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RESEARCH ARTICLE

Gaining integrated understanding of *Phaeocystis* spp. (Prymnesiophyceae) through model-driven laboratory and mesocosm studies

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Abstract Knowledge of the complex life cycle of *Phaeocystis* is a key to understanding its role in marine ecosystems and global biogeochemistry. An existing life cycle model was modified and used to integrate understanding of the *Phaeocystis* life cycle. In model-driven research, models expose gaps in our understanding, empirical studies ensue, and feedback improves understanding. Following this scheme, three facets of the life cycle model were examined here. With four exceptions, the empirical studies described have been presented in other literature citations. The first facet involved testing for the existence of a process or producing its description. These studies included: demonstration of in vitro colony division in *Phaeocystis pouchetii*, description of in vitro change in colony shape for *P. pouchetii* associated with senescence, determining which *P. pouchetii* life stage is vulnerable to viral infection and lysis, and an experiment designed to determine whether the sediment could be a source of new *Phaeocystis* colonies to overlying waters; results suggested that more-detailed investigation of benthic particles as a physical substrate for colony formation is warranted. The second facet involved investigation of process rate quantification or process control parameters. Process rate quantification included measurements of colony division rate and growth rate using mesocosm-derived colonies. Process control experiments included testing diatom frustule enhancement of *P. pouchetii* colony formation from solitary cells, and investigation of mesozooplanktonic suppression and microzooplanktonic enhancement of *Phaeocystis globosa* colony formation by planktonic grazer infochemicals. The third facet

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pertained to the molecular identification of genetic differences between single cells and colonies of *P. globosa*. These studies were designed to provide insight to the question of control factors involved in the transition between single cell and colonial life stages. The life cycle model provided a ready place to incorporate new insights and understanding from empirical studies into an existing model, and can be used to improve simulation models of the direct and indirect effects of *Phaeocystis* on global biogeochemistry.

Keywords Conceptual model · Life cycle · Mesocosm · Model-driven research · *Phaeocystis*

Introduction

The genus *Phaeocystis* contains species that may play a significant role in global biogeochemistry (Smith Jr et al. 1991; Lancelot et al. 1994; Wassmann et al. 2005). They occur over extensive areas (Stefansson and Olafsson 1991; Lancelot and Rousseau 1994), supply significant portions of global dimethyl sulphide (DMS; Gabric et al. 1999; Ayers and Gillett 2000), and play a key role in planktonic ecosystems (Verity and Smetacek 1996; Lancelot et al. 1998). Life cycle processes of *Phaeocystis* may have significant effects on ecosystem biogeochemical processes including nutrient uptake and regeneration by life forms of *Phaeocystis*, nutrient release by viral lysis, nutrient release associated with zooplankton grazing, and nutrient release by senescent colonies. Details of the current state of knowledge of the *Phaeocystis* life cycle are comprehensively reviewed in Rousseau et al. (in press, this volume). In order to integrate understanding of the *Phaeocystis* life cycle, a conceptual model was constructed (Whipple et al. 2005a), although the existence of some life stages was uncertain and the controls on many of the processes were poorly understood. This exercise highlighted experiments which could provide a better understanding of the role of the life cycle in species dynamics and ecosystem processes.

Such enhanced understanding will be incorporated into a developing conceptual model (Whipple et al. 2005a), and also into an ecosystem model in which the *Phaeocystis* life cycle is only one set of biotic compartments (Whipple et al. unpubl.). In model-driven research, models expose gaps in our understanding of the *Phaeocystis* life cycle embedded in its ecosystem; empirical studies to address these gaps ensue, and the feedback from iteration of this cycle improves understanding. This paper presents examples of how a life cycle model of *Phaeocystis* has been used to direct research on the *Phaeocystis* life cycle and its ecology, and demonstrates how insight from these studies integrated into a conceptual model context enhances and integrates such empirical findings.

Model description

The *Phaeocystis* life cycle, which has been modified from the model presented in Whipple et al. (2005a), advances clockwise around Fig. 1 from the upper left with Diploid Solitary Cells, as the starting point. These cells flow to and from New Colonies, which is the critical transition stage between solitary and colonial life forms.

After new colonies cells first divide, they transfer to the growing small colonies compartment. Maturation continues through the size classes: small → medium → large via mitosis and mucus production of colony cells. There is also back-transfer from larger to smaller size classes by colony division (Whipple et al. 2005b).

When colonies begin to lose cells, growing colonies are transferred to corresponding size-based senescent colonies. Diploid or haploid solitary cells are released from these to complete the *Phaeocystis* life cycle. Of the four colony-forming *Phaeocystis* species, haploid flagellates have been documented only for *Phaeocystis globosa* (Eilertsen 1989; Jacobsen 2002; Medlin and Zingone in press, this volume; Rousseau et al. in press, this volume). The other life cycle input to diploid solitary cells comes from transformation of new colonies cells. Syngamy has not been documented in any *Phaeocystis* species; however, it has been included in the model since it is hypothesized as necessary to create diploid solitary cells from haploid cells prior to colony formation in *P. globosa* (Rousseau et al. in press, this volume). Since this

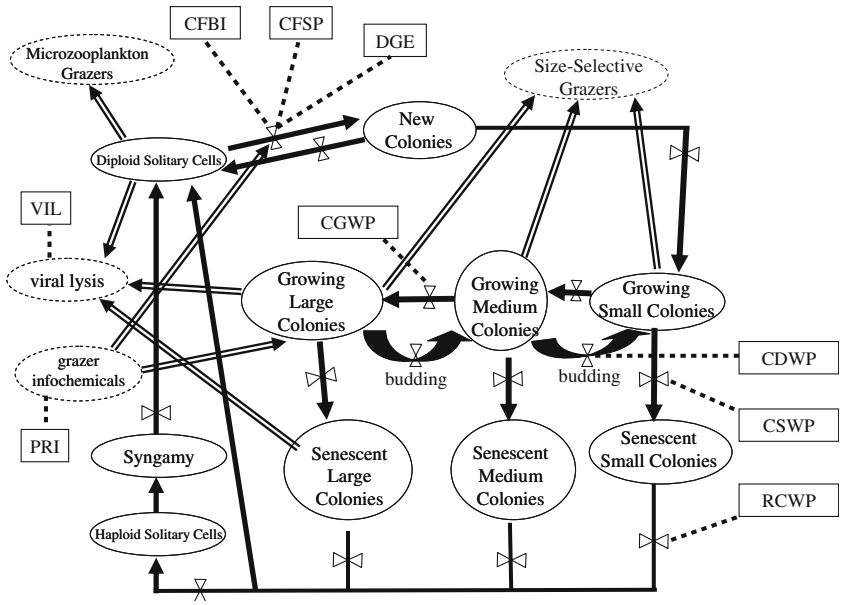

Fig. 1 *Phaeocystis* life cycle diagram. Solid-line ovals: *Phaeocystis* life cycle stages; dashed-line ovals: other ecosystem components. Solid arrows: life cycle transitions; hollow-line arrows: inputs to or outputs from *Phaeocystis*. ⋈ symbols: rate control for life cycle transitions. Rectangles with acronyms: empirical studies with dashed-lines pointing to an associated life cycle stage or life cycle transition

Names and acronyms for experiments described:

CDWP	Colony division in well plates
CFSP	Colony formation with seed particles
CFBI	Colony formation with benthic involvement
CGWP	Colony growth in well plates
CSWP	Colony senescence in well plates
DGE	Differential gene expression
PRI	*Phaeocystis* response to infochemicals
RCWP	Release of colony cells in well plates
VIL	Viral infection and lysis

model was developed to represent the genus *Phaeocystis*, haploid life stages are included, but may only occur in certain species (Whipple et al. 2005a).

Hollow-line arrows in Fig. 1 represent hypothesized flows to or from non-*Phaeocystis* components of the larger ecosystem. In some cases these serve as controls on life cycle flows, and in other cases they are boundary inputs or outputs of the *Phaeocystis* life cycle. Three main outputs from the *Phaeocystis* life cycle are represented. Two are grazing on solitary cells (upper left), and grazing on all size classes of growing colonies (upper right). The third is viral lysis (left center), which is an output process usually emphasized in viral studies. However, infection, an input process, must occur for viruses to complete their life cycle and have an effect on *Phaeocystis* cells. The last process is the input of infochemicals from zooplankton grazers (lower left) into *Phaeocystis* solitary cell-to-colony transformations or colony cells.

Case studies

The conceptual model of this paper serves two functions by: (1) providing a context for integrating what is known, and (2) defining observations and experiments to eliminate unknowns (Whipple et al. 2005a). Figure 1 graphically depicts how the *Phaeocystis* life cycle model and empirical studies conducted to address unknowns may be interrelated; each experiment within our research group described below is denoted by an acronym

Table 1 Description of the *Phaeocystis* life cycle processes and the studies associated with them that are discussed in this paper

Acronym	Name used in Fig. 1	Process description	Source of data
CDWP	Colony division in well plates	In vitro observations of colony division in *P. pouchetii*	Whipple et al. (2005b)
CFBI	Colony formation with benthic involvement	In vitro observation of colonies derived from coarse surficial material collected from shallow fjord sediments	this study
CFSP	Colony formation with seed particles	In vitro observation of colony formation from solitary cells in *P. pouchetii* with diatom frustules	Nejstgaard et al. (in press)
CGWP	Colony growth in well plates	In vitro observations of colony growth in *P. pouchetii*	Whipple et al. (2005b)
CSWP	Colony senescence in well plates	In vitro observations of colony senescence in *P. pouchetii*	this study
DGE	Differential gene expression	Differential gene expression in solitary versus colonial cells in *P. globosa*	this study
PRI	Phaeocystis response to infochemicals	Experiments to determine *P. globosa* response to presence of micro- and mesozooplankton grazers or their infochemicals	Long et al. (submitted); Long (2004)
RCWP	Release of colony cells in well plates	In vitro observations of colony cell release in *P. pouchetii*	this study
SIM	Species identification in mesocosms	18S rDNA probe assessment of *Phaeocystis* species growing in 2003 Norwegian mesocosm	this study
VIL	Viral infection and lysis	In vitro observations of viral infection and lysis for cells from intact and disrupted colonies in *P. pouchetii*	Jacobsen et al. (2005)

(e.g., VIL = viral infection and lysis; left center) indicating where it relates to the life cycle. The nine processes and their associated studies considered in this paper are shown in their life-cycle context in Fig. 1. Descriptions of these processes, their acronyms, and data sources are shown in Table 1; an entry describing verification of the *Phaeocystis* species found in the mesocosm studies (SIM) is also included. Stages or processes involved in the phenomena under investigation are described in relation to the life cycle model. Parenthesized acronyms for the Table 1 experiments appear in the subheadings of this section. In four case studies, detailed methods and results are presented; in other cases, a brief summary of methods and results will be given based on published or submitted literature sources, which may be consulted for further details. The Discussion section considers broader implications of our empirical findings for both *Phaeocystis* life cycle dynamics itself, and potential revisions of the life cycle model.

Mesocosm experiment methods

Many of the observations and experiments discussed below were performed in the context of mesocosm studies conducted in Raunefjorden at the Norwegian National Mesocosm Center, located at the marine biological field station at the University of Bergen in western Norway. Studies were conducted between 4 March and 24 March 2002 and 27 February and 2 April 2003 (Nejstgaard et al. 2006); these studies will be referred to as 2002 and 2003 mesocosm studies in the remainder of the paper. The main results of these mesocosm studies will not be presented in this manuscript; they are provided in detail in Nejstgaard et al. (2006). The purpose of this section is to provide sufficient background on the mesocosm studies so that experiments conducted in their context can be understood and interpreted.

In each study, three transparent polyethylene enclosures (4.5 m deep, 2 m diameter, ca. 11 m^3, 90% PAR) were filled with unfiltered fjord water

from 5 m depth. An airlift system completely mixed the enclosures approximately five times per day. To allow for the introduction of new species, to avoid pH changes, and to replace water removed for sampling, 10% of the water in each enclosure was renewed daily with fjord water (Nejstgaard et al. 2006). Two of the three mesocosms were amended with nitrate and phosphate that corresponded to an initial enrichment of 16 μM nitrate and 1 μM phosphate. Daily addition of 1.6 μM nitrate and 0.1 μM phosphate replaced nutrients removed by the 10% water renewal and sampling. In the 2002 experiment, based on low nutrient concentrations measured on March 12, both nutrient enriched mesocosms were augmented with additional nutrients, 8 μM nitrate and 0.5 μM phosphate on the evening of March 12. The third mesocosm in each experiment, which was left unamended, served as a control (Nejstgaard et al. 2006). Detailed description of mesocosm set-up, sampling, analytical methods, and results are described in Nejstgaard et al. (2006).

Species identification in mesocosms (SIM)

During the 2002 and 2003 mesocosm studies many of the *Phaeocystis* colonies were observed to be perfectly spherical, suggesting that there may be *P. globosa* rather than *P. pouchetii* present in western Norwegian fjords. During the second mesocosm experiment (2003) an effort was made to determine the diversity of *Phaeocystis* species present in these mesocosm studies.

Methods

Throughout the 2003 mesocosm study, water (50–500 ml) was collected every third day onto 0.8 μm Supor® membrane filters (Pall corp.) and stored at −80°C until analysis. Following enumeration of *Phaeocystis* sp. single cells and colonies, two samples were chosen for molecular determination of the composition of the *Phaeocystis* sp. community. Gene sequence analysis of *Phaeocystis* sp. small subunit ribosomal RNA (rRNA) genes from samples collected from a fertilized enclosure early in the experiment when only single cells were present (5 March 2003; single cells 15 ml^{-1}, colonies 0 ml^{-1}) and samples collected during the peak of the bloom (20 March 2003; single cells 990 ml^{-1}, colonies 41 ml^{-1}) were examined. Total DNA was extracted from filters using the Ultraclean™ soil DNA isolation (MoBio Laboratories, Inc.) as previously described (Allen et al. 2005). Small subunit rRNA genes from *Phaeocystis* sp. were specifically polymerase chain reaction (PCR) amplified using a universal 18S rRNA targeted primer UnivF-15 (5′ ctg cca gta gtc ata tgc; Frischer et al. 2002) and a *Phaeocystis* genus-specific primer PHAEO1 (5′ cgg tcg agg tgg act cgt; Lange et al. 1996). Amplification reactions were facilitated using an Applied Biosystems 9,700 thermocycler in 20 μl volumes. Each amplification reaction consisted of 8 μl Eppendorf ® MasterMix® (Eppendorf North America), 8.4 μl PCR grade distilled water, 3 μl each primer (100 ng μl^{-1}), and 3 μl of undiluted or 1:10 diluted DNA extract. Purified DNA from the extracted samples was not quantified. Reaction conditions consisted of an initial denaturation step (94°C, 3 min) followed by 35 amplification cycles (94°C, 15 s; 53°C, 15 s; 72°C, 30 s) followed by a 7 min final extension at 72°C. PCR amplicons were examined on an agarose gel to insure that only a single band was produced. Single bands were cloned into the bacterial plasmid vector PCR®2.1 using the TOPO TA Cloning® kit (Invitrogen, Carlsbad, CA) and sequenced in both directions as previously described (Frischer et al. 2002).

Results

A total of 17 clones was sequenced and the identity of each cloned sequence was confirmed to be *Phaeocystis pouchetii*, indicating that it is unlikely that any other species of *Phaeocystis* were present in these studies.

Testing for the existence of a process or producing a description of a process

Colony formation with benthic involvement (CFBI)

Benthic involvement in the life cycle of various *Phaeocystis* species has been discussed for many years in the literature. Kayser (1970) describes his

observation of benthic cells in culture as follows: "It is interesting to observe that single cells of *Phaeocystis pouchetii* exhibit a tendency to attach themselves to solid surfaces... We may assume that in the open sea single cells are attached to solid surfaces on the sea floor or to motile particles and constantly supply the free water with single cells or young colonies" (Kayser 1970, pp. 209–210). Rousseau et al. (1994, p. 26) concluded, there is "...absolutely no evidence for a truly differentiated benthic stage."

Rousseau et al. (in press, this volume) describe *P. globosa* diploid flagellates as forming new colonies within a day after adhesion to a surface (Cariou et al. 1994; Kayser 1970; Kornmann 1955; Rousseau et al. 1994). Non-living particles (Rousseau et al. 1994), culture vessel walls (Cariou et al. 1994; Kayser 1970), and diatoms (Weisse et al. 1986; Boalch 1987; Rousseau et al. 1994) have been reported as adhesion sites (Rousseau et al. in press, this volume). Attachment of cells to surfaces led to the assumption that a benthic stage, acting as an overwintering form, exists in the natural environment (Kayser 1970; Rousseau et al. in press, this volume). The general conclusion from these reports is that the diploid flagellate described in these studies is not a truly differentiated benthic form (Rousseau et al. in press, this volume).

Empirical work conducted during the 2003 mesocosm study tested whether *P. pouchetii* solitary cells or colonies would be resuspended from fjord sediment incubated with filtered water in controlled environmental chambers.

Methods

Benthic substrate, which consisted of medium-grained sands with shells and biotic remains, was hand-collected by SCUBA divers from 12 m depth in a coastal embayment near Bergen, Norway on March 14, 2003. The sediments were kept isolated from the overlying water during collection and experimental preparation, and were overlaid with filtered seawater during incubation. A set of 20 cc aliquots of drained but damp sediments were placed in the bottom of triplicate 250 ml PC bottles and gradually overlaid with 200 ml of the following water types: artificial seawater; autoclaved fjord water; 0.8 μm filtered water from mesocosm #1 (control: no nutrients added); 0.8 μm filtered water from mesocosm #2 (NO_3 and PO_4 added). The bottles were incubated under dim light (1.7 μmol m^{-2} s^{-1}, measured using a Biospherical Instruments QSL−100 meter with a spherical light collector; 12:12 L:D) at 3°C in a walk-in cold room and sampled at day 0 (start of incubation) and at day 7. The abundance, diameter, and cell density of *P. pouchetii* colonies were measured microscopically.

Results

Phaeocystis pouchetii colonies was observed in all sediment-water treatments after seven days of incubation (Fig. 2). The highest number of colonies developed in bottles containing filtered water from mesocosm 2 (M2) treatments, which had received daily nitrogen and phosphorus additions (Fig. 2a). M2-water samples also contained colonies with the largest diameter, and highest number of cells per colony (Figs. 2b,c).

Existence of colony division in P. pouchetii (CDWP) Using in vitro observations of mesocosm-derived *P. pouchetii* colonies in well plates in 2002, Whipple et al. (2005b) reported that 10–12% of wells contain colonies that divided at least once. They also observed that division of colonies was only observed after many hours had elapsed. This may indicate that colonies undergo a period of colony enlargement by cell division and mucus production before they divide to produce daughter colonies (Whipple et al. 2005b). This pattern of colony multiplication was also reported for *P. globosa* (Verity et al. 1988).

Identification of molecular markers that distinguish between single cells and colonies A first-order hypothesis-generating question associated with the multiform life cycle of *Phaeocystis* sp. is the identification of cellular and genetic differences between single and colonial cells. To begin to address this question in the context of the complex *Phaeocystis* life cycle, a differential display PCR approach was used to identify genes whose expression was regulated in colonial versus single cells. These studies were conducted with

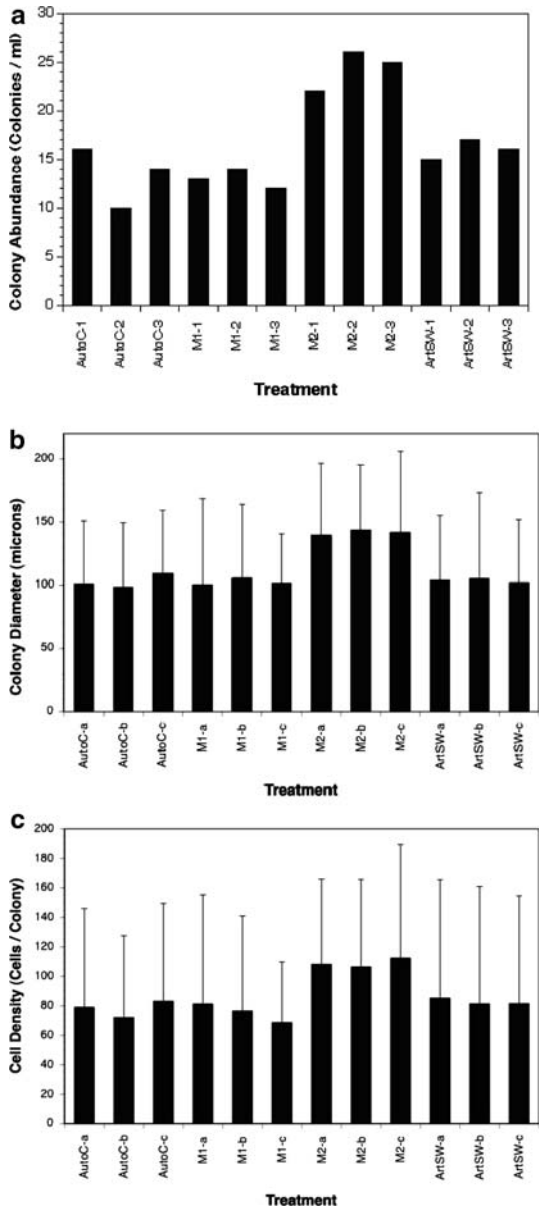

Fig. 2 Results of surficial material collected from shallow fjord sediments experiment. Treatment key: ArtSW#: artificial seawater; AutoC#: autoclaved fjord water; M1- #: 0.8 μm filtered water from mesocosm #1 (= no nutrients added); M2- #: 0.8 μm filtered water from mesocosm #2 (NO_3 and PO_4 added). (**a**) Abundance of *P. pouchetii* colonies (colonies ml^{-1}); histograms represent colony estimates for each replicate bottle. (**b**) Colony diameter (μm); error bars represent standard deviations of estimates within each bottle calculated from triplicate counts. (**c**) Cell density (cells $colony^{-1}$); error bars represent standard deviations of estimates within each bottle calculated from triplicate counts

laboratory cultures of *P. globosa* (CCMP 1528, Provasoli-Guillard National Center for Culture of Marine Phytoplankton, USA).

Methods

Three independent cultures of *P. globosa* CCMP 1528 were grown for one week at 20°C, 14:10 light:dark, in L1-Si media (Guillard and Hargraves, 1993). Under these conditions both single cells and colonies were produced in each culture. Single cells were separated from colonies by reverse osmosis with a 10 μm mesh sieve and hand pump. Cells and colonies were separately collected onto 25 mm, 5 μm Durapore filters (#SVLP02500, Millipore, Bedford, MA). Approximately 7.5×10^6 single cells and 5×10^4 small colonies (ca. 1 mm) were immobilized per filter. RNA was extracted from each sample using the Qiagen RNeasy Plant Mini Kit (Cat# 74903, Qiagen, Valencia, CA) following the manufacturer's recommended procedures (Qiagen, Valencia, CA). Purified RNA extracts of replicate samples from each culture were combined (6 extractions for each cell type). RNA was quantified by fluorometry using RiboGreen (Molecular Probes, Inc.) and purified RNA was stored at −80°C until analysis.

Differentially expressed genes were identified by differential display following cDNA synthesis from ca. 1 μg of total RNA using the Advantage® cDNA PCR kit (Cat# K1905–1, Clontech Laboratories, Inc., Palo Alto, CA). Differential display analysis was facilitated using reagents and procedures associated with the commercially available delta differential display kit® (Cat# K1810–1, Clontech Laboratories, Inc., Palo Alto, CA). Amplification of cDNA was accomplished with all 90 unique combinations of 10 arbitrary 25-mer primers (P primers) and 9 Oligo(dT) primers (T primers) supplied in the delta differential display kit. Differential display amplification reactions were conducted in 20 μl volumes with 2 μl 10X Klen Taq PCR reaction buffer, 14.2 μl PCR grade distilled water, 0.2 μl dNTP mix (5 mM each), 0.4 μl 50X Advantage® KlenTaq, 0.2 μl ^{33}P dATP, and 1 μl of a 1:40 or a 1:10 dilution of cDNA template. Amplification conditions were as

Table 2 Differentially expressed gene fragments from *P. globosa* colonial and single cells

Cell type	No. of bands	Con[a]	Partially regulated		Fully regulated	
			Up[a] reg	Down reg[a]	On	Off
Cells	1365	1193	32	32	117	16
Colonies	1254	1193	7	7	7	15

[a] Con, constitutively expressed; up reg, upregulated; down reg, downregulate

recommended by Clontech and consisted of three low-stringency PCR cycles (1st cycle: 94°C, 5 min; 40°C, 5 min; 68°C, 5 min), second and third cycles (94°C, 30 s; 40°C, 30 s; 68°C, 5 min), and 25 additional high-stringency cycles (94°C, 20 s; 60°C, 30 s; 68°C, 2 min) followed by a dwell cycle for 7 min at 68°C. Amplification was performed in a dedicated Amplitron thermocycler (Barnstead/Thermolyne, Dubuque, IA). Following confirmation of PCR amplification of cDNA by agarose gel electrophoresis, 5 µl of PCR product was mixed with gel loading dye (2 ml 10× TAE, 7.5 ml 40% sucrose, 830 µl 6× bromophenol blue, 100 ml distilled water) and 3 µl was loaded onto a 7M urea, 6% acrylamide gel (553 × 381 × 4 mm) with 1× TBE buffer and electrophoretically separated at 1500V, 50W for 2.5–3 h. The gel was transferred to filter paper, covered with plastic wrap and dried on a vacuum gel dryer (Fisher Scientific, Pttsburgh, PA) for 1 h at 80°C. After drying, bands were detected by autoradiography in a film cassette with a ^{33}P intensifying screen. Films were exposed for 4–6 days at −80°C. All bands were manually assigned into three primary categories; constitutively expressed, differentially expressed, or fully regulated. Bands categorized as constitutively expressed were those that appeared at approximately the same density in both colonial and single cells. Bands identified as differentially expressed were those whose autoradiographic density was substantially different between colonial and single cells. Fully regulated bands were those that were either present or absent in one cell type compared to the other.

Following identification of differentially expressed genes, the seven genes that were present in colonial cells but not in single cells were cloned and sequenced. Differentially expressed PCR bands were excised from the dried gel as recommended by Clontech. Briefly, bands were cut from the gel/filter paper, placed in 40 µl of DNase- and RNase-free water, covered with two drops of sterile mineral oil and heated at 100°C for 5 min. The filter paper was then discarded. The PCR product was re-amplified with the same primers originally used to amplify the band of interest. Following amplification, PCR amplicons were examined on an agarose gel to insure that only a single band was produced. Single bands were cloned into the bacterial plasmid vector PCR®2.1 using the TOPO TA Cloning® kit (Invitrogen, Carlsbad, CA) and sequenced in both directions using the M13 priming sites as previously described (Allen et al., 2005). After sequence assembly and editing using the Vector NTI Suite 8 software package (InforMax, Bethesda, MD), putative gene identities were determined using BLASTn and BLAST searches for short nearly identical matches (NCBI, http://www.ncbi.nih.gov)

Results

A total of 1,365 expressed gene fragments derived from single cells and 1,254 expressed gene fragments in colonial cells were observed in these studies (Table 2). Of these expressed fragments, 117 unique fragments were observed in single cells that were not observed in colonial cells and seven fragments that were expressed in colonial cells but absent in single cells were detected. Of the 172 differential expressed genes observed in flagellated cells, 87% of them (149) were either up regulated or turned on compared to colonial cells. In comparison, only 23% (14) of the 61 differentially expressed genes observed in colonial cells were upregulated or turned on relative to single cells.

The seven gene fragments that were observed only in colonial cells were cloned and sequenced,

Table 3 Differentially expressed genes associated with *P. globosa* colonies

Clone	Size (bp)	Genbank accession no.	Closest blast hit Genbank accession no.	Identity	E-value	Predicted function
PgC-3	490 bp	DQ886384	AAP94719	*Emiliania huxleyi* NADH Dehydrogenase subunit I	4×10^{-14}	Electron transport
PgC-10	342 bp	DQ886385	ZP00777402	UDP-3-0-acyl N-acetylglucosamine deacetylase (bacterial)	1×10^{-37}	Lipid assembly
PgC-19	213 bp	DQ886386	AY741371	*Emiliania huxleyi* strain CCMP 373 rRNA gene	9×10^{-50}	Protein synthesis
PgC-13	384 bp	DQ886387	XP639957	Hypothetical protein DDB01862282(*Dictyostelium discoideum*)	2×10^{-6}	Similar to putative stress responsive protein Srg6
PgC-5	261 bp	DQ886388	AF100332	Dendrobium grex Madame Thong-IN putative 21D7 protein (ovg29)	6×10^{-5}	Differential expressed gene during orchid floral transition
PgC-6	486 bp	DQ886389	AF107586	Dendrobium grex Madame Thong-IN putative cell control protein (otg4)	4×10^{-4}	Differentially expressed gene during orchid flora transition gene involved in cell division

although one did not produce useable sequence information (Table 3). The size of these fragments ranged from 213 to 490 bp with an average of 362 ± 114 bp. Of the genes that were putatively identified, one was involved in energy production (PgC-3) with closest similarity to the nicotinamide adenine dinucleotide (NADH) dehydrogenase subunit I. PgC-10 was most closely related to a bacterial UDP-3-0-acyl N-acetylglucosamine deacetylase coding gene involved in lipid assembly, and PgC-19 was most similar to the large subunit ribosomal gene from *Emiliania huxleyi*. Interestingly, the remaining three fragments appear to be related to stress proteins or genes associated with flowering in higher plants. PgC-13 was most similar to a hypothetical stress response protein from the amoeba *Dictyostelium discoideum*. Both PgC-5 and PgC-6 appeared to be related to genes associated with flowering in an Orchid species. The sequences of these gene fragments have been deposited in Genbank with accession numbers DQ886384-9.

Determination of life stage vulnerable to viral infection in P. pouchetii (VIL) Viruses and the processes of viral infection and lysis have been characterized for *P. pouchetii* and *P. globosa* (Bratbak et al. 1998a, b; Brussaard et al. 2004, 2005; Jacobsen 2000, 2002; Jacobsen et al. 1996; Veldhuis et al. 2005). Determining which stages of the life cycle are vulnerable to viral infection and lysis is critical for understanding the effects of viruses on *Phaeocystis* life cycle dynamics and ecology. Most previous work with *P. pouchetii* (Bratbak et al. 1998b; Hamm et al. 1999; Jacobsen 2000; Jacobsen et al. 1996) and *P. globosa* (Brussaard et al. 2004; Brussaard et al. 2005) indicated that solitary cells are vulnerable to viral infection and lysis while colony cells are not. However, in one set of experiments, Baudoux and Brussaard (2005) showed that colonial *P. globosa* cells can be infected by the PgV virus. To determine which life stages of *P. pouchetii* are vulnerable to viral infection and lysis, Jacobsen et al. (2005) conducted a set of experiments during our 2003 mesocosm study. In one set of experiments *P. pouchetii* solitary cells were incubated with different amounts of virus additions and a control containing no viruses (Jacobsen et al. 2005). In a second set of experiments, colony cells that were detached from their colony matrix were incubated with different amounts of virus additions and a control containing no viruses; for comparison solitary flagellate cells were also included in this experiment. Colonies were shaken strongly in order to detach the cells from the mucus; this method is effective in producing colonial cells free of their mucus matrix because, in contrast to

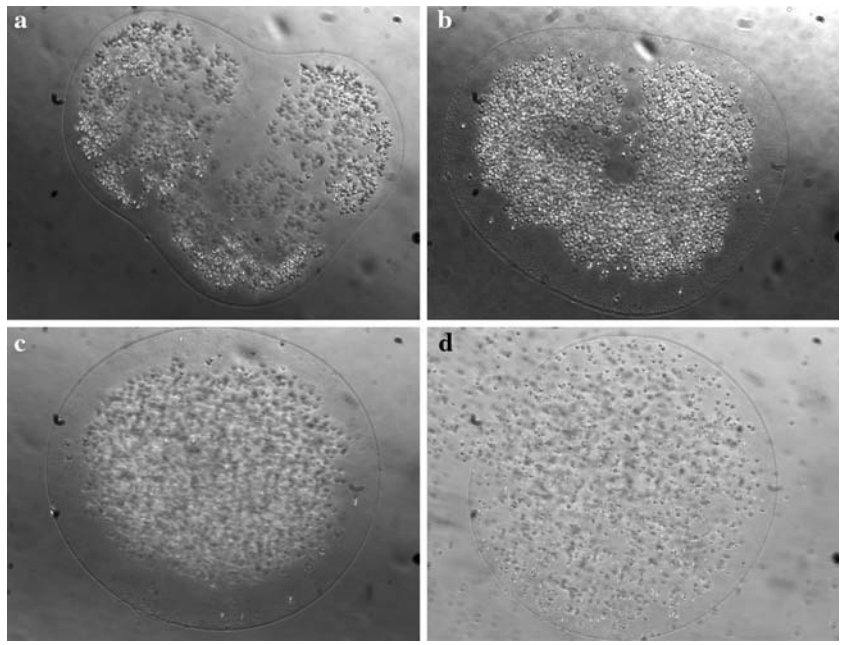

Fig. 3 A time sequence of a single *P. pouchetii* colony isolated and maintained in a well plate from the 2003 mesocosms (see Mesocosm Experiment Methods); data format: longest linear dimension and date and time of photo (**a**) 880 mm ; 26 March 2003, 11:33 (**b**) 920 mm ; 27 March 2003, 11:57 (**c**) 930 mm ; 28 March 2003, 09:41 (**d**) 890 mm ; 29 March 2003, 09:19; Cells within colony skin and in well plate observed to be motile at this time

P. globosa, colonies of *P. pouchetii* easily rupture (Jacobsen et al. 2005).

Intact colonies incubated with viruses showed no evidence of viral infection or lysis (Jacobsen et al. 2005). In experiments where colonial cells were detached from their colonies, detached colonial cells showed no evidence of cell lysis, whereas the solitary flagellated cells were completely lysed during the experimental period (Jacobsen et al. 2005).

Colony shape changes accompany colony senescence in P. pouchetii (CSWP; RCWP) The senescence of *Phaeocystis* colonies has been observed under natural, mesocosm, and laboratory conditions (Davidson and Marchant 1992; Lancelot and Rousseau 1994; Rousseau et al. 1994; Veldhuis et al. 1986; Verity et al. 1988). Solitary-cell release from senescent colonies at bloom termination has also been documented (Kornmann 1955; Rousseau et al. 1994; Verity et al. 1988). However, there is little detailed understanding of general colony-senescence and bloom-termination processes. In an in vitro study of *P. pouchetii*, colonies were isolated in well plates from the 2003 mesocosms, observed over a three-day period, and digital micrographs were taken to record colony morphology and measurement of dimensions.

During this study, a single colony was photographed over time that shows a change in morphology from the typical lobular *P. pouchetii* shape to a spherical shape (Fig. 3a–d). The time interval required for the observed shape change was two days. Approximately one day after the observed shape change, the colony was observed to contain motile cells and had also released some cells into the medium (Fig. 3d). While the possibility exists that spherical colonies were *P. globosa*, we found no evidence for the presence of *P. globosa* in these studies (see section in this manuscript entitled Species Identification in Mesocosms). Further investigation is needed to document the observed senescence phenomenon in other contexts, and to rigorously describe and quantify the observed morphological changes.

Quantification or investigation of a process or control parameter

Process rate quantification

Division (CDWP) and growth (CGWP) of P. pouchetii colonies Whipple et al. (2005b) reported that in vitro median colony division rates of mesocosm-derived *P. pouchetii* colonies ranged from 0.21–0.28 divisions day^{-1}. They also found that these colonies required an average of

3.5–4.9 days to complete one division; culture data from Cariou (described in Rousseau et al., 1994) indicated that *P. globosa* colony division occurred after 4–5 days, indicating similarity to *P. pouchetii* (Whipple et al. 2005b). Whipple et al. (2005b) reported that median growth rates of colonies, measured as maximum linear dimension, ranged from near zero to 7 μm h^{-1}.

Control parameters

Effect of diatom frustules on colony formation rate in P. pouchetii (CFSP) The transition from solitary cells to colonial forms is a critical event in the *Phaeocystis* life cycle. Many blooms consist primarily of colonial life stages (but see Wassmann et al. 2005). Even after nearly a century of study, no definitive set of conditions has been established as necessary for colony formation from solitary cells. Diatom frustules have been considered as particle nuclei for colony formation, including a number of observations of small *Phaeocystis* colonies attached to the setae of diatom frustules (especially *Chaetoceros*) (Boalch 1987; Lancelot and Rousseau 1994; Rousseau et al. 1994). Rousseau et al. (1994) stated that "...experimental work under controlled laboratory conditions (Rousseau and Davies, unpublished data), gives strong evidence that any microscopic particles, either biological (e.g. diatoms), organic or mineral (sand, glass wool) may act as substrate for colony development" (Rousseau et al. 1994, p. 35). Peperzak et al. (2000b) suggested that, in addition to providing a substrate that counteracts local turbulence, diatoms may influence colony formation by secreting vitamin B1, a possible growth stimulator, or depleting vitamin B12, a postulated growth inhibitor. These observations have led to the hypothesis that diatom frustules might enhance colony formation rate.

During the 2002 mesocosm study, an experiment was conducted to determine if in vitro *P. pouchetii* colony formation rates would be enhanced by the presence of diatom frustules added to culture medium; detailed methods and results appeared in Nejstgaard et al. (in press). Colony formation rates were estimated using water from a nutrient-amended mesocosm that was filtered to remove *Phaeocystis* colonies but allow solitary cells to pass. Diatomaceous earth was added to colonies in treatment well plates, while control well plates received only filtered mesocosm water (Nejstgaard et al. 2006). In all the experiments, new colonies increased approximately linearly over time. The colony formation rate for the filtered mesocosm water ranged from 1.3–1.9 colonies ml^{-1} d^{-1}; rates for water with diatomaceous earth ranged from 2.6–2.8 colonies ml^{-1} d^{-1}. Linear regression slopes for the filtered water versus water with diatomaceous earth were significantly different (Nejstgaard et al. 2006). The general conclusion of Nejstgaard et al. (2006) was that diatomaceous earth enhanced the in vitro colony formation rate for *P. pouchetii*.

Infochemical effect on colony formation due to mesozooplankton and microzooplankton (PRI). Long and colleagues conducted a series of experiments to test the ability of *P. globosa* solitary cells to respond to grazer-associated signals by altering colony formation or colony size. Colony formation was assessed when *P. globosa* encountered either microzooplankton or mesozooplankton grazers directly, only the chemical signals from these grazers, or no grazer cues (Long 2004; Long et al. submitted). In initial experiments with mixed zooplankton, the three dominant grazers were adult copepods of taxa *Acartia tonsa* and *Pseudodiaptomus pelagicus* (size ca. 1mm) and the heterotrophic dinoflagellate *Noctiluca scintillans*; the size of *P. globosa* colonies was about 50 μm in diameter. In other experiments, *A. tonsa* and *Euplotes* spp. were used alone as separate grazer treatments.

Two types of containers, both of which consisted of connecting compartments separated by a membrane and allowed for chemical exchange between connecting compartments while preventing algae and grazers from moving between compartments were used. *P. globosa* cells were added to both compartments while micro- or mesozooplankton grazers were added to only one compartment in grazer treatments. One set of containers was 1 L plastic bottles with sides removed and covered with Nitex mesh® (1 μm) using superglue. These mesh bottles were placed inside 2 l glass beakers that allowed for chemical exchange across 260 cm^2 of mesh. The second set of containers, called 'communication chambers',

was constructed from two 0.64 cm thick, clear, acrylic, capped tubes (each 8.89 cm in diameter and approximately 7.94 cm in length, for a final volume of 500 ml each) that screwed into each other and were separated by a 0.8 μm polycarbonate membrane filter (Long 2004; Long et al., submitted).

Phaeocystis globosa cells were added to both compartments while grazers were added to only one in grazer treatments. This allowed for simultaneous assessment of the direct and indirect effects of grazers on colony formation compared to grazer-free controls (Long 2004; Long et al., submitted). To enhance exchange of chemical signals between experimental chambers, mesh bottles were placed in bench top orbital shakers and rotated at 50 rpm; each bottle was also gently lifted twice a day until half of its volume was displaced from one chamber to the other (Long 2004; Long et al., submitted). Additional details of the setup of the experiments, sampling methods, and results can be found in Long (2004) and Long et al. (submitted).

In the initial mixed zooplankton experiments, Long et al. (submitted) found that crustacean mesozooplankton, dominated by adult copepods *A. tonsa* and *P. pelagicus*, significantly suppressed colony formation. In other experiments, Long et al. (submitted) found that infochemical cues alone from the mesozooplankton *A. tonsa* alone suppressed colony formation. Long et al. (submitted) also observed that the colony suppression effect of *A. tonsa* was density dependent. In experiments with the microzooplankton *Euplotes* spp., Long et al. (submitted) reported that infochemical cues from this ciliate enhanced *P. globosa* colony formation.

Discussion

Solitary cell transformation to colonies

Diatom frustules enhance *P. pouchetii* colony formation in vitro (Nejstgaard et al. 2006), a process that may therefore accelerate the in situ transformation of solitary motile cells into new colonies. This is the critical step in the formation of colony-based *Phaeocystis* blooms, and thus is essential in the construction of quantitative ecosystem models of *Phaeocystis*. Future research must elaborate details of the control of colony formation rate by diatom frustules, such as dose response, species differences (*Phaeocystis* or diatoms), and how this control might change over the course of a bloom. One method by which these results could be represented in mechanistic models is to include a colony formation rate algorithm that varies colony formation rate with respect to the density of diatom frustules in the *Phaeocystis* environment.

The results of chemical cue experiments point to a potentially important role of consumer organisms in influencing the *Phaeocystis* life cycle. Tang (2003) found that grazing by microzooplankton resulted in an increase in the proportion of cells in the colony life stages, and increased the mean size of colonies; Long et al. (submitted) also found that infochemical cues from ciliates (*Euplotes* spp.) enhanced colony formation in *P. globosa*. In contrast, Long et al. (submitted) found that chemical signals from the mesozooplankton *Acartia tonsa* suppressed colony formation in *P. globosa*. Because of the different responses observed for meso- versus microzooplankton grazers, Long et al. (submitted) postulated that *P. globosa* might be able to detect grazing, identify the grazer type, and respond with enhancement or suppression of colony formation or growth of existing colonies. This type of induced response by *Phaeocystis* implies a sophisticated control mechanism for colony formation where chemical signals are received, processed, and a response is made dependent on the information contained in the signal. Further empirical work might include identification of chemicals in the signal, and determination of specificity of the signal for grazer taxa. The Tang (2003) and Long et al. (submitted) results would allow for control structures to be added to mechanistic models involving detection of grazers, identification of sender, and appropriate response of *Phaeocystis* solitary cells or colonies to grazers. In addition, the results of Long et al. (submitted), that the colony suppression effect of *A. tonsa* is density-dependent, gives the first data for producing a dose-response algorithm for the effects of these grazer-derived infochemicals in plankton models containing the *Phaeocystis* life cycle stages. These sort of

controls demonstrate one aspect of the biocomplexity that organism semiotics can generate in ecosystem models.

Experimental results using coarse surficial material collected from shallow fjord sediments indicate that benthic particles may function as sites for colony formation. The colonies that appeared in the overlying water during the incubation were derived from cells or very small colonies associated with the sediments. It is inferred that these cells or small colonies were naturally associated with these sediments, however the possibility cannot be excluded that they were artificially mixed into the sediments at time of collection. These studies were not designed to prove or disprove the occurrence or existence of a true benthic life cycle stage, which has not been found since its original hypothesized existence (Kornmann 1955; Kayser 1970). Rather, the most parsimonious explanation is that these coarse sediments functioned as attachment sites for the initiation of new colonies, very much like diatoms and diatom frustules in the water column (Rousseau et al. 1994; Jacobsen 2002; Nejstgaard et al. 2006). Colonies were present in the fjord throughout the mesocosm study period (Nejstgaard et al. 2006). These results suggest that shallow sediments underlying illuminated waters during the period of *Phaeocystis* colony growth may serve as colony formation sites. The ecological contribution of colonies derived from such a mechanism must await a thorough seasonal study that includes sampling before and after the period of colony appearance in the water column. This finding suggests that some form of benthic involvement should be included in *Phaeocystis* life cycle models; these experiments do not provide evidence for a distinct benthic life cycle stage of *P. pouchetii*. Mechanisms that provide for benthic sediments providing colony formation sites could be provisionally included in models of *Phaeocystis* life cycles. Empirical studies to provide details of these sediment mechanisms are required to further elaborate conceptual or mechanistic models.

Comparison of gene expression profiles between single and colonial *Phaeocystis* cells indicated that a larger array of genes were expressed during the single stage life cycle phase than during the colonial life cycle stage, implying increased biochemical complexity associated with single cells. While the differential display analyses conducted as part of these studies in no way represent a comprehensive characterization of molecular differences between the colonial and single cell life cycles, intriguingly several of the genes that were expressed in colonial *Phaeocystis* cells and not in flagellated single cells appear to be related to stress, cell signaling, and complex developmental transitions in higher plants. Furthermore, the presence of genes in *Phaeocystis* most closely associated with phylogenetically diverse organisms including bacteria, amoeba, and higher plants, in addition to the closely related haptophyte *Emiliania huxleyi*, suggest that the genome of *Phaeocystis* is likely composed of a complex mosaic of genes and regulatory elements similar to other microalgae, including diatoms, whose genomes have recently been fully sequenced (Montsant et al. 2005).

The studies described here also demonstrate the tractability of *Phaeocystis* sp. to genomic and transcriptomics studies in the future. With relatively little modification it was possible to adapt commercially available RNA purification kits for work with plant cells to produce RNA suitable for constructing cDNA libraries from both single cells and colonies of *Phaeocystis globosa*. Several previous reports have demonstrated that genomic and plastid DNA suitable for molecular studies is also relatively easily obtained from a variety of *Phaeocystis* species (Zingone et al. 1999; Lange et al. 2002). As is occurring in all fields of biology, access to molecular information from individual organisms and communities of organisms is yielding tremendous insights into the physiology, life history, and ecology of organisms including microalgae for which molecular information is available (Allen 2005, Walker et al. 2005). Undoubtedly, important new insights into the life history of *Phaeocystis* will be gained when complete genomic and comprehensive gene expression data become available.

Colony proliferation

Documentation of colony division in vitro provides evidence for two types of colony proliferation in *P. pouchetii*. First, colonies may form

from solitary cells and grow by mitosis and secretion of extracellular mucus to form the large colonies observed in most blooms. Relationships between cell number and biomass has been documented for *P. globosa* (Rousseau et al. 1990) and *P. pouchetii* (Verity et al. in press); such data would enhance the usefulness of *P. pouchetii* colony size measurements. Second, existing colonies may undergo division and may also increase in size after division by mitosis and secretion of extracellular mucus (Whipple et al. 2005b). The latter pathway allows the number of colonies to increase faster than would be possible if all colonies were produced from solitary cells, because daughter colonies produced by colony division start out with hundreds of cells, and may attain a large size much faster than colonies established with a solitary cell. With the possibility of colony division, colonies that form from solitary cells constitute a source population from which large numbers of multicellular colonies may be derived by successive colony growth and colony division stages. Modeling this mode of colony proliferation could provide insight into its possible effects on *Phaeocystis* bloom dynamics. Further work will be needed to determine the extent to which this pattern is observed in natural *Phaeocystis* blooms.

Colony senescence and solitary cell release

Observation of spherical colonies with supporting genetic data, identifying them with high confidence as *P. pouchetii*, indicates that colony shape alone should be confirmed with other methods to unambiguously identify the species of *Phaeocystis* colonies observed in the field. The observed in vitro morphological changes of colonies of *P. pouchetii* suggest that there may be many changes in colony cell activities and physiology that would be required to perform this morphological change during colony senescence. It is not known in detail what changes the colony cells undergo during senescence; however, this colony shape change observation points to a period where major changes may be occurring. Further investigation of the colony cell characteristics during this shape-change period might provide further insight into the senescence processes in *P. pouchetii*. Qualitative shape changes, as described in these results, cannot usefully be incorporated into *Phaeocystis* life cycle models; however, if details of cellular physiology during senescence were to be discovered by further study of this colony stage, they would likely prove useful for model development.

Colony or solitary cell losses: viral infection and lysis

If blooms form predominantly from colonies, *P. pouchetii* can escape viral lysis by forming colonies. Until colonies undergo senescence and release solitary flagellate cells, which would be predicted to be vulnerable to viruses, *P. pouchetii* colony cells would be protected from viral infection. Transparent exopolymer particles (TEP), which appear to bind viruses (Brussaard et al. 2005), introduce another control that might be especially significant during senescence of *Phaeocystis* colonies at the end of blooms.

Jacobsen et al. (2005) report that detached colonial cells of *P. pouchetii* appear to be non-infectable. This suggests that the colony cells may have or acquire some inherent resistance to viral infection that does not involve the colony integument. Because the detached colonies would be surrounded by colony mucus that contains TEP, the PgV particles might have been bound to TEP, reducing their ability to infect the detached cells. This potential confounding factor should be addressed in future experiments. Jacobsen (2002) proposed that a colony integument pore size too small to admit virus particles might be a mechanism for prevention of viral infection of colonies. Results reported in Jacobsen et al. (2005) indicate that another mechanism might be responsible for the lack of colony cell lysis observed for *P. pouchetii*. These experiments were not designed to elucidate the specific viral infection mechanisms, and further investigation is needed to confirm these preliminary observations and investigate mechanisms of lysis resistance (Jacobsen et al., 2005). Ruardij et al. (2005) incorporated viral lysis into a simulation model of *P. globosa*; if the results of Jacobsen et al. (2005) were incorporated into a simulation model of *P. pouchetii*, viral lysis would only involve solitary cells and once cells were in

their colonial form they would be invulnerable to viruses.

Summary and conclusions

Several compartments, process flows, and their controls that were included in Whipple et al. (2005a) were empirically investigated to fill gaps in our understanding. Two major aspects of the conceptual model were involved.

The first involved testing for the existence of a life cycle process or producing a description of such a process. Five examples discussed here include (**a**) demonstration of in vitro colony division in *P. pouchetii* (CDWP); (**b**) description of in vitro change in colony shape for *P. pouchetii* associated with senescence (CSWP); (**c**) determining which life stage of *P. pouchetii*, solitary cells or colonies, is vulnerable to viral infection and lysis (VIL); (**d**) demonstration of potential benthic involvement in the *P. pouchetii* life cycle (CFBI); (**e**) demonstration of differential gene expression in solitary versus colonial cells of *P. globosa* (DGE).

The second facet involved quantification of processes or regulatory controls. Two examples of process rate quantification for *P. pouchetii* are: (**a**) in vitro colony division rate (CDWP) and (**b**) in vitro colony growth rate (CGWP). There were also two examples of investigating process controls. The first was diatom frustule enhancement of colony formation from solitary cells in *P. pouchetii* (CFSP), and the second was suppression of colony formation in *P. globosa* by infochemicals released by mesozooplanktonic grazers, and enhancement of colony formation by infochemicals released by microzooplankton grazers (PRI).

The significance of investigating these features of *Phaeocystis* ecology in the context of a *Phaeocystis* life cycle model is that new insights and understanding are readily accommodated in a pre-existing organized scheme of established facts and hypotheses. This new understanding, made coherent by the life cycle model, can improve ecosystem simulation models (e.g., Canziani and Hallam 1996; Verity 2000; Lancelot et al. 2005; Ruardij et al. 2005) designed to receive the life cycle information and relate it to the broader ecosystem. From this perspective, *Phaeocystis* is a definitive example of a complex adaptive system (CAS) (Waldrop 1992; Kauffman 1993, 1995) incorporated within its broader ecosystem, also seen as a complex adaptive system. Using models, conceptual or ecosystem, to drive empirical research that is then incorporated into new model structures can ultimately provide better insight into the direct and indirect effects of *Phaeocystis* populations on such important contemporary problems as global biogeochemical cycles, as well as illuminate the roles of *Phaeocystis* in its organized ecosystems.

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