found. Nor has work been published on the source and fate of offshore, deep-water blooms of *Pseudo-nitzschia* spp., e.g. in waters off Scotland, Ireland, the Mediterranean, and western North America. In these cases, cells (resting or vegetative) may be accumulating at depth, at a pycnocline. There, they may perhaps be transported long distances under conditions of cold, low irradiance, and high nutrients, to inoculate the water column at a different location and time of year.

One approach for shallower water is to incubate sediments at times of the year when vegetative cells are absent from the water column, in order to germinate any resting stages. This has been attempted in the Bay of Naples and the UK, but apparently with little success. If resting stages can be identified, then attempts should be made to develop molecular probes against them. Other physiological studies are required, e.g. to determine conditions for resting stage formation and excystment, DNA content and other unique physiological characteristics, and to develop physiological stains for vitality or growth potential (use of flow cytometry). Efforts should also be made to coordinate the analysis of sediment samples collected for dinoflagellate cysts, in order to also examine them for diatom frustules, especially if resting stages can be identified.

Physiological condition of cells

After the appropriate cell size is attained, a second condition for sexualization of pennate diatoms is that the cells must be in good physiological condition and usually growing rapidly. In the case of centric diatoms, by contrast, cells may have to be in a poor physiological condition, e.g. through nutrient limitation; this difference with pennates requires more study. Novel methods must be developed and applied in order to better determine the physiological condition of diatom cells in the field. Some information may be gathered on pennates such as *Pseudo-nitzschia* spp. simply by examining the number of cells per chain by light microscopy. Actively growing cells form long chains, which then break into smaller chains and then into single cells as the division rate slows and then stops. A single, physiologically inactive, cell may sink out of the water column more rapidly than a chain of cells. It may thus reach the sediment where it may form a protective resting stage, or sink to a pycnocline where it may be transported to a more favourable growing area. Tools for measuring cell size and colony length are thus required. Automated methods for these measurements were discussed (as per the discussion on cell size measurements, above), e.g. flow cytometry and image analysis.

Mating compatibility

All understanding of HAB organisms is ultimately based on the existence of sound taxonomy. Species-level classifications depend in part on morphology and increasingly

on gene sequence data, which can then be used to develop molecular probes for identification. However, another important approach is to apply reproductive data and determine if clones of the same or different morphology are compatible and can produce viable offspring (provided the cell sizes and conditions are appropriate). For example, clones of *Pseudo-nitzschia pseudodelicatissima* from the Black Sea and from the CCMP culture collection are unable to interbreed, even though they were identified morphologically as the same species. The Discussion Group advocated carrying out more mating compatibility experiments, and combining this information with morphological and molecular studies.

Gaps in Knowledge

- Presence or absence and characteristics (physiological, morphological, genetic) of resting stages of HAB diatom species.
- Methods to identify resting stages in natural populations.
- Molecular techniques to identify sexual stages in field samples.
- Location of overwintering populations of HAB diatoms.
- Sexual life cycle dynamics and size spectrum dynamics in natural populations and their genetic and ecological consequences.

Recommendations

- Select appropriate sites and species and investigate the dynamics of life history stages (particularly auxospores and resting stages) and size spectra in nature at high temporal resolution (on the order of 2 weeks) over time scales appropriate to the likely duration of the diatom's life cycle (considerably greater than one year).
- Develop molecular probes and image analysis techniques to detect life history stages of HAB diatoms.
- Apply a combination of morphological, molecular, genetic, and reproductive compatibility approaches to clarify species boundaries and population structure of HAB diatoms.

LIFE CYCLES IN DINOFLAGELLATES. J. LEWIS AND K. OLLI

Different life history stages of dinoflagellates have been identified as playing key roles in initiation, maintenance and termination of HABs (Montresor, this volume). The initiation of a bloom relies on an inoculum of cells. In terms of life cycles, we can identify two alternative possibilities for over wintering populations that provide inocula for blooms:

1. Species that form resistant benthic resting stages (cysts).
2. Species that survive as a small (resting) planktonic population *in situ* or elsewhere.

Maintenance of blooms relies on cell losses being balanced by cell gains. Life cycle stages may have a role to play in survival (for example formation of temporary cysts) and hence the maintenance of blooms. The termination of some blooms has been documented to be caused by the formation of resting stages.

Detailed life history studies have only been carried out for relatively few dinoflagellate species (some 15 species). Basic information on life histories should be gathered for HAB species where it is not presently available. Acquiring life history information for a wide range of species should be a further goal to allow us to better understand patterns in the class. It should also be noted that some species may be more amenable to manipulation and would act as good model species for detailed investigation. Such investigations need to document the detail of all parts of the life cycle and where possible give timings of various events. In this context light microscopy with video recording is helpful.

There is some uncertainty in basic definitions and terminology. Firstly, how do we define gametes? In some cases gametes can be distinguished from vegetative cells by size and/or behaviour. Often, gametes are smaller in size and have a different swimming pattern. This, however, is not general. In many cases gametes cannot be morphologically distinguished from vegetative cells. Are they chemically different? Are there qualitative differences between gametes and vegetative cells? Recent investigations have found the gamete stage to be reversible, e.g. a morphologically differentiated gamete is capable of resuming vegetative growth once re-suspended into fresh medium. Secondly, not all “small cells” are necessarily gametes. In *Dinophysis* and *Karenia* small cells can multiply vegetatively, but at least in the case of *Dinophysis*, can also take part in the sexual process (Reguera, this volume). Thirdly, there is a lack of clarity in the terminology used with regard to temporary cysts (syn. pellicle cysts, ecdysial cysts). New observations have been made on temporary cyst cycling in natural populations (Garcés, this volume). Temporary cysts can no more be considered a culturing artefact, and should now be recognized as stages in the life history of several coastal species. It is important to clarify the terminology associated with such stages because even though they are morphologically similar, they may have different functions in a population (division stages, resting stages, resistant stages – e.g. in passage through guts). The role of temporary cysts in population and bloom dynamics needs to be more clearly understood.

The taxonomy of species is always the first point of investigation in new harmful phenomena. The fact that HAB species are the subject of intensive investigation has led to their taxonomic revision. Recently, such revision has included molecular information. It is clear that such research is fundamental in maintaining quality comparative information in a wider geographic context. Additionally, for those species with a cyst stage it is necessary to have detailed taxonomic information on all stages of the life cycle. There are, for example, species which are likely to form cysts but where the cyst stage is not known. Detailed knowledge of life histories also allows resolution of taxonomic confusion where different life history stages have been identified as different species, e.g. *Dinophysis*.

Laboratory cultures provide a convenient, cost-effective system for improving our understanding of life cycles in dinoflagellates. Improvements in laboratory culture techniques in recent years have allowed us to manipulate more species through their various life cycle stages. Further improvements in culture technology will be required before some species can be maintained and manipulated in the laboratory. Species that have been maintained for long periods of time have been recorded as suffering changes. If mutation rates were known, it would be possible to predict the likely longevity of cultures. Where possible, fresh isolates should be used for experimental purposes. New evidence suggests that the source of isolates may affect behaviour in culture (Bolch, this volume). This phenomenon needs wider evaluation. Furthermore, genetic variability means that results from one culture are unlikely to be representative of a natural population. Where possible, more than one isolate should be used for experimental purposes. Findings from culture should be, where possible, validated by field observations. We recommend a practical workshop on culture methods.

We advocate a critical revision of sampling techniques and sampling designs. Standard oceanographic sampling methods (e.g. water bottles at discrete depths) do not suffice to uncover the accumulation of cells in thin layers. Such overlooked cell aggregations can be instrumental for transition in life cycle stages (e.g. threshold density for gametes to fuse). Additionally, this patchiness is important in the impact of the HAB. The improvement in water column video monitoring systems (“cam scan”) could be important in this regard.

Currently, we have no satisfactory methods to measure cell fluxes between planktonic and benthic habitats. Sediment traps are widely used to study the vertical flux of particles in the water column, and when used with caution have provided a useful tool to estimate cyst flux into the benthos (Heiskanen 1993). However, reliable and simple methods to estimate *in situ* germination rates of cysts are urgently needed. The detection and enumeration of cysts in sediments is presently time consuming and could be further improved. Furthermore, there is no methodology to determine viability of cysts apart from germination studies (germination traps).

Even more, the recurrent nature of many harmful blooms is not in accordance with the apparent lack of over wintering stages in many occasions, either benthic or planktonic. This contradiction may well be due to our inability to choose adequate sampling strategies and this hampers our elementary understanding of bloom dynamics and life cycles of these species.

In summary, the following have been identified as priority areas:

- Investigation of life histories of HAB species including a wide range of dinoflagellates.
- Development of molecular markers for gametes.
- Role of temporary cysts in life cycles.
- Investigation of mutation rates in cultures.
- Practical workshop on culturing dinoflagellates.
- Development of methodologies suitable for detailed water column monitoring.
- Development of technologies to allow determination of *in situ* germination rates.
- Development of markers to denote viable cysts.
- Search for overwintering stages of species where a resting stage is not known.

LIFE CYCLES IN HAPTOPHYTA. C. LANCELOT AND V. ROUSSEAU

The microalgal class Prymnesiophyceae Hibberd in the division Haptophyta includes some well-known HAB species, all of which occur in European waters. Among the haptophytes implicated in HAB events are ichthyotoxic species (C*hrysochromulina polylepis*, *C. leadbeateri*, *Prymnesium parvum, Phaeocystis pouchetii)* and high-biomass colonial *Phaeocystis* (*P. globosa* in nutrient-enriched coastal areas). The Prymnesiophyceae also includes the coccolithophorids (haptophytes covered with calcified scales) which are generally not considered as HAB species but are seen as important agents in climate regulation (e.g. *Emiliana huxleyi*). Some non-blooming coastal coccolithophorid species, including members of the genera *Pleurochrysis* and *Ochrosphaera*, are however suspected to have the capability of producing toxins (I. Probert, unpublished results).

Although knowledge of the haptophytes has expanded with recent focus on HAB species and the coccolithophorids, information is still needed on the basic biology and particularly the life cycles of this group as a whole. The discussion focussed on three main areas: life cycle mechanisms, haptophyte toxins, and the ecological relevance of haptophyte life cycles.

Life cycle mechanisms

Despite the fact that very few complete life cycles have been entirely elucidated in haptophytes, there is growing evidence that haplo-diploid life cycles with alteration of morphologically distinct stages are widespread (possibly ubiquitous) in the Prymnesiophyceae. Current knowledge of haptophyte life cycles results mainly from culture studies in which ploidy levels have been demonstrated by various methods (chromosome counting, flow cytometry, etc.), but the processes of meiosis and syngamy have rarely been observed. In some cases, field observations complement our knowledge, particularly in *Phaeocystis*, with observations of stages which have not been seen in culture.

The life cycles of known haptophyte HAB species vary in their complexity, involving alternation of two or more morphologically distinct stages. In this volume, the current state of knowledge in the genera *Phaeocystis* (V. Rousseau, L. Peperzak), *Chrysochromulina* and *Prymnesium* (B. Edvardsen) are reviewed.

The coccolithophorid genera *Pleurochrysis* and *Ochrosphaera* alternate haploid scale-bearing cells and diploid coccolith-bearing cells. In the coccolithophorid genus *Pleurochrysis*, meiosis and syngamy have been directly observed (Gayral & Fresnel, 1983). Also, alternance of holococcolith-heterococcolith has been demostrated in several cocoloithophors (Cross *et al*. 2000).

The main points to arise from this part of the discussion were:

- Identification of complete life cycles, including ploidy analysis, is required in more members of the Prymnesiophyceae in order to determine whether patterns exist across group. Improvements in culture techniques are required to be able to maintain and manipulate a wider range of haptophytes in culture.

- Direct evidence for a haplo-diploid sexual life cycle involving meiosis and syngamy is needed. These processes are rare and probably short-lived events. Knowledge of the triggering factors for phase changes would increase the likelihood of observing these phenomena in culture (at present we have very little information on potential phase change triggering factors in haptophytes).
- Are complex mating systems involved in gamete recognition? Clonal cultures of haploid stages of *Prymnesium* and some coccolithophorids have been observed to undergo the transition to the diploid stage (i.e. homothallism can occur), but information is at present too scarce to form any solid conclusions.
- Field and culture observations need to be cross validated (e.g. can the different flagellate stages be produced in cultures of *Phaeocystis*? Do *Chrysochromulina* and *Prymnesium* benthic stages actually exist in nature?).

Haptophyte toxins

The toxins involved in haptophyte HAB events have only been elucidated extensively in *P. parvum*. Further studies are required to determine the nature of toxins in other haptophyte species: is one toxin type common to all toxic haptophytes, or are different toxins involved as in the dinoflagellates? Preliminary tests showing that *Pleurochrysis* and closely related littoral coccolithophorid genera are toxic to *Artemia* require validation.

Evidence from some cultured clones of *Chrysochromulina* reveals that toxin production may differ between life cycle stages (B. Edvardsen, this volume). Pure cultures of different ploidy stages of clonal cultures are required to determine whether this is a common phenomenon. Little information is available on the genotypic vs phenotypic control of toxin production in haptophytes and the extent to which cultivated cells maintain their ability to produce toxins over time.

Life cycles and ecology

The relevance of the haplo-diploid life cycle to haptophyte ecology was discussed. Unlike diatoms (diploid life cycle) and dinoflagellates (haploid life cycle), both stages of the haptophyte haplo-diploid life cycle are capable of independent asexual division. Generally a haplo-diploid life cycle is considered as an adaptation to an environment which is seasonally variable or contains two different niches. Preliminary evidence from autecological studies of pure cultures of haploid and diploid stages of certain oceanic coccolithophorid species suggests an ecological differentiation between stages. The diploid stage adopts a strategy similar to diatoms (*r*-selected: high growth rates, use of inorganic nutrients, resistance to turbulence...), while the haploid stage, like dinoflagellates, is better adapted to stable nutrient-poor conditions (*K*-selected: low growth rates, motility, mixotrophic nutrition...). Some time-series distribution studies of coccolithophorids in nature do provide support for such ecological niche separation. Do life cycles play key roles in the ecology of haptophyte HAB species? No autecological differences have been observed in cultures of the two phases of *Prymnesium parvum*, and in our experience no clear niche separation is evident in nature. The two flagellate stages reported in *Chrysochromulina polylepis* have different environmental

requirements and could be adapted responses to changing conditions in the water column.

Systematic autecological studies on both stages of haptophyte HAB species would not only provide useful ecological information towards interpreting / predicting natural bloom events, but may also provide evidence on the factors inducing phase transitions. From our discussion, the factors *suspected* to be of potential relevance for phase transitions are: inorganic and organic nutrient depletion, light quality and intensity, infochemicals (diatoms, grazers), turbulence and endogenous factors / biological clock (it was noted that phase changes in cultures maintained in constant conditions throughout the year often occur at similar times of the year).

One intriguing question about HAB haptophytes is the extent to which toxin production, colony formation (see Lancelot *et al.*, this volume), or coccolith production correspond to an adaptive or defence strategy for developing blooms.

Summary of priorities for future research

- The identification of all life stages (including benthic stages) in the field is a prerequisite for a complete construction of the life cycle of the different species. The combination of flow cytometric, electron microscopic and genetic techniques applied to samples taken at high temporal and depth resolution (including sediment) is necessary to ensure the identification of the life stages and avoid taxonomic confusion.
- Cultures of the identified life cycle stages would provide material for the identification of the processes leading to the reconstruction of the whole life cycle and the understanding of its controlling mechanisms. These include syngamy, meiosis and mating systems (never studied in most species), the formation and germination of *Prymnesium* and *Chrysochromulina* cysts and possibly *Phaeocystis* amoeboid stages, the process of *Phaeocystis* colony formation and termination. The factors triggering the switch between the different life stages have also to be identified.
- These laboratory cultures would provide material for ecophysiological characterization of the different stages. This would contribute to an understanding of possible ecological niche separation of the different stages in the natural environment and of how the harmful events associated with these species (toxicity, foam, anoxia) are related to their life cycles.

DIFFERENT MOLECULAR TECHNIQUES TO EXAMINE LIFE CYCLES.

B. EDVARDSEN AND L. GUILLOU

Introduction

DNA technologies have been increasingly employed during the last decade in order to assess phylogeny, geographical variation and distribution, and species- and group-specific detection and quantification of harmful algal bloom (HAB) species. Nevertheless, the continued development of molecular methods, and particularly the variety of polymerase chain reaction (PCR)-based techniques, means an increasing array of methods is available to investigate genetic diversity, phylogeny and life cycles of microorganisms. We review some of the available molecular techniques applicable to studying HAB life cycles in cultures and field samples and provide recommendations and future needs.

Molecular and cellular techniques and challenges

The current molecular methods available are each suited to examining particular aspects of life cycles, cellular physiology or genetic diversity at different levels.

At the cellular level

Some harmful algae, including alternate life cycle stages, cannot be identified accurately from morphological characters alone, especially when they cannot be maintained in culture. In addition, it is difficult to detect HABs at very low concentrations in natural plankton populations. With sufficient genetic background data, molecular techniques can allow, without prior cultivation, a precise identification of all life cycle stages of HAB species despite markedly different morphology. When applied to field samples, these techniques can detect both vegetative cells and resistant cysts that are either free living in the water column or associated with sediment. DNA amplification followed by sequencing of single cells or clonal cultures is a very informative method (Guillou & Biegala, this volume), but can be both time consuming and expensive for routine monitoring purposes. Nevertheless, this approach is essential to gather background DNA sequence information needed to develop species- or genotype-specific oligonucleotide probes for geographical or life cycle studies in natural marine environments. When molecular probes have been designed, one can detect the presence of whatever stage, even if very rare, in natural samples, e.g. by PCR amplification of total DNA by specific primers or by fluorescent *in situ* hybridization (FISH) (Guillou & Biegala, this volume). FISH is also suited for quantification of specific taxa. Flow cytometry and artificial neural networks may be used in combination with molecular probes, but reports on their applicability on natural samples are at present limited.

A useful approach for examining and demonstrating sexual life cycles is ploidy analysis (Edvardsen, this volume). Algal cells are stained with DNA-specific fluorochromes which can then be detected and quantified using fluorescence microscopy or flow cytometry.

At the population level

Information about intraspecific genetic variability is important for identification (e.g. different genotypes in the resting stage pool stored in the sediments), in order to study the succession of populations during a bloom or to compare geographically different populations of the same species. Sufficient variation for global scale studies is often present in DNA sequences of non-coding intergenic spacer (IGS) regions, intergenic transcribed spacers (ITS), or the hypervariable domains (e.g. between D1 and D2) of the large ribosomal subunit RNA gene (LSU-rDNA). This approach can be used on single cells from cultures, cysts or cells from field samples. When higher variability is needed, fingerprinting methods such as RAPD, AFLP, RFLP and microsatellites can be applied. However, they usually require a high number of clonal cultures and population genetic analysis of the data can be problematic (see Table 6).

In situ growth rates (e.g. using Carpenter 1988), as well as cell size distributions and their influence on life cycle transition, are also important components to be integrated in field studies of populations.

At the molecular level

We currently have virtually no information on the genetic control and gene expressions during the transition between different life cycle stages (encystment / excystment, syngamy, meiosis, auxosporulation) of HABs. The study of genetic regulation of these different events will be an important step in the characterization of life cycles. The identification of genetic markers for specific physiological conditions that can be related to life stages transitions (e.g. functional genes, genes activated by stress) is also one of the challenges for the years to come. A genome sequence database for a dinoflagellate and a haptophyte (in addition to those for a chlorophyte and a diatom that are under way), may provide key tools for the study of genetic control at the cellular level. Protein identification and characterization (Proteomics) may also be a valuable tool.

Table 6. Molecular techniques and their applications for life cycle studies of microalgae. C = cellular level; P = population level; M = molecular level.

Technique	Level of study	Application, description of the method[1]	Advantages, resolution
Species detection			
Direct amplification by PCR from entire cells	C, P	Allows genetic characterization of a particular life cycle stage.	Can be employed on clonal cultures and cells isolated from field samples (living or fixed).
Specific PCR	C, P	Destructive method. Qualitative and semi-quantitative. Amplification from entire cells or extracted DNA using specific primers. When amplification occurred, the target species was present.	Nested-PCR amplification may allow detection of <10 cells·L^{-1}. Can be employed on cultures, field samples, and sediments.
Dot Blot Hybridization	C, P	Destructive method. Qualitative and semi-quantitative. Specific hybridization using oligonucleotides on RNA or amplified DNA immobilized on membrane.	Allows the detection of a few ng of DNA extracted from cultures or field samples.
FISH (Fluorescent *in situ* Hybridization)	C, P	Non-destructive method. Specific fluorescent oligonucleotides are used to bind target rRNA inside the cell. As ribosomes are present in numerous copies, the target cells become fluorescent and easily detectable by epifluorescence microscopy.	Can be employed on cultures and field samples.
Genetic polymorphism in populations			
RAPD (Random Amplified Polymorphic DNA)	P	Destructive method. PCR amplification of the genome by using an arbitrary 10 bp primer.	Must be employed on clonal cultures. Some doubts about reproducibility. DNA quality is crucial. Data analysis is problematic. Genetic variation detected is usually high.
AFLP (Amplification Fragment Length Polymorphism)	P	Destructive method. The total genome is digested by two selected restriction enzymes. Adaptors, specific to the restriction enzymes, are fixed at the fragment extremities. The fragments are then amplified by PCR. This method can also be used on cDNA.	Must be employed on clonal cultures. DNA quality is crucial. Data analysis is problematic. Genetic variation detected is usually high.
RFLP (Restriction Fragment Length Polymorphism)	P	Destructive method. The DNA is digested by restriction enzymes and the banding patterns from the different fragments are then compared.	Must be employed on clonal cultures if tested on total genomic DNA. In this case, a large amount of DNA is needed. Level of genetic variation is often low.

Continued

Technique	Level of study	Application, description of the method[1]	Advantages, resolution
DGGE (Denaturing Gradient Gel Electrophoresis)	P	Destructive method. Electrophoresis of amplified DNA on a denaturing gradient gel. The fragments, initially double stranded, are partially denatured according to their nucleotide composition, which results in differences in migration.	Allows separation of DNA types which differ by only one nucleotide difference. The bands can be extracted, re-amplified and sequenced for characterization. Fragment length is limited to 500 bp. Can be employed on cultures and field samples.
SSCP (Single Strand Conformation Polymorphism)	P	Destructive method. Amplified DNA is denatured by heating and rapidly cooled to avoid DNA pairing. The single strand DNA is visualized on non-denaturing gel and migrates according to its tri-dimensional conformation.	The bands can be extracted, reamplified and sequenced for characterization. One DNA type may have several tri-dimensional conformations. Can be employed on cultures and field samples.
Microsatellites and SSR (Simple Sequence Repeat)	P	Destructive method. Repetition of mono, di- tri- or tetranucleotides are used as markers for DNA polymorphism in total genome.	SSR method must be employed on clonal cultures. Development of microsatellites is usually time consuming and expensive. Amenable to standard population genetic analyses.
HMA (Heteroduplex Mobility Assay)	C	Destructive method. Amplified DNA is denatured by heating and then slowly renatured. Pairing of heteromorphic DNA is then visualized on non-denaturing gel.	Rapid method to check the homogeneity of a PCR product.
Gene expression			
Differential display and related techniques	M	Separation by electrophoresis of cDNA differentially expressed, and previously amplified by PCR.	Little RNA material is needed. Can be used to characterize rare transcripts of mRNA.
EST (Expressed Sequence Tag)	M	Random sequencing of partial cDNA.	Allows characterization of the commonest mRNA transcripts.
cDNA array or DNA chips (transcriptomics)	M	DNA network of a total genome or using specific oligonucleotides. Different applications: genetic expression, detection of micro-organisms, detection of mutant or sequencing by hybridization.	Technique in development which uses detection by fluorescence or by electric properties of the DNA. Its applicability to field samples needs to be tested and validated.
Others			
Genome sequencing	M		
Proteomics	M		

[1]Destructive methods, leading to cell disruption do not allow combining morphology and genetic characterizations. Nevertheless, they can be applied on clonal cultures and during special events such as monospecific blooms.

Recommendations and future needs

- Combine morphology and molecular probes during identification of different life cycle stages.
- Investigate mating capability.
- Investigate genetic intraspecific variability to test probes for geographically isolated populations.
- Obtain more sequences to design more probes and validate available probes.
- Improve detection methods for natural samples.
- Use flow cytometry for *in situ* growth rates and cell sizes.
- Develop fingerprinting markers for population studies.
- Identify functional genes.
- Carry out genome projects on haptophyte and dinoflagellate species.
- Protein identification and characterization.

Many molecular techniques and combinations of techniques are already available and well developed in other more extensively studied organisms. Technical and analytical expertise has been developed in other related fields (e.g. *Saccharomyces* genetics) or disciplines (higher plant genetics). We need to adapt such existing methods for microalgae and plankton species and populations. Sources of funding to adapt methods and to develop expertise in new methods are limiting factors.

KEY AREAS FOR RESEARCH ON RESTING STAGES IN COASTAL SEDIMENTS OF EUROPE. B. DALE AND M. MONTRESOR

Introduction and main aims

Many HAB species include benthic resting stages in their life cycle. It is becoming evident that the alternation between planktonic life stages in the water column and benthic stages in the sediments is one of the crucial factors determining the occurrence, timing and development of HABs.

The detection and quantification of HAB species in the water is often hampered by the inherent limits of the standard procedures used for phytoplankton sampling. HAB species can accumulate in thin layers within the water column, be present only during restricted time intervals, form patches of limited spatial extension, or occur episodically. The presence of species producing benthic life stages may be more readily detected from investigations of the sediments. Mapping cyst beds in surface sediments thus provides a tool for assessing biogeographic distribution and dispersal of some HAB dinoflagellate species, in order to relate their distribution to known environmental parameters and to assess risk potential for establishing new aquaculture activities in coastal areas.

A number of resting stages have the potential to fossilize and produce a record in the sediments of their presence and abundance over extended time intervals. The analysis of changes in such benthic stage abundances over time scales of tens or hundred of years in cored bottom sediments provides a tool for tracking phytoplankton responses to climatic changes and/or cultural eutrophication. This approach has proved successful in studies along the Norwegian coast (Dale, this volume).

Approaches suggested

1. *Assessment of the biogeographic distribution of HAB species along European coasts*.

Mapping the distribution of benthic stages of HAB species (dinoflagellates, raphidophyceans, diatoms, haptophytes, cyanophyceans) in surficial sediments would allow the establishment of a baseline for monitoring spreading events, introduction of new species, and human-assisted dispersal. The site selection should cover European coasts along a wide latitudinal gradient, including key environments with different topographic/physical characteristics (e.g. fjords, Baltic Sea, Mediterranean Sea, Black Sea, Atlantic coasts), and should include accumulation sites (e.g. harbours) and areas designated for aquaculture activities.

2. *Identification of key areas affected by HAB events along the EU coasts*.

These sites should represent “case” studies for the population dynamics of selected HAB species blooming in different environmental settings. The following questions need to be addressed: when are resting stages produced? how many? how many resting stages are viable in the sediments at any given time? and what is the timing of their germination? Key areas selected to address such ecological studies would benefit from the existence of long-term physico-chemical and planktonic data sets, since these

otherwise represent the most useful information for detecting species timing and recurrences, and relating them with environmental and biotic factors.

3. *Reconstruction of long-term trends of HAB species with fossilizable resting stages in sediment cores.*

The assemblage composition of HAB benthic stages in core samples, integrated with the signal provided by other biological and chemical proxies, will provide a tool for reconstructing species trends and abundances through changing environmental conditions (climate, eutrophication, etc.). Possible "proxy" stations should be identified from different regions that offer particularly detailed records (i.e. sites with anoxic bottom conditions such that fine-laminated sediment layers remain undisturbed by bioturbation). These sites would provide optimal conditions for integrated multidisciplinary research (i.e. biologists, micropaleontiologists, sedimentologists, geochemists).

Methodologies - tools

Much of the basic research on resting stages in sediments so far may be characterized as preliminary observations. For this to be integrated into more comprehensive surveys as outlined above, standard methods will required, and for some tasks innovative new procedures will have to be developed. Future needs include:

- Standardization of methods for the collection, detection and quantification of resting stages in sediments;
- Development of new advanced techniques for sampling life stages in the water column and in the sediments (e.g. to detect thin phytoplankton layers in the water column or to estimate germination rates of benthic resting stages);
- Development of molecular tools for automatic detection of HAB species and their different life stages (e.g. cysts and spores).

In addition, it should be noted that much of the expertise needed for this work is currently held by just a few specialists. European and international research in general would therefore benefit from the development of a website including life cycle information for phytoplankton species, a taxonomic guide book for the identification of benthic resting stages and their correspondence with the planktonic stages, reference collections of benthic stages (e.g. slides) and development of advanced training courses.

THE IMPORTANCE OF LIFE CYCLES IN THE ECOLOGY OF HARMFUL ALGAL BLOOMS.

A. ZINGONE, E. GARCÉS, T. WYATT, B. SILVERT AND C. BOLCH

Although some critical factors which influence bloom formation have been described for some species, we have only a provisional understanding of the mechanisms that promote and sustain harmful algal blooms. Life cycles have important implications for the occurrence and bloom dynamics of harmful species. For example, heteromorphic life cycles allow a species to survive different environmental conditions, and some resistant stages may contribute to the dispersal of a species. The transitions between different life stages play an important role during different phases of harmful blooms. Sexual events in the course of life cycles are at the base of the genetic structure and diversity of populations. Some life stages can confer protection from viruses, grazers or parasite attacks.

Physical, chemical and biological factors in the transition among different stages

Important questions centre on how environmental factors affect transitions between life-stages. Evidence from cultures indicates the importance of nutrient limitation, yet other experiments (Olli, this volume) demonstrate the opposite, while field evidence is controversial or lacking. There is also the possibility that a fraction of the population is able to keep the internal pool of nutrients low under conditions when dissolved nutrients are available (Probert, this volume), which highlights the role of biological control on nutrient fluxes.

A more general, controversial question is the role of eutrophication in HABs. Fast nutrient recycling is an important though difficult to measure process in the maintenance of natural populations, which can explain blooms occurring at low nutrient concentrations as well as the evidence that most often blooms decline before nutrients become depleted. The role of nutrients in bloom dynamics of *Phaeocystis* in the North Sea is however still controversial. N-source (e.g., ammonium, nitrate) preferences vary among species, and the N-source preference of a species may shift depending on availability. Different forms of N can also influence the dominance of the different stages of a species, at least in the case of *Phaeocystis*. Another important issue is that harmfulness of some species can be enhanced in particular nutrient conditions, as in the case of *Pseudo-nitzschia* in Atlantic Canada, which apparently produces domoic acid when under nutrient stress.

Laboratory experiments are extremely important to understand the mechanisms of life stage transitions, yet they fail to reproduce all the environmental condition and should be validated whenever possible by field observations. As an example, cyst germination rates could be influenced by their sedimentary environment, which is usually not taken into account in laboratory experiments.
The transitions between the different stages in species with complex life cycles can be triggered by several factors, but the relative role of endogenous and exogenous control is still largely unknown. As an example, does a turbulent environment favour cell encounter rates and sexual reproduction? Or is chemical communication responsible for active cell aggregation? Do resting cysts rely on resuspension by bottom currents before germination? Or, rather, do the seasonally changing light or other environmental

signals trigger excystment at the right time? Or are shifts regulated by internal clocks, whereby specific metabolic pathways act as 'Zeitgeber'?

HABs are the result of the active or passive accumulation of nuisance species in selected areas. While passive aggregation is largely controlled by hydrographic factors, active accumulation strongly depends on life strategies of single species. “Infochemicals” are being discovered with increasing frequency in planktonic communities. These are cell signalling chemicals produced by organisms which influence the behaviour of individuals of their own species and other organisms. Mounting evidence suggests that these mechanisms are also important for micro-algae and HAB species (e.g. bioassay evidence using grazer and bacterial conditioned algal growth media).

So far, it is mainly physical and chemical factors that have been taken into account when interpreting environmental conditions triggering HABs and life-stages transitions. However, there is nowadays evidence of the importance of biological control by phytoplankton of the physical and chemical environment. Some examples include biological contributions to nutrient concentrations and fluxes, the role of biological products in altering the rheological properties of water, attenuation of light by algae, and heat absorption and warming by layers of cyanobacteria and other planktonic organisms.

Gaps in knowledge

There is no general theory for bloom initiation that can be applied to any individual species. Even in the case of *Alexandrium* spp., where cysts are generally considered important to bloom initiation (Zingone, this volume), there are contradictory results that limit our understanding. The respective roles of cysts and resting stage, over-wintering and advected populations may be different among species, sites and years, which would explain the high diversity and low predictability of bloom dynamics (e.g. Moita & Amorim, this volume).

How much inoculum, either from cysts or seeding cells, is required to generate a bloom? Is the magnitude of a bloom limited by the number of resting stages available? Mechanical mixing (e.g. by ships) and resuspension (e.g. by dredging) may increase the availability of cysts for inoculation of the water column. In sheltered environments such as harbours, a small inoculum may be enough, but in large and energetic coastal areas a larger amount may be necessary due to increased dispersive losses. It has been hypothesised that a minimum cell concentration (e.g. less than 100 cells·L^{-1}) is required below which the population is not viable (Wyatt, this volume). However, the amount of the inoculum should rarely be a limiting factor, since cells may aggregate either actively or by taking advantage of physical processes.

Sites which are more suitable for cyst accumulation and hence act as sources of inocula for HABs (e.g. Raine, this volume) should be identified and localized. Retention zones, such as gyres and harbours, can retain cells and cysts and may increase the opportunity and incidence of HABs, e.g. harbours retaining *G. catenatum* cysts along the Portuguese coast.

There is not enough information on how different life stages interact with the physical, chemical and biological environment, so as to maximize their concentration, extend the duration of a bloom and ensure the perpetuation of the local population. Some life stages, such as temporary cysts, could play an essential role in this context (Garcés, this volume). Cysts and spores could be a means to avoid predation or attack by viruses, bacteria and parasites. The colonial stage of *Phaeocystis* can act as a grazing deterrent (Lancelot *et al*. this volume).

Sexual processes, active aggregation, ageing of the population and cell death (apoptosis) can contribute to the decline of a bloom, yet the respective roles of these mechanisms are not known for any species. Some species are capable of massive encystment (Olli, this volume), and the fate of resting stages following a bloom can be relevant to the subsequent blooms (Wyatt, this volume). Again, the roles of endogenous versus exogenous factors need clarification. As an example, encystment may be triggered by physical factors (temperature and irradiance decrease), by high densities, by the presence of compatible mating-types, or by endogenous clocks. A working hypothesis is that intrinsic factors unique to each species control encystment and excystment, whereas the timing and scale of response may be modulated by the environment.

In addition to their possible roles in bloom initiation and defence, resting stages are considered to contribute to the natural or artificial dispersal and introduction of harmful species in new areas (e.g. translocation with shellfish, ballast water). Much importance has been given so far to the phase when non-indigenous aquatic species are introduced. However, biological invasions involve several other processes with scarcely known aspects, including e.g. colonization mechanisms, competition and survival in the new site.

Recommendations

- Ecological studies on both harmful and non-harmful species should be promoted in order to better understand the dynamics of HABs. There is also a need to review existing data and publications for evidence of mechanisms underlying bloom dynamics that could be applied to HABs.
- Hypotheses generated by laboratory experiments on harmful species should be validated in field studies to ascertain the actual role of different environmental parameters in life-stage transitions. The ecological interactions of each life stage with the physical chemical and biological environment should be investigated for the different harmful species.
- Field studies on HAB dynamics in key areas should be set up to understand the role of life stages at different steps of a bloom (initiation, growth, stationary phase and decline). Life stages of a single species of interest should be sampled and quantified at the appropriate scales before bloom initiation and throughout the bloom phases. Databases resulting from these studies should be made available, in appropriate formats, for integration into models.
- The respective roles of resting stages of harmful species as means to i) avoid predation or attack by viruses, bacteria and parasites, ii) preserve genetic

diversity, iii) inoculate future blooms and iv) promote species dispersal, should be ascertained.

- Techniques should be developed to sample specific structures such as microlayers, bottom-water interfaces and other physical and biological discontinuities. Techniques are also needed to identify and quantify different life stages of a species and their physiological status and *in situ* growth rates.
- New interdisciplinary sampling procedures should be used in field studies. Multiscale physical-chemical-biological interactions in life-stage transitions require that the scale of the relevant biological processes dictate the scale of sampling for environmental parameters.
- The role of biological control in different phases of blooms should be explored at different levels. The presence of endogenous mechanisms which could be responsible for life-cycle transitions should be tested. In addition, the effects of phytoplankton on environmental conditions (light, nutrients, temperature and rheological properties of sea water), should be taken into account to explore mechanisms which can trigger transitions and/or enhance blooms.
- Species-specific models (Gentien, this volume) have to be designed which integrate life cycles in order to advance our understanding of population dynamics and improve prediction capabilities of HABs.

CONCLUSIONS and GENERAL RECOMMENDATIONS

A large amount of literature was reviewed within LIFEHAB (a the list of more than 400 titles included here). Notwithstanding this bulk of scientific information, a main conclusion of the Workshop was that there are still many open questions concerning the basic features and ecological role of life cycles in HAB species and, in phytoplankton in general. It is widely accepted that notable differences in life histories exist among species belonging to distinct algal divisions. Less understood is that significant differences in e.g. resting stage germination rates and timing, and in general in life-cycle strategies, may exist among closely related species, and even within a single species at one site or across its biogeographic range. Most of our knowledge on life histories derives in fact from laboratory studies of a limited number of algal strains. Conditions used to induce life-cycle transitions in the laboratory may not be the ones effective in the natural environment. As an example, nutrient depletion has been widely used to induce dinoflagellate cyst production in culture, yet cysts are regularly produced in non-limiting nutrient conditions in nature, including the early phases of bloom development. It is clear that we still know very little about the biology and life histories of microalgae and, moreover, we have only just begun to unravel the links among the different life history stages within an ecological framework.

The extensive discussion which took place during the Workshop highlighted a number of research needs which are common to all algal groups, as well as specific requirements related to life-cycle modalities peculiar to distinct algal divisions. The main gaps identified in our knowledge and priority research lines are summarized in the following:

Characterization of species

At present, phytoplankton taxonomy is mainly based on certain morphological features of selected stages of species life cycles. Knowledge of the morphological and functional complexity of life cycles, and of the modes of sexual reproduction, is needed in order to provide a more comprehensive framework for the definition of distinct taxa. In addition, morphological studies coupled with molecular systematics, breeding data and biogeographic distributions can give general insights into speciation and evolution mechanisms of phytoplanktonic organisms, which is the crucial theoretical basis for testing and/or implementing the current phylogenetic framework.

Elucidation of life cycles?

Basic information on life histories should be gathered for a wide range of species to gain information on general life-cycle patterns within the different algal groups. Improved culture techniques should allow reconstruction of whole life cycles and description of the different stages. These techniques are also required for the physiological characterization of the distinct stages, which will contribute to the understanding of their potential ecological roles. Mating systems are practically unknown for most species, as well as ploidy status for groups with a haplo-diploid life cycle (Haptophyta, Raphidophyta). The use of advanced techniques (i.e. flow

cytometry, molecular techniques) should allow the identification of ploidy stages, markers for specific physiological conditions, genetic control and regulation at the cellular level, and triggers of the transitions among the different stages.

Life histories and toxicity

There is increasing evidence for the presence of different amounts / composition of toxic compounds in different life stages in both dinoflagellates and haptophytes. Furthermore, there is increasing evidence of economically damaging toxic events (e.g. effecting commercial shellfish) in winter when there is no evidence of toxic motile stages in the plankton. It is thus crucial to unravel the mechanisms and effects of toxin production in different life-cycle stages, which requires more sensitive methods for the quantification and detection of toxin profiles at the single-cell level. Toxin production has been reported for both freshwater dinoflagellates and cyanophyceans. This can be a serious threat due to the use of freshwater bodies for drinking water supply. It is therefore recommended that life cycle research be extended to freshwater harmful species.

How to couple life histories and *in situ* population dynamics?

The main challenge is to understand how distinct stages of the life cycles interact with environmental and biological variables so as to produce harmful blooms, contribute to the persistence and expansion of harmful species, and to the make-up of their genetic diversity. Particularly relevant is knowledge of the endogenous and exogenous mechanisms controlling the alternations between one stage and another. Morphologically and physiologically different life stages can enhance the ecological success of a species in expanding and diversifying its performances in relation to sinking, defence from grazing and pathogens, resistance to adverse and extreme conditions, etc. For species forming benthic resting stages, the mapping and quantification of "cyst beds", coupled with knowledge of mechanisms and environmental conditions for bloom inoculation and spreading, can be used for building provisional models at different scales. On a short-term and local scale, models based on this information can improve prediction of the start of a bloom and of its dispersal routes. At a larger scale, knowledge of the geographic distribution and density of cyst beds of harmful algae can help identify risk areas under different ecological and hydrographical scenarios, providing useful information for the management of coastal areas.

What kinds of methodological and technological development are needed?

One of the major needs and challenges is the development and validation of molecular probes for rapid and reliable identification of HAB species and their different life history stages in the natural environment. Genetic intraspecific variability, as well as different physiological-related responses of the target organisms, should be investigated in order to design and validate molecular probes.

Technological advances are required for improving *in situ* detection methods and instrumentation, e.g. for the localization of the different life stages in thin accumulation layers in the water column, and for the quantification of life cycle transitions (encystment, excystment, auxosporulation). Accurate detection and

quantification methods should also be developed for benthic dormant stages, which can act as inoculae for the motile, growing phase.

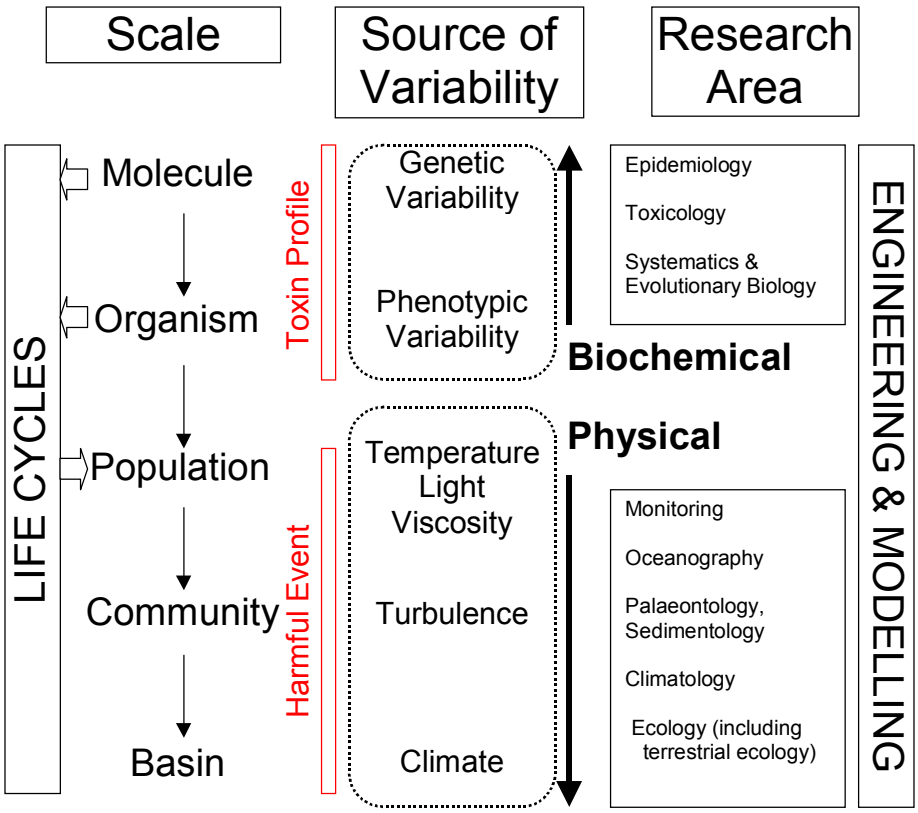
How can different disciplines contribute to the development of knowledge on HAB life cycles?

The mechanisms underlying life cycles of harmful algae and their negative effects encompass a wide range of biological, physical and ecological scales, from genes to populations, from the interstitial environment in the sediment to large-scale movements of water masses, from cell-to-cell relationships to ecosystem interactions. It is evident that the study of these phenomena requires the implementation of *ad hoc* interdisciplinary research (Box 4).

The cooperation among different research disciplines, coupled with the application of new technologies, and with the development of new models, represents a special challenge for future research.

Box 4 - Interdisciplinary research for life cycles.

Flows of information required to determine the mechanisms underlying HAB population dynamics. The complexity of the biological processes involved in life cycles and toxin production requires research and information at the molecular, organism and population scales, and needs to be coupled with the effects of environmental conditions. The situation is made more complex due to inherent variability at all levels. Expertise can, however, be drawn from a very wide variety of disciplines and activities, from medicine through to terrestrial biology. Underlying the science, at all levels, is the contribution of engineering and modelling. The interdisciplinary nature of research necessary for integrating life cycle studies into ecological, oceanographic and other sciences is clear. The diagram does not include the research required pertaining to socio-economic consequences of HABs.



Graph by Robin Raine.

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APPENDIX 1. Life cycle of diatom HAB species that cause or may cause problems in European coastal waters.

Species	Harmful effect	Life history		References
		rs	Auxosp	
Amphora coffeaeformis ?*	Domoic acid	+		Anderson 1975; 1976
*Coscinodiscus concinnus**	Bird mortality		n.o.	
*C. centralis**	Bird mortality		n.o.	
*C. wailesii**	Mucilage; high biomass effect on nori	+		Nagai & Imai 1999 Nagai *et al*. 1999
*Ceratulina pelagica**	Shellfish and finfish kills		n.o.	
*Chaetoceros concavicornis**	Fish kills		n.o.	
*C. convolutus**	Fish kills		n.o.	
Corethron sp.*	Fish kills		n.o.	
*Guinardia delicatula** (= *Rhizosolenia delicatula*)	Mucilage clogs fishnets		n.o.	
*Leptocylindrus minimus**	Fish kills	+		Hargraves 1990
*Minutocellus pseudopolymorphus**	Strong smell in water on beach		n.o.	
Nitzschia navis-varingica	Domoic acid		n.o.	
*Pseudo-nitzschia multiseries**	Domoic acid		+	Davidovich & Bates 1998a, b Hiltz *et al*. 2000 Kaczmarska *et al*. 2000
*P. australis**	Domoic acid		n.o.	
*P. delicatissima**	Domoic acid		n.o.	
*P. fraudulenta**	Domoic acid		n.o.	
*P. multistriata**	Domoic acid		n.o.	
*P. pseudodelicatissima**	Domoic acid		+	Davidovich & Bates 1998a, b
*P. pungens**	Domoic acid ?		n.o.	
*P. seriata**	Domoic acid		n.o.	
P. turgidula ?	Domoic acid		n.o.	
Rhizosolenia chunii	Shellfish kills; gives bitter taste		n.o.	
*Skeletonema costatum**	High biomass	+		Hargraves & French 1975
*Tabularia affinis** (= *Synedra affinis*)	Epiphyte on nori		n.o.	
*Thalassiosira mala**	Mucilage on gills kills shellfish		n.o.	

* Species recorded in European waters, even if harmful events attributable to the species have not been reported in the area. References are given for reports of life cycle events only.

rs = resting spore; Auxosp = auxospore (including gametes and zygotes); n.o. = not observed

APPENDIX 2. Life cycle of dinoflagellate HAB species that cause or may cause problems in European coastal waters.

Species	Harmful effect	Life history				References
		rc/tc/dc	he/ho	Col	sc	
*Akashiwo sanguinea** *(=Gymnodinium sanguineum)*	Ichthyotoxic ?				+	Silva & Faust 1995
Alexandrium acatenella	PSP					
A. andersoni	PSP	rc				Montresor *et al*. 1998
A. angustitabulatum	PSP ?					
*A. catenella**	PSP	rc/tc	he	+		Adachi *et al*. 1999 Delgado 1999 Fukuyo *et al*. 1982 Hallegraeff *et al*. 1998 Sawayama *et al*. 1993 Sawayama *et al*. 1993 Yoshimatsu 1981 Yoshimatsu 1984
A. cohorticula	PSP	rc		+		Fukuyo & Pholpunthin 1990
A. hiranoi	goniodomin	rc/tc				Kita & Fukuyo 1988 Kita *et al*. 1985 Kita *et al*. 1993
*A. margalefi**	ichthyotoxic					
*A. minutum** (incl. *A. lusitanicum)*	PSP	rc/tc				Blanco 1990 Bolch *et al*. 1991 Probert *et al*. 1998 Silva & Faust 1995 Garcés, pers. comm.
A. monilatum	ichthyotoxic	rc		+		Walker & Steidinger 1979
*A. ostenfeldii**	spirolide (SST)	rc/tc				Jensen & Moestrup 1997 Mackenzie *et al*. 1996

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Species	Harmful effect	Life history				References
		rc/tc/dc	he/ho	col	sc	
*A. tamarense** (=*A. excavatum*) (incl. *A. fundyense*)	PSP	rc/tc	he			Adachi *et al*. 1999 Anderson 1980 Anderson 1998 Anderson & Keafer 1987 Anderson & Lindquist 1985 Anderson & Morel 1979 Anderson & Wall 1978 Anderson *et al*. 1983 Anderson *et al*. 1984 Anderson *et al*. 1987 Benavides *et al*. 1983 Castell Perez *et al*. 1998 Dale 1977 Destombe & Cembella 1990 Doucette *et al*. 1989 Fritz *et al*. 1989 Fukuyo *et al*. 1982 Ichimi *et al*. 2001 Sako *et al*. 1990 Turpin *et al*. 1978 Watras *et al*. 1982 Wyatt & Jenkinson 1997 Yentsch *et al*. 1980 Yentsch & Mague 1980
A. tamiyavanichi	PSP			+		Balech 1995
*A. taylori**	discolouration	rc/tc/dc	ho ?		+	Garcés *et al*. 1998 Giacobbe & Yang 1999
*Amphidinium carterae**	Ichthyotoxic haemolytic		ho			Cao Vien 1967 Cao Vien 1968
*A. operculatum**	haemolytic ichthyotoxic ?					
A. operculatum v. *gibbosum*	cytotoxic					
*Cochlodinium polykrikoides**	ichthyotoxic					
*Coolia monotis**	cooliatoxin	rc/dc	ho		+	Faust 1992 Silva & Faust 1995

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Species	Harmful effect	Life history				References
		rc/tc/dc	he/ho	col	sc	
*Dinophysis acuminata**	DSP	rc ?			+ *D. skagii*	Bardouil *et al*. 1991 Mackenzie 1992 Reguera & González-Gil 2001
*D. acuta**	DSP	rc ?			+ *D. dens*	Hansen 1993 Mackenzie 1992 Moita & Sampayo 1993 Reguera & González-Gil 2001 Reguera *et al*. 1990, 1995
*D. caudata**	DSP	rc ?			+ *D. diegensis*	Moita & Sampayo 1993 Reguera *et al*. 1990, 1995 Reguera & González-Gil 2001
*D. fortii**	DSP				+ *D. lapidistri-giliformis* ?	Fukuyo 1993 Uchida *et al*. 1999
*D. mitra**	DSP					
*D. norvegica**	DSP				+ *D. crassior*	Hansen 1993
*D. rapa**	DSP					
*D. rotundata**	DSP				+ *D.* cf. *parvula* ?	Reguera & González-Gil 2001
D. sacculus (incl. *D. pavillardi*)*	DSP				+ *D. micro-strigiliformis* ?	Bardouil *et al*. 1991 Delgado *et al*. 1996 Giacobbe & Gangemi 1997 Hansen 1993
*D. tripos**	DSP	rc ?			+ *D. diegensis var. curvata* ?	Moita & Sampayo 1993 Reguera & González-Gil 2001
Gambierdiscus australes	Ciguatoxin-/maitotoxin-like toxins					
G. pacificus	Ciguatoxin-/maitotoxin-like toxins					
G. polynesiensis	Ciguatoxin-/maitotoxin-like toxins					
G. toxicus	Ciguatera					Van Dolah *et al*. 1995
G. yasumotoi	maitotoxin-like toxins					
*Gymnodinium catenatum**	PSP	rc/tc	ho/he			Anderson *et al*. 1988 Blackburn *et al*. 1989 Blackburn *et al*. 2001 Bravo 1986 Bravo & Anderson 1994
*G. pulchellum**	ichthyotoxic					

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Species	Harmful effect	Life history				References
		rc/tc/dc	he/ho	col	sc	
*Gyrodinium corsicum**	ichthyotoxic					
Heterocapsa circularisquama	Ichthyotoxic	tc				Nagasaki *et al*. 2000 Uchida 2001
Karenia brevis (= *Gymnodinium breve*)	NSP	rc ?	ho/he	+	+ (gametes)	Steidinger *et al*. 1998 Walker 1982 Van Dolah & Leighfield 1999
K. brevisulcata	ichthyotoxic					
*K. mikimotoi** (= *Gymnodinium mikimotoi*, *G. nagasakiense*, *Gyrodinium aureolum partim*)	ichthyotoxic				+	Partensky & Vaulot 1989 Partensky *et al*. 1988 Partensky *et al*. 1991 Uchida 2001 Yamaguchi 1992
*Karlodinium micrum** (= *Gymnodinium micrum*)	ichthyotoxic					
K. veneficum (= *Gymnodinium veneficum*)	ichthyotoxic					
*Noctiluca scintillans**	discolouration anoxia				+ (gametes)	Sato *et al*. 1998 Schnepf & Drebes 1993 Zingmark 1970
Ostreopsis heptagona	Ciguatera ?					
O. lenticularis	ostreotoxin					
O. mascarenensis	toxic compound					
*O. ovata**	toxic compound					
O. siamensis	ostreocine/ palytoxin					
Pfiesteria piscicida	neurotoxic ichthyotoxic	rc			+	Burkholder & Glasgow 1997a Burkholder & Glasgow 1997b Steidinger *et al*. 1996
P. shumwayae	neurotoxic ichthyotoxic	rc				Glasgow *et al*. 2001
Prorocentrum arenarium	DSP					
P. belizeanum	DSP					
P. borbonicum	toxic compound					

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Species	Harmful effect	Life history				References
		rc/tc/dc	he/ho	col	sc	
*P. cassubicum**	DSP					
P. concavum	DSP ?					
P. emarginatum	DSP ? haemolytic ?					
P. hoffmannianum	DSP					
*P. lima**	DSP	rc/dc				Faust 1993a Faust 1993b
P. maculosum	DSP					
P. mexicanum	haemolytic toxins					
*P. minimum**	ichthyotoxic	tc				Grzebyk & Berland 1996
*Protoceratium reticulatum**	yessotoxin	Rc				Braarud 1945
*Protoperidinium crassipes**	azaspiracid					
Pyrodinium bahamense v. *compressum*	PSP	rc/tc	he ?	+		Azanza-Corrales & Hall 1993 Matsuoka *et al*. 1989 Usup & Azanza 1998

* Species recorded in European waters, even if harmful events attributable to the species have not been reported in the area.

Synonyms are reported only for species whose names have changed in the last years.

A few non-toxic, high-biomass species are included.

References are given for reports of life cycle events only.

rc = resting cysts; tc = temporary cysts; dc = division cysts; he = heterothallic; ho = homothallic; col = colonies; sc = small cells

APPENDIX 3. Life cycle of haptophyte HAB species that cause or may cause problems in European coastal waters.

Species	Harmful effect	Life history					References
		Col	m/nm	haplod	sex	rs	
*Chrysochromulina leadbeateri**	ichthyotoxic		1+	?	n.o.	n.o.	Edvardsen 1998
*C. polylepis**	ichthyotoxic, haemolytic		3/1?	+	n.o.	n.o.	Edvardsen & Medlin 1998 Edvardsen & Vaulot 1996 Edvardsen 1998 Paasche *et al.* 1990
Ochrosphaera neapolitana	toxic to *Electra pilosa*		2/2	+	n.o.	calcareous cyst	Gayral & Fresnel-Morange 1971 Jebram 1980
*Phaeocystis globosa**	high biomass (foam, anoxia)	+	3/1	+	n.o.	n.o.	Cariou *et al.* 1994 Kornmann 1955 Peperzak *et al.* 2000 Rousseau *et al.* 1994 Vaulot *et al.* 1994
*P. pouchetii**	high biomass toxic to cod larvae	+	1+/1	+	n.o.	amoeboid	Carentz 1985 Eilertsen 1985
Pleurochrysis carterae	toxic to *Artemia* ?		1/1	+	+	n.o.	Gayral & Fresnel 1983
Prymnesium calathiferum	toxic to *Artemia*		1/0	?	n.o.	n.o.	Chang & Ryan 1985
*P. faveolatum**	toxic to *Artemia*		1/0	?	n.o.	n.o.	Fresnel *et al.* 2001
*P. parvum/patelliferum**	ichthyotoxic, haemolytic		2/1	+	n.o.	cyst	Larsen 1999 Larsen & Edvardsen 1998 Padan *et al.* 1967 Pienaar 1980
*P. zebrinum**	toxic to *Artemia*		1/0	?	n.o.	n.o.	Billard 1983

* Species recorded in European waters, even if harmful events attributable to the species have not been reported in the area.

col = colonies; m/nm = motile/non motile stages; haplod = haplodiplontic; sex = sexuality; rs = resting stage; n.o. = not observed

APPENDIX 4. Life cycle of raphidophyte HAB species that cause or may cause problems in European coastal waters.

Species	Harmful effect	Life history			References
		Vegetative cells	Small [1] cells	Resting stages	
*Chattonella antiqua**	ichthyotoxic	Diploid [2]	+	Cyst	Imai & Itoh 1986; 1988
*C. marina**	ichthyotoxic	Diploid [2]	+	Cyst	Imai & Itoh 1986; 1988
C. aff. *minima**	ichthyotoxic	?	?	?	
C. aff. *verruculosa**	ichthyotoxic	?	?	?	Lu & Göbel 2000 Backe-Hansen *et al.* 2001
*Fibrocapsa japonica**	ichthyotoxic	Diploid	?	Cyst	Yoshimatsu 1987
*Heterosigma akashiwo**	ichthyotoxic	Diploid	?	Cyst and non-motile cells in mucus	Imai *et al.* 1993 Tomas 1978 Yamochi & Joh 1986
*H. inlandica**	ichthyotoxic	?	?	?	ICES-IOC HAEDAT

* Species recorded in European waters, even if harmful events attributable to the species have not been reported in the area.

[1] Haploid small cells resulting from meiosis.

[2] Vegetative cells resulting from diploidization of germinated haploid cysts or directly from a germinated diploid cyst (see Imai *et al*. 1998 and Nakamura *et al.* 1990).