Susceptibility of colonies and colonial cells of *Phaeocystis pouchetii* (Haptophyta) to viral infection

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ABSTRACT: Viruses play a significant role in the microbial food web, as controlling agents in community composition and succession, and in termination of blooms. The flagellated stage of the polymorphic *Phaeocystis pouchetii* (Hariot) Lagerheim was previously shown to be readily infected by the species-specific PpV-virus. In the present study, we investigated if colonial cells of *P. pouchetii* were susceptible to virus infection and if the growth stage of the host population affected viral infectivity, based on 2 types of observations: incubation experiments with natural *P. pouchetii* colonies and added viral concentrate, and monitoring viral abundance during 2 different growth seasons in large outdoor mesocosm experiments. In the incubation experiments, colonial cells of *P. pouchetii* at various growth stages, embedded in and detached from the mucus, were inoculated with different concentrations of PpV-AJ96. Viral lysis of colonial *P. pouchetii* cells was not observed, regardless of virus concentrations and stage of *P. pouchetii* colony growth. However, flagellated cells of *P. pouchetii* were readily infected with the same virus strain. In the mesocosm sampling studies, the development of populations of PpV-like particles along with a bloom of *P. pouchetii* was followed in 2 separate growth seasons. These studies showed a dynamic PpV-like abundance over time that was closely linked to the host population. PpV-like viruses were present and readily detected in both mesocosm experiments in which *P. pouchetii* appeared. The results from these experiments suggest that colonial cells of *P. pouchetii* were not infected by the virus strain PpV-AJ96 and that the colonial stage of *P. pouchetii* provides protection against viral infection.

KEY WORDS: *Phaeocystis pouchetii* · Colonies · Virus

INTRODUCTION

Viruses are abundant in marine ecosystems, where they significantly affect microbial food webs (e.g. Fuhrmann 1999, Suttle 2000, Brussaard 2004). Viruses infecting phytoplankton have been demonstrated to play an important role in bloom dynamics (e.g. Zingone 1995), as controlling agents in community composition and succession (Wommack & Colwell 2000, Larsen et al. 2001), as well as in the termination of phytoplankton blooms (Bratbak et al. 1993, Nagasaki et al. 1994, Castberg et al. 2001, Jacquet et al. 2002, Brussaard 2004).

Species from the genus *Phaeocystis* Lagerheim have a worldwide distribution and may form dense blooms (e.g. Lancelot et al. 1994) that can alter material flow among trophic levels and export from the upper ocean (Wassmann et al. 1990, Lancelot et al. 1998). Transitions between colonial and solitary cell stages are the rule within the genus (e.g. Kornmann 1955, Rousseau et al. 1994, Jacobsen 2002). The gelatinous colonial stage is readily recognized as it produces prodigious blooms and has therefore received attention in numerous investigations worldwide (e.g. Davidson & Marchant 1992, Lancelot et al. 1994, Nejstgaard et al. 2006). Blooms of the colonial

stage of *P. pouchetii* occur regularly in spring in Norwegian waters (Eilertsen et al. 1981, Jacobsen 2000, Wassmann et al. 2005). Several studies (e.g. Jacobsen et al. 1996, Bratbak et al. 1998) have shown the flagellated stage of *P. pouchetii* to be extremely susceptible to viral mortality, which could thereby explain the apparently insignificant abundance of this morphotype in Norwegian waters.

A virus specific for *Phaeocystis pouchetii* (PpV-AJ96), isolated and maintained in culture for over a decade (Jacobsen et al. 1996), is capable of lysing flagellated cells of *P. pouchetii* (Jacobsen et al. 1996, Jacobsen 2000). Both flagellated cells and non-flagellated colonial cells of the closely related species *P. globosa* Scherffel are also susceptible to viral infection when not embedded in mucus (Baudoux & Brussaard 2005). It has been shown that the outer membrane of colonies of *P. globosa* possesses 1 to 4.4 nm large pores, which are smaller than the size of the *P. globosa* viral particle (PgV) and may thus act as mechanical protection against viral infection (Hamm et al. 1999).

The purpose of the present study was to elucidate, for the first time, if colonial cells of *Phaeocystis pouchetii* were susceptible to virus infection, and also if the growth stage of the host population influenced infectivity. Both intact colonies and colonial cells detached from the mucus were inoculated with different concentrations of PpV-AJ96 at various growth stages. In addition, the development of the virus population during a bloom of *P. pouchetii* was followed in 2 separate mesocosm experiments (2002 and 2003).

MATERIALS AND METHODS

The present study includes 2 types of experiments: (1) infection experiment using wild *Phaeocystis pouchetii* colonies and cultured *P. pouchetii*-specific viruses, and (2) sampling of *P. pouchetii* and *P. pouchetii* viral-like particles during 2 growth seasons in large outdoor mesocosms.

Infection experiment. Four virus-infection experiments (Expts 1 to 4) were conducted in 2003 to see if viruses infected colonial cells embedded in mucus and/or detached from the mucus. The virus strain used in Expts 1 to 4 (PpV-AJ96) was obtained from the virus collection at the Department of Biology (University of Bergen, Norway). The virus was isolated in 1995 and has proven specific for *Phaeocystis pouchetii* (Jacobsen et al. 1996). Prior to each colony infection experiment, a fresh virus lysate was counted by flow cytometry (FCM) in order to give an accurate number of viruses added. Keeping *P. pouchetii* as colonies in a laboratory culture is difficult and to our knowledge

there is no such culture available. Accordingly, we used *P. pouchetii* colonies from various stages of bloom development collected during a mesocosm experiment in 2003, in which colonies of *P. pouchetii* dominated the phytoplankton community (Nejstgaard et al. 2006). The colonies used in the infection experiments described below were obtained from mesocosm NPb (see 'Mesocosm sampling' for further details).

Expt 1: Expt 1 was conducted in the early exponential stage of the *Phaeocystis pouchetii* mesocosm bloom, starting on Day 10 (see Fig. 4). A sample of mesocosm water (4 ml) containing an average number ($\pm$SD) of 1.5 ± 0.5 colony ml^{-1} (n = 5) and a mean of 112 ± 9 cells colony^{-1} was added to each of 12 wells in 5 Nunc culture plates. The number of cells per colony was estimated according to Verity et al. (2007a). Each of the 12 wells in the 5 Nunc culture plates were then inoculated with PpV-AJ96 corresponding to a final concentration of 10^8, 10^7, 10^6, 10^5 and 0 ml^{-1}, respectively, with a virus-to-host ratio ranging from 0 to 892 857. The multiplicity of infection (MOI) was not measured, but the infectivity of the viruses employed in this study was earlier reported to be 1 to 10 % (Bratbak et al. 1998). Colonies of *P. pouchetii* rupture easily in contrast to *P. globosa*; hence, to obtain detached colonial cells, a 100 ml mesocosm water sample was shaken vigorously and a 4 ml sample was added to each of 12 wells in 1 Nunc culture plate containing an average number of 214 ± 87 cells ml^{-1}. In addition, 4 ml of an exponentially growing culture of flagellated cells (strain AJ01, Jacobsen et al. 1996) containing an average number of $10.5 \times 10^4 \pm 8721$ cells ml^{-1} was added to each of 12 wells in 1 Nunc culture plate. Each of the 12 wells in these 2 Nunc culture plates was then inoculated with PpV-AJ96 corresponding to a final concentration of 10^8 ml^{-1}. Every plate was inspected and *P. pouchetii* counted each day for 5 d. Colonial *P. pouchetii* was counted under a dissection microscope (Wild M10). Flagellated and detached colonial cells were inspected and counted under a Leitz Dialux-20 light microscope with phase contrast at 400× magnification using a Fuchs Rosenthal haematocytometer with a counting error of ±10 % (Andersen & Throndsen 2003).

Expt 2: Expt 2 was conducted in the exponential stage of the *Phaeocystis pouchetii* mesocosm bloom starting on Day 18. A 2 ml mesocosm water sample containing an average of 49 ± 5 colonies ml^{-1} and an average of 154 ± 12 cells colony^{-1} was added to each of 12 wells in 5 Nunc culture plates. Each of the 12 wells in the 5 Nunc culture plates was then inoculated with PpV-AJ96 corresponding to a final concentration of 10^8, 10^7, 10^6, 10^5 and 0 ml^{-1}, respectively. The initial concentration of detached colonial and flagellated cells contained an average of 336 ± 16 and $13 \times 10^4 \pm 13 588$

cells ml^{-1}, respectively. The virus-to-host ratio ranged from 0 to 16 233. For experimental design, sampling procedures, inspection and counting: see description of Expt 1.

Expt 3: Expt 3 was conducted in the early stationary stage of the *Phaeocystis pouchetii* mesocosm bloom starting on Day 25. A 1 ml mesocosm water sample containing an average of 96 ± 16 colonies ml^{-1} and an average of 210 ± 16 cells colony^{-1} was added to each of 12 wells in 5 Nunc culture plates, each of which were then inoculated with PpV-AJ96 corresponding to a final concentration of 10^8, 10^7, 10^6, 10^5 and 0 ml^{-1}, respectively. The initial concentration of detached colonial and flagellated cells contained an average of 532 ± 21 and 10.5×10^4 ± 8721 cells ml^{-1}, respectively. The virus-to-host ratio ranged from 0 to 4762. For experimental design, sampling procedures, inspection and counting: see description of Expt 1.

Expt 4: Expt 4 was conducted at the collapse of the *Phaeocystis pouchetii* mesocosm bloom starting on Day 32. A 1 ml mesocosm water sample containing an average of 205 ± 18 colonies ml^{-1} was added to each of 12 wells in 5 Nunc culture plates. Unfortunately, the number of cells per colony was not counted in this experiment; therefore, the virus-to-host ratio is not given. Each of the 12 wells in the 5 Nunc culture plates was then inoculated with PpV-AJ96 corresponding to a final concentration of 10^8, 10^7, 10^6, 10^5 and 0 ml^{-1}, respectively. The initial concentration of detached colonial and flagellated cells contained an average of 727 ± 18 and 12×10^4 ± 22 500 cells ml^{-1}, respectively. For experimental design, sampling procedures, inspection and counting: see description of Expt 1.

Mesocosm sampling. Sampling for *Phaeocystis pouchetii* and *P. pouchetii* viral-like particles was part of major mesocosm studies carried out in March to April 2002 and 2003 at the Marine Biological Field Station, University of Bergen, Norway (Nejstgaard et al. 2006, Verity et al. 2007a,b). Two sets of experiments, each consisting of three 11 m^3 enclosures, were mounted on floating frames moored to a raft (see Nejstgaard et al. 2006 for further details). Two of the enclosures were initially fertilized with nitrate (NaNO$_3$) and phosphate (KH$_2$PO$_4$), corresponding to increases of 16 µM nitrate and 1 µM phosphate, and one served as a control without nutrient addition. Each day, 10% of the enclosure volume was renewed along with 10% of the initial nutrient load. All of the enclosures were amended with the same initial nutrient concentration, except for the controls. In the 2002 experiment, both nutrient-enriched enclosures were augmented with 8 µM nitrate and 0.5 µM phosphate on Day 9 due to low nutrient concentrations. The mesocosms were named control, NP and NPF in 2002 and control, NPa

and NPb in 2003, where NP corresponds to nitrate (N) and phosphate (P) amended mesocosms in both years, and F corresponds to added flagellated cells (in 2002 only; see below).

To investigate if higher initial abundance of flagellated cells would induce more or earlier colony formation, a 40 l culture of *Phaeocystis pouchetii* flagellated cells (8.25×10^4 cells ml^{-1}) was added to mesocosm NPF (only in 2002), resulting in a final concentration of flagellated cells of 300 cells ml^{-1}. The initial native flagellated population of *P. pouchetii* was ca. 200 cells ml^{-1}. The culture of *P. pouchetii* flagellated cells was originally isolated from Raunefjorden in 2001 and grown in f/2-Si media (Guillard 1975) at a salinity of 31‰.

The development of virus populations was followed along with blooms of *Phaeocystis pouchetii* in the mesocosm experiments in both years, except in the control mesocosm in 2002. Flagellated cells and colonies of *P. pouchetii* were counted as described in Nejstgaard et al. (2006), and viruses were counted by means of FCM. For FCM analyses, seawater samples were fixed with glutaraldehyde (0.5% final concentration), frozen in liquid nitrogen and stored at –70°C (Marie et al. 1999b). After thawing, the samples were stained with SYBR Green I (Molecular Probes) and analyzed according to Marie et al. (1999a) using a FACSCalibur FCM (Becton Dickson). The discrimination of virus subtypes was based on groupings observed in scatter plots of SSC signal versus green DNA-dye (SYBR Green) fluorescence (see Fig. 3). Datafiles were analyzed using CYTOWIN (Vaulot 1989, available at www.sb-roscoff.fr/Phyto/index.php) and EcoFlow (version 1.0.5, available from the authors).

Water samples from mesocosms NPF (2002) and NPb (2003) were collected on a regular basis and used to inoculate growing cultures of *P. pouchetii* (AJ01), to test for presence of functionally infective *P. pouchetii* viruses. These samples were also analyzed by FCM for populations of virus and bacteria.

RESULTS

Infection experiments

The results from the virus infection experiments in which intact colonies were infected with PpV-AJ96 showed no reduction in the number of colonies present (Fig. 1). There were no signs of cell lysis within the colonies during the different stages of growth (Expts 1 to 4). On visual inspection the cells appeared healthy, and the number of colonies stayed relatively stable from the start of each experiment until termina-
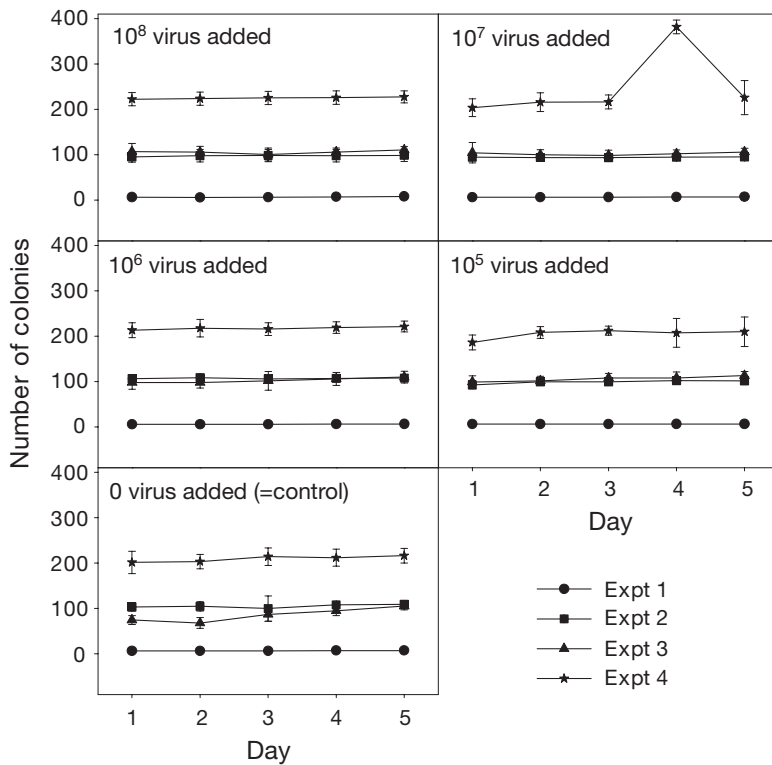
Fig. 1. *Phaeocystis pouchetii*. Time series during 4 different infection experiments with colonial cells of *P. pouchetii* embedded in mucus at different virus concentrations added (0 to 10^8 virus ml^{-1}) and at different growth stages of the host population. Numbers are mean colonies per well ±SD, n = 12

tion 5 d later. None of the dilution treatments or the different growth stages of the host populations were affected by virus addition (10^8 to 10^5 ml^{-1}), and they were similar to the controls (Fig. 1). One exception, however, was the colony abundance in the 10^7 virus addition of Expt 4, which increased on Day 4, probably caused by a counting error as the abundance decreased the following day (Day 5). The virus infec-

tion experiments with colonial cells detached from the mucus also did not reveal reductions in cell numbers caused by viral lysis. In contrast, the flagellated cells of *P. pouchetii* infected with PpV-AJ96 were completely lysed within 3 d (Fig. 2). Although the infection experiments were ended after 5 d, all of the Nunc plates were inspected after an additional 10 d in order to detect a possible viral infection of the colonial cells. This was never observed (results not shown).

Mesocosm studies

A *Phaeocystis pouchetii* virus (PpV) was readily detected in mesocosm water in which *P. pouchetii* appeared (Fig. 3). In order to confirm the presence of PpV-specific viruses in the mesocosm water we inoculated a flagellated *P. pouchetii* culture (strain AJ01) with water from mesocosms NPF (2002) and NPb (2003). The cultures lysed within 2 d and the virus groups could be observed in FCM dot plots (Fig. 3A,C). A virus population with similar FCM signature to the previously isolated PpV-AJ96 (not shown) and PpV from these lysates (Fig. 3A,C) was observed in water samples directly from the mesocosms as well (Fig. 3B,D) and is hereafter called PpV-like particles.

PpV-like viruses appeared during and after blooms of *Phaeocystis pouchetii* in both mesocosm experiments (Figs. 3 & 4). In the 2002 experiment, the PpV-like particles increased considerably as the bloom of *P. pouchetii* progressed (Fig. 4). This was also seen in the 2003 experiment, but the virus proliferation was delayed and more prominent after the bloom of *P. pouchetii* (Fig. 4).
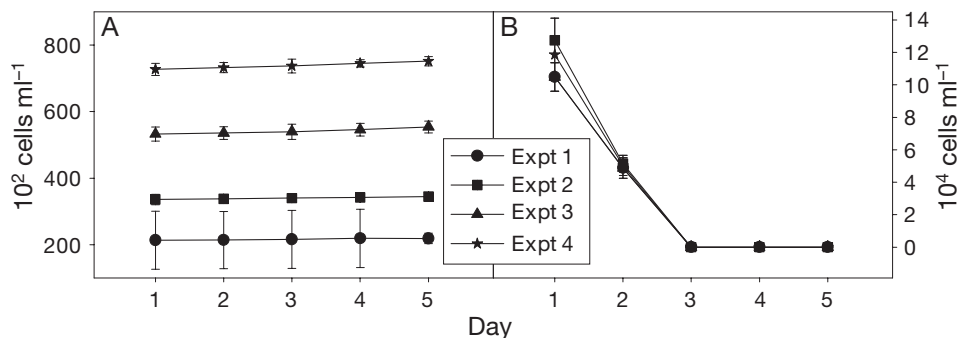

Fig. 2. *Phaeocystis pouchetii*. Time series during 4 different infection experiments adding viruses (10^8 ml^{-1}) to (A) detached colonial cells and (B) flagellated cells of *P. pouchetii* with host population in different stages of growth. Numbers are mean cells ml^{-1} ±SD, n = 12
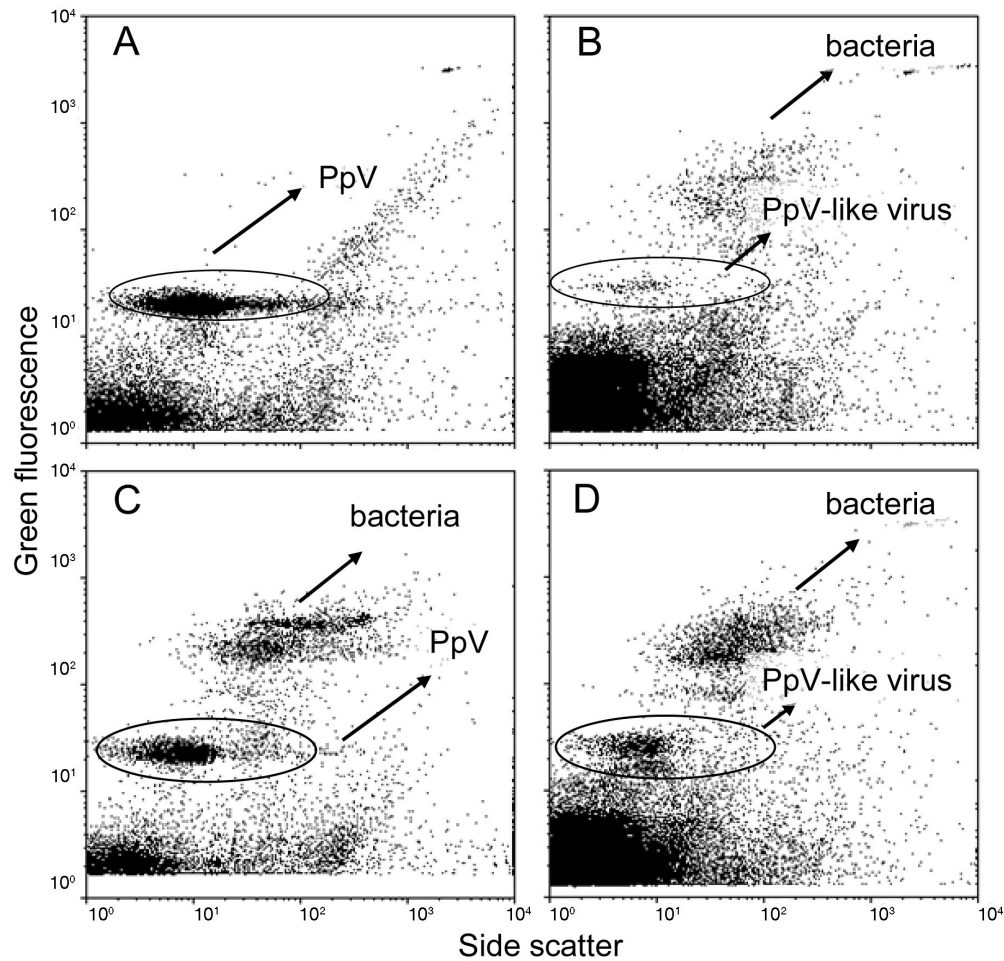
Fig. 3. Biparametric flow cytometry plots showing populations of viruses and bacteria (A,C) in lysates and (B,D) in mesocosm samples. (A) Virus and bacteria in a lysate after inoculating *Phaeocystis pouchetii* culture with water from mesocosm NPF (26 March 2002). (B) Virus and bacteria in mesocosm NPF on 26 March 2002. (C) Virus and bacteria in a lysate after inoculating *P. pouchetii* culture with water from NPb (20 March 2003). (D) Virus and bacteria in mesocosm NPb on 3 April 2003. PpV: *P. pouchetii* virus

DISCUSSION

The results from the virus infection experiments imply that colonial cells of *Phaeocystis pouchetii* embedded in or detached from the mucus, regardless of the growth stage of the population, were not affected by viral lysis. One probable cause could be that the outer membrane of the colonies acts as mechanical protection against, for example, viruses and makes the cells inaccessible to viral infection (Hamm et al. 1999), permitting colonies to bloom. Brussaard et al. (2005) showed that the colonial form of *P. globosa* is an excellent mechanism to prevent viral infection. Modeling suggests that the relevant issue may be the size of the *P. globosa* colonies, which reduces the chance of infection per cell, as opposed to the mucus acting as a barrier (Ruardij et al. 2005), since

when the diameter of the colonies increased the viral infection became insignificant. From this hypothesis, colonies should therefore be more susceptible to virus infection at an early stage of the bloom when the diameter is fairly small (e.g. Verity et al. 2007a,b), but this was not observed for *P. pouchetii* in the infection experiments. Even when the virus-to-host-cell ratio was higher when infecting colonies (8929 to 892 857) compared to the virus-to-host-cell-ratio when infecting the flagellated cells (1000), viral infection of the colonies was not promoted.

Detached colonial cells of *P. pouchetii* were not susceptible to viral infection. In contrast, Baudoux & Brussaard (2005) showed that one strain of *P. globosa* (detached colonial cells) was infected by the PgV virus and that it had a strain-specific spectrum of infection. They also showed that flagellated cells of *P. globosa*
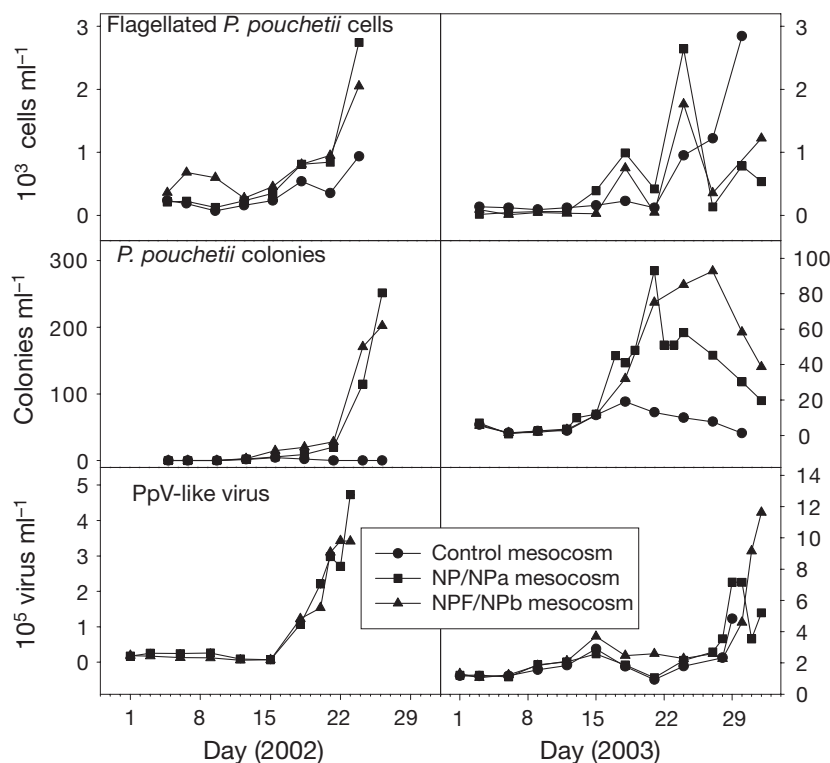
Fig. 4. Time series of flagellated cells and colonies of *Phaeocystis pouchetii* and PpV-like particles in 2 mesocosm experiments (2002 and 2003). Control = no nutrients added; NP, NPF, NPa, NPb = nutrients added

then form blooms of colonies under non-limiting conditions (e.g. Nejstgaard et al. 2006) in order to escape grazing by zooplankton (Nejstgaard et al. 2007). Brussaard et al. (2005) showed that the 2 morphologies of *P. globosa* cells (flagellated and colonial) were differentially regulated by viral control. Under non-limiting nutrient and light conditions blooms of colonies were formed. Viruses were found to be a significant loss factor, but could not prevent bloom formation. Under conditions that restricted colony formation and favoured the proliferation of flagellated single cells, viruses were found to prevent bloom formation. Flagellated cells of *P. pouchetii* can grow at relatively high rates (Jacobsen & Veldhuis 2005, Wassmann et al. 2005) outside the blooming period in natural waters under different growth conditions, implying that viral infection of flagellated populations may take place at a relatively constant rate and regulate population growth.

The mesocosm studies showed a dynamic PpV-like abundance over time that was closely linked to the host population. A peak in PpV-like particles coincided with the peak of both colonies and flagellated cells in 2002, while in 2003 both colonies and flagellated cell collapses were linked to increased viral abundance.

It has been speculated that blooms of *Phaeocystis pouchetii* may be terminated by species-specific virus infection (Jacobsen 2000). However, there is strong evidence that viral lysis does not terminate colonial blooms of *P. pouchetii* (Nejstgaard et al. 2006) and that viruses do not infect colonial *P. pouchetii* cells (present study). At the peak of the *P. pouchetii* mesocosm blooms, flagellated cells were observed inside of the colonies, followed by proliferation of flagellated cells, which were possibly triggered by nutrient limitation (Whipple et al. 2005). This may explain the observed increase in free flagellated cells in the mesocosms, coinciding with the peak of the colonies. Both (1) the release of flagellated cells and (2) nutrient limitation preventing production of new colonies may therefore explain the decrease in colony numbers. Once the flagellated cells were liberated they became susceptible to viral infection, resulting in the observed increase in PpV-like particles. The modeling results of Ruardij et al. (2005) suggested that the fraction of infectious PgV was highest when single cells of *P. globosa* dominated the population.

were readily infected by the PgV and that the susceptibility of one strain was not constant over time. This is also in accordance with Nagasaki & Yamaguchi (1998) and Nagasaki et al. (1999), who showed that some clones of *Heterosigma akashiwo* were resistant to viral infection and had different virus sensitivity. Thus it is possible that the lack of infection of colony cells in the present study reflects viral strain specificity. However, this seems unlikely given that (1) different strains of *P. pouchetii* were susceptible to viral infection in culture (e.g. Jacobsen et al. 1996), and (2) the results represent natural *P. pouchetii* populations undoubtedly containing multiple genetic lines (e.g. Verity et al. 2007c), and occurring over a 2 yr period.

Thus it is parsimonious to conclude from available evidence that only the flagellated cells of *Phaeocystis pouchetii* are infected by the PpV and therefore PpV only have the ability to affect bloom dynamics when the host organisms appears in the flagellated stage. A constant low abundance of viruses can theoretically prevent massive blooming of flagellated cells and thereby prevent colony formation from the flagellated cells (e.g. Jacobsen 2002). However, a small fraction of the flagellated population in nature may be, or even may develop, resistance to viral infection as shown by Thyrhaug et al. (2003). Such resistant populations can

In conclusion, there were no signs of lysis of colonial *Phaeocystis pouchetii* cells embedded in mucus, regardless of virus concentration and growth stage of the host population. The flagellated cells were, however, readily infected. This suggests that the colony stage of *P. pouchetii* provides protection against viral infection and that the flagellated stage is vulnerable to viral infection and lysis. In an ecological perspective, colony formation by *P. pouchetii* may thus act as a defense mechanism against viral infection and/or grazing, and thereby escape a major mortality source. The extreme susceptibility of the flagellated cells can also be an explanation of the apparently insignificant abundance of this morphotype in Norwegian waters.

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