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ORIGINAL PAPER

Haemolytic activity of live *Phaeocystis pouchetii* during mesocosm blooms

Marion van Rijssel · Anne-Carlijn Alderkamp · Jens C. Nejstgaard · Andrey F. Sazhin · Peter G. Verity

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Abstract Chemical defence is a potential mechanism contributing to the success of *Phaeocystis* species that repeatedly dominate the phytoplankton in coastal areas. Species within the genus *Phaeocystis* have long been suspected of imposing negative effects on co-occurring organisms. Recently a number of toxins have been extracted and identified from *Phaeocystis* samples, but it is not clear if they do enhance the competitive advantage of *Phaeocystis* species.

In the present study the cytotoxic impact of live *Phaeocystis pouchetii* to human blood cells in close proximity, regardless of the nature of the responsible mechanism, was quantified using a bioassay. Haemolytic activity was measured during blooms of *P. pouchetii* in mesocosms. These environments were chosen to mimic natural conditions including chemically mediated interactions that could trigger defensive and/or allelopathic responses of *Phaeocystis*.

Haemolytic activity correlated with *P. pouchetii* numbers and was absent during the preceding diatom bloom. Samples containing live *P. pouchetii* cells showed the highest activity, while filtered sea water and cell extracts were less haemolytic or without effect. Dose-response curves were linear up to 70% lysis, and haemolysis in samples containing live *P. pouchetii* cells reached EC_{50} values comparable to known toxic prymnesiophytes ($1.9 * 10^7$ cells l^{-1}). Haemolytic activity was enhanced by increased temperature and light. The results indicate that unprotected and thus presumably vulnerable cells present in a *P. pouchetii* bloom may lyse within days.

Keywords Allelopathy · Chemical defence · Prymnesiophyte · PUFA · PUA

M. van Rijssel (✉) · A.-C. Alderkamp
Department of Marine Biology, Centre for Ecological and Evolutionary Studies, University of Groningen, P.O. Box 14, 9750AA Haren, The Netherlands
e-mail: m.van.rijssel@rug.nl

J. C. Nejstgaard
Department of Fisheries and Marine Biology, University of Bergen, P.O. Box 7800, 5020 Bergen, Norway

A. F. Sazhin
P. P. Shirshov Institute of Oceanology, Russian Academy of Science, 36 Nakhimovski Prospekt, 117997 Moscow, Russia

P. G. Verity
Skidaway Institute of Oceanography, 10 Oceans Science Circle, Savannah, GA, 31411, USA

Introduction

Phaeocystis is an algal genus which regularly dominates vernal blooms in coastal regions all over the world, especially in temperate and higher-latitude waters. These almost monospecific

blooms (Lancelot et al. 1987) have a great impact on the local marine food web because they produce the bulk of the primary production in springtime (Arrigo et al. 1999; DiTullio et al. 2000; Schoemann et al. 2005, introduction of this issue). There is likely a combination of mechanisms behind the bloom forming capacity of this genus. For example, *Phaeocystis* is able to take advantage of eutrophication (Lancelot et al. 1987; Cadée and Hegeman 2002), resulting in high biomass production. Blooms are typically dominated by the colonial form of these species and adjustment of colony size could be a response to escape grazing pressure, thereby reducing population losses [Jacobsen and Tang 2002; Tang 2003, see however Nejstgaard et al. (this issue) for a discussion on this mechanism]. Envelopment of the cells by a colonial mucous layer could be another mechanism to reduce losses because only the motile cells seem to be susceptible to viral lysis (Brussaard et al. 2005, this issue).

Also, *Phaeocystis* has been suspected for a long time of having a negative effect on co-occurring organisms. Penguins died after consumption of krill that fed on *P. antarctica* (Sieburth 1960, 1961). Schools of herring seem to avoid *Phaeocystis* blooms (Savage 1930), and mass mortality of caged fish occurred during a *P. globosa* bloom in the China Sea (Huang et al. 1999). Cod larvae died in the presence of natural densities of *P. pouchetii* (Eilertsen and Raa 1995; Aanesen et al. 1998) and negative effects of *Phaeocystis* were recorded on the bryozoan *Electra pilosa* (Jebram 1980). Bacterial consumption rates of acrylate in field samples increased when the > 20 μm fraction containing *P. globosa* colonies was removed (Noordkamp et al. 2000). And then there is the so-called legend of *Phaeocystis* unpalatability (Huntley et al. 1987) that says that healthy colonies are not consumed due to some sort of chemical deterrence (Estep et al. 1990). The observed negative effects of *Phaeocystis* presence on other organisms may be a key to its bloom forming capacity; chemical deterrence could be a way to reduce grazing (Nejstgaard et al., this issue) as well as inhibiting competitors (allelopathy), thereby increasing fitness.

Up to now, three toxic components that could be involved in chemical deterrence have been identified in *Phaeocystis* species: acrylate, a polyunsaturated aldehyde, and a haemolytic glycolipid. (1) Acrylate is produced by *Phaeocystis* (Guillard and Hellebust 1971; Sieburth 1960) upon enzymatic cleavage of dimethylsulphoniopropionate (DMSP, Stefels and Dijkhuizen 1996) and accumulates in mM concentrations in the colonial mucous layer (Noordkamp et al. 1998). During growth, however, acrylate is unlikely to cause harmful effects on nearby living cells because it is not excreted from the colonies (Noordkamp et al. 2000). Additionally, the concentration of acrylate present in the water column is not expected to exceed the 4 μM observed in senescent cultures (Noordkamp et al. 2000). This is much lower than the mM range of $L(E)C_{50}$ values reported for marine organisms (Sverdrup et al. 2001). Therefore, acrylate is not a likely component to be involved in allelopathy. Acrylate produced by *Phaeocystis* could, however, have a negative impact on grazers (and their consumers) when *Phaeocystis* cells accumulate in their guts. In these acidic environments the high concentrations of acrylate will be in the protonated toxic form (below pH 4.25). A grazing-activated chemical defence system based on the conversion of DMSP into DMS and acrylate upon cell damage was already described for another prymnesiophyte, *Emiliania huxleyi* (Wolfe et al. 1997).

(2) The isolation and identification of an unsaturated aldehyde from *P. pouchetii* (Hansen et al. 2004) may indicate a line of defence that was recently revealed for diatoms (Paffenhöfer et al. 2005, and references therein). Membrane lipids are converted into mildly toxic polyunsaturated fatty acids (PUFAs) by a grazing-activated enzymatic conversion. In the presence of reactive oxygen species (ROS) PUFAs may be converted into highly toxic polyunsaturated aldehydes (PUAs). In laboratory tests these PUAs negatively affect copepod fecundity and egg-hatching, and induce apoptosis in sea urchin embryos and cytotoxicity in human cell lines (Pohnert and Boland 2002). Precursors for PUAs, such as the PUFA eicosapentaenoic acid (EPA), are abundantly present in *P. globosa* (Hamm and Rousseau 2003). Although these haemolytic PUFAs (Arzul et al. 1998; Fu et al. 2004) provide essential nutrition

for copepods high concentrations may be harmful (Jüttner 2001). Extracted culture fluid of *P. pouchetii* containing PUAs and corresponding with densities of 10^6 cells per ml completely blocked DNA replication in sea urchin embryos (Hansen et al. 2003; Hansen et al. 2004). Extraction of cells or sea water yielded fractions that were mildly haemolytic as well as anaesthetic upon injection in flies (Stabell et al. 1999).

(3) The massive *P. globosa* bloom of 1997 in the coastal waters of southeast China induced fish mortality and the economic losses were substantial (Huang et al. 1999). A haemolysin that was isolated and characterised as a glycolipid with a digalactose and a PUFA (heptadecadienoyl) group was found to be responsible for the fish mortality (He et al. 1999) by induction of pores in the cell membrane of target cells (Peng et al. 2005). Both the isolated toxin and supernatant of the *P. globosa* cultures inhibited cultures of other microalgae (J.-S. Liu pers. comm.).

Now that evidence is accumulating about toxic substances produced by *Phaeocystis* species, field studies are needed to assess if these toxins are used in chemical warfare against predators and/or competitors (allelopathy). In this study we tested the hypothesis that *P. pouchetii* excretes a lytic agent. The potential impact of live *P. pouchetii* to cells in close proximity was quantified in a bioassay during a *P. pouchetii* bloom in a mesocosm experiment. Red blood cells were selected as model targets representing unprotected cells, and lysis was quantified by monitoring dissolved haemoglobin. *Phaeocystis* blooms in mesocosms, with natural plankton communities containing all trophic levels up to macrozooplankton, were used to simulate in situ conditions including the chemically mediated interactions (Hay and Kubanek 2002, and references therein) that could trigger defensive responses of *P. pouchetii*.

Material and methods

Mesocosm set up

Haemolytic activity was studied in *P. pouchetii* blooms in experiments conducted from 27 February (experimental day 1) to 3 April 2003 at the marine biological field station in Raunefjorden, outside Bergen, Norway (60°16′N, 05°14′E). Three transparent floating polyethylene enclosures were used [4.5 m deep, 2 m diameter, ca 11 m^3, 0.12 mm thick walls with 90 % penetration of photosynthetically active radiation (PAR)]. Details of the location and general mesocosm design can be found in Svensen et al. (2001) and at the website of the University of Bergen (http://www.ifm.uib.no/LSF/inst2.html).

The mesocosms were filled on 27 February by pumping nutrient-poor fjord water from 5 m depth. The water column was well mixed with an airlift system, pumping 40 l min^{-1} (Jacobsen, 2000). In order to allow the introduction of new species, to avoid substantial pH changes due to primary production, and to replace water sampled during the mesocosm experiment, 10% of the water was renewed daily in each mesocosm from 3 March by pumping (peristaltic) fjord water from about 2.5 m depth outside the mesocosms. On 3 March, mesocosms M2 and M3 were enriched with nitrate ($NaNO_3$) and phosphate (NaH_2PO_4) to final concentrations of 16 µM and 1 µM, according to the Redfield ratio, to stimulate the development of a bloom of *P. pouchetii*. Mesocosm M1 received no nutrients and served as a control. Nutrient outflow with renewal of water was compensated for by daily additions of nutrients (1.6 µM nitrate and 0.1 µM phosphate per mesocosm in M2 and M3). On 20 March daily nutrient replacement in M2 was stopped to induce a fast decline of the *P. pouchetii* bloom.

Sampling

Sampling was performed at least every third day, in the morning, 4 h after sunrise, using 15 l carboys that were filled at the surface of the mesocosms. Samples were taken gently to avoid disruption of the colonies. Carboys were stored in the dark at ambient temperature (ca. 4°C). Cells were collected within 1 h from 250 ml subsamples filtered over GF/F filters (47 mm, Whatman) using gravity only. The GF/F filtrate was collected and stored in the dark at 4°C until analysis on the same day. The filters were transferred into test tubes containing 5 ml artificial seawater at 31.5 PSU (ambient salt concentration) which

contained 23.4 g NaCl, 9.35 g $MgCl_2 \cdot 6H_2O$, 0.50 g $CaCl_2.2H_2O$, 3.05 g Na_2SO_4, and 0.81 g K_2SO_4 per litre (Admiraal and Werner 1983). To release water soluble cell content, cells on the filter were disrupted by three sonic bursts (5 s, amplitude 90, 28 Watts) with a probe (Sonics Vibratell^{TM}), followed by centrifugation (30 min, 8000 × *g*). The supernatant (hereafter referred to as extract) was ready to be tested for haemolysis. After experimental day 24 it was no longer possible to filter the 250 ml using gravity and sampling on filters was stopped.

Phytoplankton analysis

Phytoplankton abundance and species composition were determined in 60 ml samples that were fixed with glutaraldehyde (0.5% final concentration) and stored at 4°C. Samples were settled onto black-stained Nuclepore filters with 0.4 µm pore size and then frozen. Cells were counted at 200×, 400× and/or 600–1250× magnification on a light and epifluorescence Olympus or LUMAM-P8 microscope. Cellular carbon of the various phytoplankton species was determined using the conversion described by Menden-Deuer and Lessard (2000). Samples for chlorophyll *a* (Chl *a*) were filtered onto 0.45 µm cellulose-acetate filters (Sartorius AG, Germany), immediately extracted in 90% acetone overnight, and analyzed according to Parsons et al. (1984) on a Turner Designs 10-AU fluorometer.

Erythrocyte lysis analysis (ELA)

Whole mesocosm samples, GF/F filtrate and extracts (up to 23 March 2003) were diluted 1:1 with blood cell suspension. For whole-sample transfer, every pipette tip used was clipped with a pair of scissors to have a > 3 mm diameter inlet, making sure to include colonies while sampling. The blood cell suspension was prepared by adding five drops of fresh human blood to 30 ml buffer (Eschbach et al. 2001), centrifugation (5 min, 4500 × *g*) and addition of the same volume of buffer to the pellet. Standard incubations were done for 24 h in triplicate in test vials (2.5 ml, Eppendorf) at 15°C, 7 µmol photons $m^{-2}\,s^{-1}$. After incubation, intact blood cells and phytoplankton were removed by centrifugation (5 min, 4500 × *g*) and the supernatant was transferred into a cuvette and measured at 414 nm to quantify haemoglobin. Artificial seawater diluted 1:1 with ELA buffer was the 0% control, a sonified blood suspension diluted with artificial seawater was used as 100% lysis control. For comparison with other haemolytic studies a dose response curve with saponin (Sigma) was made to estimate the EC_{50} value for this control substance.

In addition to standard incubations, tests were performed. Dose-response curves were made with dilution series of whole mesocosm samples in artificial sea water. The effect of the incubation temperature (15°C) was also compared with a series at ambient temperature (4°C, all sample handling and incubation in cold room). Effects of light conditions were tested by incubations in the dark (wrapped in aluminium foil), 7 and at 40 µmol photons $m^{-2}\,s^{-1}$.

Data analysis

Results of regression analysis (Excel 2003, Microsoft Office) are presented as significant with the symbol P (<0.05); P^{*} (<0.01), P^{**} (<0.001), P^{***} (<0.0001), or by the actual P value.

Results

Bloom description

In the beginning of the experiment a diatom bloom dominated by *Chaetoceros socialis* developed in all mesocosms. Maximal densities of 4.5, 5.9 and 5.2×10^6 cells per litre were measured on day 12 of the experiment for M1, M2, and M3, respectively (ca. 4.5 ug l^{-1} Chl *a* in all mesocosms, Fig. 1, Table 1). After day 15, *P. pouchetii* blooms developed in all mesocosms, but were more pronounced in the two nutrient-enriched mesocosms (M2 and M3) compared to the control bag (M1). In M1 maximal *P. pouchetii* numbers were $9\cdot10^6$ cells per litre representing 55% of total carbon biomass at 2–3 µg total Chl *a* l^{-1}. In M2, that was fertilized until experimental day 21, maximal *P. pouchetii* numbers were 4.3×10^7 cells per litre (92% of total biomass at 23 µg Chl *a* l^{-1}), and in M3 5×10^7 cells

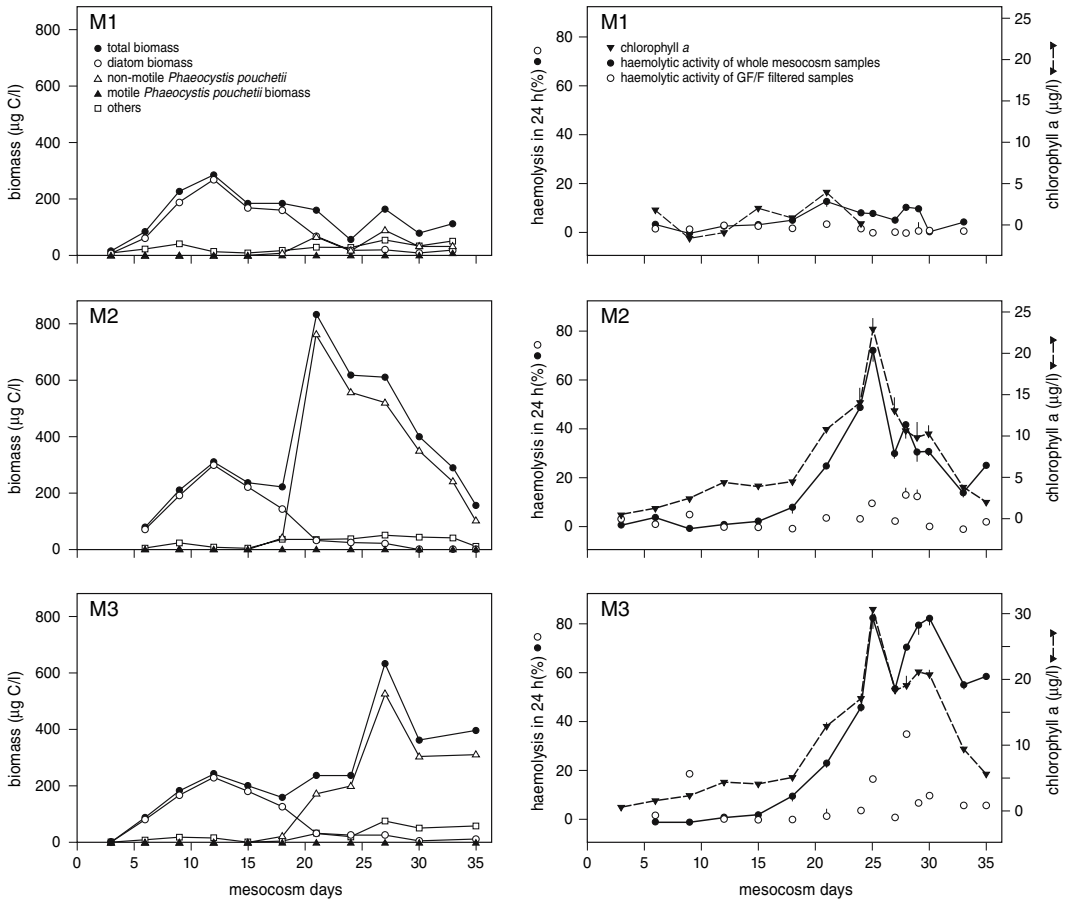

Fig. 1 Left-hand panel: development of biomass for different phytoplankton groups during the mesocosm incubations. M1, control bag; M2, fertilized until day 21; M3 continuously fertilized. Total biomass is the sum of carbon of diatoms, non-motile *Phaeocystis pouchetii*, motile *Phaeocystis* and others. Right-hand panel: development of chlorophyll *a* and haemolytic activity in the mesocosms of both whole mesocosm samples and GF/F filtered samples. Bars represent standard errors

per litre (84% of biomass at 30 µg Chl *a* l^{-1}). First the diatoms and then *P. pouchetii* completely dominated the phytoplankton community both in numbers (Table 1) and in biomass (Fig. 1).

Haemolytic activity

The haemolytic activity measured in whole-mesocosm samples was absent, or very low, during the diatom blooms (Fig. 1). In M1 haemolytic activity in whole mesocosm samples was low during the experiment, increasing from zero at the beginning of the mesocosm experiment to about 10% lysis in the period between day 21 and 29. In M2 and M3 the haemolytic activity was higher. In M2 it exceeded 10 % at day 21, reaching a peak of 70% lysis at day 25, followed by a slow decrease. Also in M3 values above 10% were observed from day 21 onwards, reaching a maximum of 81% lysis at day 30, followed by a slow decrease. The high value at day 25 seemed to be a high value within an increasing trend.

After day 15 the haemolytic activity followed the Chl *a* concentration in the nutrient enriched mesocosms (Figs. 1 and 2). The correlation was: lysis (%) = 3.40 × Chl *a* (ug l^{-1}) − 4.96, $n = 42$, $r^2 = 0.90$, P^{***}). Most of the haemolytic activity was associated with living cells, since only 14% of the whole mesocosm sample signal (total lysis) was measured in the GF/F filtrate: filtrate lysis (%) = 0.141 × [total lysis (%)] + 0.63, $n = 42$, $r^2 = 0.30$, P^{**}, Figs. 1 and 3).

Table 1 Cell densities (numbers × 1,000 per litre) enumerated by microscopy for the groups of plankton in three mesocosms in experiments conducted from 27 February (experimental day 1) to 3 April 2003 at the marine biological field station in Raunefjorden, outside Bergen, Norway (60°16′N, 05°14′E)

↓Categories of plankton in three mesocosms experimental day→	0	6	9	12	15	18	21	24	27	30	33	35
MESOCOSM 1 control, no NP additions												
Diatoms	80	1492	5103	7229	4557	4401	1700	465	511	306	467	
Motile *Phaeocystis*	239	134	120	90	120	157	225	120	949	1220	2842	
Non-motile *Phaeocystis*	7	15	45	0	195	358	6275	1210	8262	3119	2958	
Small phototrophic flagellates	1379	5314	18937	5294	709	2626	4300	1728	2603	1369	1457	
Large phototrophic flagellates and ciliates	2	2	1	1	1	2	2	1	26	0	103	
Small heterotrophic flagellates	596	356	909	121	741	801	1195	1141	2254	2617	5587	
Large heterotrophic flagellates and ciliates	3	5	26	35	13	11	15	31	98	39	96	
MESOCOSM 2 NP enriched until day 21												
Diatoms	159	1945	4996	8039	5916	3874	760	642	723	104	15	627
Motile *Phaeocystis*	60	15	45	60	60	390	990	420	2644	134	782	537
Non-motile *Phaeocystis*	0	0	30	60	105	2213	41810	30510	28606	19285	13216	5920
Small phototrophic flagellates	331	1486	7073	2436	1240	4678	4120	3685	2006	876	1114	455
Large phototrophic flagellates and ciliates	1	0	1	1	2	1	9	15	4	3	1	1
Small heterotrophic flagellates	105	241	242	114	48	840	302	241	3360	2640	2719	2834
Large heterotrophic flagellates and ciliates	25	3	40	5	6	26	53	27	80	51	133	6
MESOCOSM 3 NP continuously enriched												
Diatoms	108	2101	4389	6196	5174	3481	1022	684	724	207		474
Motile *Phaeocystis*	90	90	11	45	30	26	748	45	1763	353		1222
Non-motile *Phaeocystis*	0	0	7	30	165	1465	15687	18750	48654	28138		29017
Small phototrophic flagellates	561	5201	11018	8591	2810	1014	3150	2220	1830	1014		1216
Large phototrophic flagellates and ciliates	0	0	1	1	0	1	1	1	52	47		113
Small heterotrophic flagellates	39	76	63	16	19	19	50	138	1985	2016		1535
Large heterotrophic flagellates and ciliates	16	13	8	5	8	11	49	24	184	106		204

Diatoms, completely dominated by chain forming *Chaetoceros socialis*, 4–30 μm; motile *Phaeocystis* cells, about 3 μm; non-motile *Phaeocystis* cells, 5–6 μm; Small phototrophic flagellates (dominated by small flagellates other than motile *Phaeocystis* and only a few small *Scrippsiella* sp., 2–9 μm; large phototrophic flagellates and ciliate *Myrionecta rubra* (10–54 μm), small heterotrophic flagellates (2–9 μm), large heterotrophic flagellates and ciliates (10–69 μm)

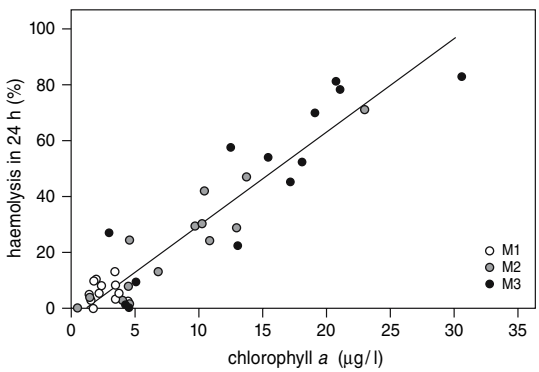

Fig. 2 Correlation between haemolytic activity of whole mesocosm samples and their chlorophyll *a* content for the three mesocosms

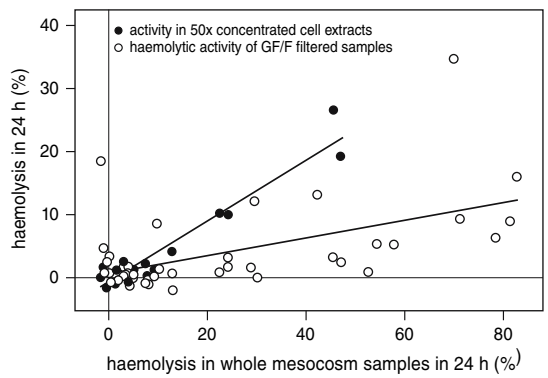

Fig. 3 The relation between the haemolytic activity measured in whole-mesocosm samples and the haemolytic activity in GF/F filtrate of the same samples, or the activity in the 50-times-concentrated cell extracts

In the period 17–23 March, haemolytic activity of the extracts was less than half of activity of whole-mesocosm samples (Fig. 3). Bearing in mind that the extracts are concentrated 50 times compared to the whole-mesocosm samples, the conclusion must be that the effect of the whole samples is not simply caused by components

Table 2 Densities of the different phytoplankton groups (see Table 1) of 31 mesocosm samples were related to the haemolytic activity of the samples as well as to the densities of non-motile *Phaeocystis pouchetii* using linear regression

Density of phytoplankton groups	Haemolytic activity		Non-motile *Phaeocystis*	
	Slope	*P*	Slope	*P*
Non-motile *Phaeocystis*	pos	<0.0005		
Large phototrophic flagellates and ciliates	pos	0.0056	pos	0.0230
Large heterotrophic flagellates and ciliates	pos	<0.0005	pos	<0.0005
Diatoms	neg	0.0011	neg	0.0008
Motile *Phaeocystis*	zero	0.1500	pos	0.0054
Small phototrophic flagellates	zero	0.0590	zero	0.1580
Small heterotrophic flagellates	zero	0.2490	zero	0.2120

released by lysing algae but rather being produced in the presence of intact cells. The activity of the soluble cellular contents was only 1% compared to the activity of the whole sample (extract lysis (%) = 0.010 × [total lysis (%)] −0.015, $n = 22$, $r^2 = 0.930$, P^{***}).

Correlation of haemolysis with phytoplankton groups

Correlations with haemolysis were determined for the most abundant phytoplankton groups. Haemolysis for whole mesocosms samples correlated with total *P. pouchetii* numbers (lysis (%) = 0.001203 * [cell density ml^{-1} sample] + 3.43, $n = 31$, $r^2 = 0.674$, P^{***}). For the non-motile *P. pouchetii* cell abundance, i.e., cells from colonies this regression equation was almost the same (% lysis = 0.001248 × [cell density ml^{-1} sample] + 3.63, $n = 31$, $r^2 = 0.677$, P^{***}, Fig. 4, Table 2). Interestingly, motile *P. pouchetii* numbers (Fig. 1) did not correlate with the haemolytic activity. This was most evident in M1 with low haemolytic activity and highest densities of motile cells (Table 1, Fig. 1). The relation between diatoms and haemolytic activity was negative because the haemolysis started just after the diatom bloom (P^{**}). There was no correlation between the haemolytic activity and heterotrophic flagellates or the small phototrophic flagellates (mainly unidentified flagellates and a few small developing cells of *Scrippsiella trochoidea* with their distinctive chloroplasts, Table 2). There was, however, a positive correlation between the haemolytic activity and the abundance of both the larger heterotophs (P^{*}) as well as the larger phototrophs (P^{*}). The abundance of these groups, however, were positively correlated with the non-motile *Phaeocystis* cells abundance (heterotrophs, P^{***} and phototrophs P, Table 2) which complicates assessment of which groups were responsible for the haemolytic activity. The phototrophs in M1 and M3 reached comparable densities while M1 had much lower haemolytic activity (Fig. 5a). In addition, in M2 the phototrophs displayed low densities in the second half of the mesocosm period while haemolytic activity was high. These observations suggest that the larger phototrophs were not responsible for haemolytic activity. In all bags the larger heterotrophs were present during the diatom blooms and increased during the second half of the experiment, while there was hardly any haemolytic activity present during the diatom blooms and the *P. pouchetii* period in M1 (Fig. 5b). Therefore, the larger heterotrophs are unlikely candidates for haemolysis as well.

Dose response curves at different temperatures

Dose-response curves from dilution series of samples taken at March 24 from the three mesocosms showed a linear relationship between 10 and 70% lysis. For the whole data set the activity at 4°C was approximately half the activity measured at 15°C (value at 4°C = 0.44 × [value at 15°C] + 3.24, $n = 21$, $r^2 = 0.963$, P^{***}, Fig. 6).

Light effects

Aanesen et al. 1998 measured a light dependent negative effect of *P. pouchetii* on fish larvae. To

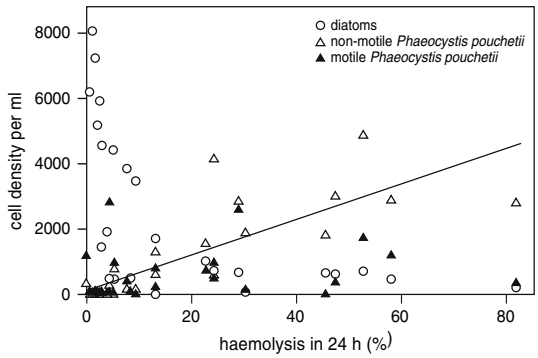

Fig. 4 Relation between the abundance of phytoplankton and the haemolytic activity measured in whole mesocosm samples. The regression line displayed is for non-motile *P. pouchetii* (i.e., cells present in colonies)

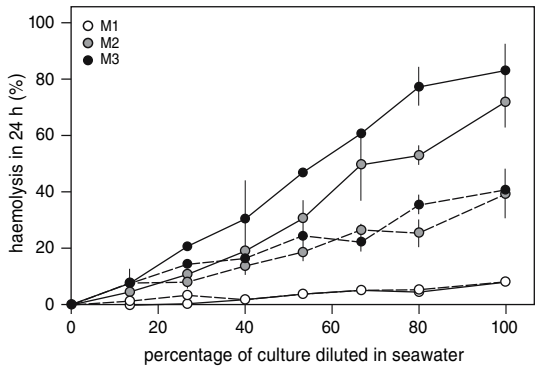

Fig. 6 Dose response curves for whole mesocosm samples taken at 24 March, 2003. Dilution series were measured at ambient temperature (4°C, dotted lines) and at standard experimental temperature (15°C, solid lines). Bars represent standard deviation

test possible influence of light intensity the haemolytic activity of whole mesocosms samples was analyzed not only at 7 but also at 40 µmol photons $m^{-2}\,s^{-1}$ on experimental day 21. For the three mesocosms the haemolytic activity increased twofold at higher light conditions (13 and 28% lysis for M1, 24 and 57% for M2; 23 and 55% for M3,). A similar experiment also including a dark incubation was performed on day 24 (Fig. 7). There was no difference between the dark and standard light conditions. Similar to the experiment on 20 March, at 40 µmol photons $m^{-2}\,s^{-1}$ the haemolytic activity was higher compared to 7 µmol photons $m^{-2}\,s^{-1}$, although not twice the amount observed in the previous experiment. The measurements at high light in this case could be an underestimation because they are higher than 70% where the response is no longer linear (Fig. 6, previous section). Extracts were also more active at the high-light conditions (data not shown).

Discussion

Haemolytic activity was observed in samples during *Phaeocystis pouchetii* blooms in mesocosms. The activity measured using incubations of whole mesocosm samples mixed with blood suspensions was proportional with the amount of sample added. The haemolytic activity in the mesocosms correlated best with the density of non-motile *P. pouchetii* but also with the abundance of the larger phototrophs and heterotrophs. The last two were also positively related to *P. pouchetii* numbers. Biomass during the *P. pouchetii* bloom was dominated by non-motile *P. pouchetii* cells and

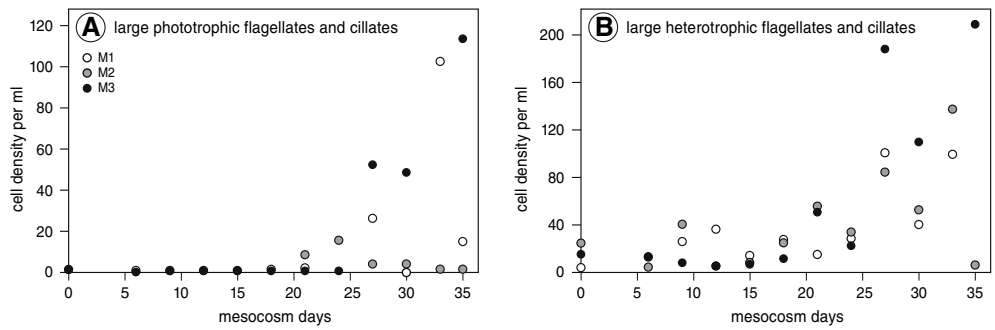

Fig. 5 Relation between haemolytic activity of whole mesocosm samples and abundance of (**A**) large phototrophic flagellates and ciliates (10–54 µm) and (**B**) large heterotrophic flagellates and ciliates (10–69 µm)

Table 3 Overview of haemolytic studies compared to the results obtained for non-motile *Phaeocystis pouchetii* using the EC_{50} saponin values of the tests used (adapted from de Boer et al. 2004)

Species	Microalgae EC_{50} density [10^7 cells l^{-1}]	Cell volume [μm^3]	Microalgae EC_{50} biovolume [10^9 μm^3 l^{-1}]	Reference
Alexandrium tamarense	0.0152	18700[a]	3	2
Phaeocystis pouchetii	1.9	65–110[a]	1–2	This study
Prymnesium parvum	0.3–24.7	280[b]	1–69	2,3,4,5,6
Fibrocapsa japonica	1.7–6.3	3800–4400[b]	64–277	1
Chrysochromulina polylepis	116–170	270[b]	310–460	5

[a] Volume calculated from cell dimensions or [b] Given cell volumes. References: (1) De Boer et al. 2004, (2) Eschbach et al. 2001, (3) Fistarol et al. 2003, (4) Johansson and Granéli 1999, (5) Simonsen and Moestrup 1997, (6) Stolte et al. 2002

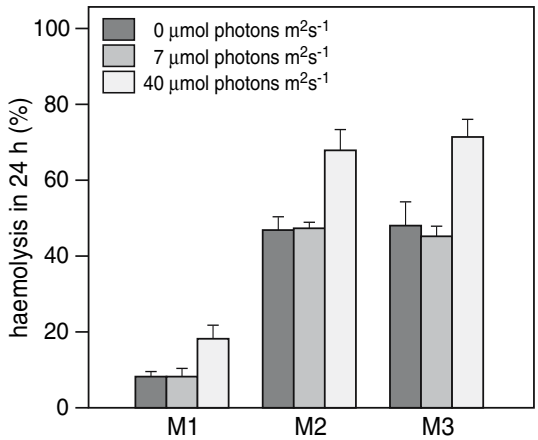

Fig. 7 Haemolytic activity observed in mesocosm samples (mesocosms day 24) incubated at different light intensities. Bars represent standard deviation

close examination of trends in densities of the other plankton groups revealed that these were unlikely to be the haemolytic agents. The non-motile *P. pouchetii* cells present in colonies were responsible for haemolytic activity, although it cannot be excluded that motile *P. pouchetii* cells (almost negligible in their biomass contribution) were also able to produce lytic substances. Haemolytic activity was absent during the diatom blooms that preceded the *P. pouchetii* blooms.

From the relationship between cell numbers and haemolytic activity, an EC_{50} cell density was estimated to 1.86×10^7 *P. pouchetii* cells l^{-1}. This value compares favourably with the EC_{50} densities of other algae that produce harmful algal blooms (Table 3). However, values for the other algae were measured with cell extracts where the EC_{50} values expressed on a biovolume basis indicate an internal toxicity level relevant for the assessment of grazing effects. The values in this mesocosms study were obtained with live cells, whereas *P. pouchetii* extracts contained only one percent of the total activity measured with live samples. Consistent with this, low haemolytic values were reported earlier for methanol extracts of *P. pouchetii* (Stabell et al. 1999) and extracts of *P. pouchetii* cells were not inhibitory to growth of yeast cells. Fractions derived from *P. pouchetii* culture water containing the PUA 2-*trans*-4-*trans*-decadienal, however, blocked sea urchin embryo development as well as the growth of yeast cells (Hansen et al. 2003, 2004).

Approximately 14% of the haemolytic activity in whole-mesocosm samples was present in GF/F filtrate, while almost no activity was extracted from the broken cells. It is unlikely that the bulk of the activity was bound to the cell membranes because a substantial part of the cell debris was still present in the extract (own observation). An alternative explanation would be that the live *P. pouchetii* cells produce an unstable component which greatly enhances haemolysis. In accordance with this is the observation that haemolytic activity did not accumulate over time in the mesocosms, but followed daily variations of chlorophyll *a*. A mechanism that involves physical contact between *Phaeocystis* colony and the blood cell could also explain the activity of live *Phaeocystis*.

During high-light conditions an increased mortality of cod larvae incubated in seawater from a *P. pouchetii* bloom has been observed (Eilertsen and Raa 1995; Aanesen et al. 1998). Similarly, light enhanced the effect of *P. pouchetii*-contain-

ing mesocosm samples on blood cells and may have been caused by the same toxic principle that killed the cod larvae with their exposed gills. It is tempting to speculate on the mechanism involved. Perhaps the haemolytic compound itself reacts in a light dependent manner. Light dependent haemolytic cytotoxins were recently identified in raphidophyte cultures (Kuroda et al. 2005). An alternative explanation is the following. During the mesocosms blooms the PUA that was identified earlier for *P. pouchetii* (Hansen et al. 2004) may have been converted into more toxic derivates by ROS produced by living cells: a cascade reaction sequence described earlier in diatoms (Jüttner 2001). This scenario provides an explanation for the action of living cells as well as the effect of light. The light-dependent production of liable ROS such as superoxide seems to be a common feature among microalgae, including prymnesiophytes (Marshall et al. 2005).

Haemolytic activity displayed by *P. pouchetii* was almost exclusively related to active cells and not to compounds within the cells. This situation seems to be different from the haemolytic glycolipids extracted from *P. globosa* isolated from ichthyotoxic blooms in Chinese coastal waters (He et al. 1999), the glycolipids from the prymesiophyte *Chrysochromulina polylepis* (Yasumoto et al. 1990), or the polyethers found for *Prymnesium* species (Legrand et al. 2003, and references therein). Because of the low haemolytic activity of the cell extracts, it is doubtful if predators on *P. pouchetii* in the mesocosm would experience negative effects after consumption of this prey, although healthy colonies are avoided by some copepods (Estep et al. 1990). Organisms such as bacteria and phytoplankton that co-occur with *Phaeocystis*, however, may be inhibited by this lytic action. If so, actively growing *P. pouchetii* colonies in this way further improve their competitive advantage, perhaps contributing to subsequent dominance.

Haemolytic activities observed in the nutrient-enriched mesocosms were higher than those measured in the control bag and values to be expected during blooms in the field. From the data it was possible to estimate the lysis rate at ambient temperature in a natural bloom, based on cell densities or chlorophyll *a* present. The 24 h of exposure to 7 μmol photons m^2 s^{-1} was on the same order of magnitude as the average daily received illumination in the mesocosms and the field (cf. Nejstgaard et al. 2006). Cell densities reported during *P. pouchetii* blooms of 1–2 × 10^7 cells per litre (Schoemann et al. 2005) would lead to a daily lysis rate of 12–29%, whereas the reported Chl *a* values between 5–10 ug l^{-1} would lead to a 16–29% lysis rate. These rates indicate that unprotected cells like the blood cells used in this study, would lyse within days during a *P. pouchetii* bloom. Live *P. pouchetii* colonies are highly haemolytic and the mechanism seems to be fundamentally different from the haemolytic harmful algal bloom species studied so far.

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