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Effect of different salinities on growth and intra- and extracellular toxicity of four strains of the haptophyte *Prymnesium parvum*

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ABSTRACT: The present study investigates the effect of brackish (7 PSU) and marine (26 PSU) salinity on physiological parameters and intra- and extracellular toxicity in 4 strains of *Prymnesium parvum* Carter. The different *P. parvum* strains were grown in batch cultures in 2 trials under different experimental conditions to test the development of intra- and extracellular toxicity during growth. The response of *P. parvum* toxicity to salinity was validated using 2 protocols. Intraspecific variations in growth rate, maximal cell density (yield) and cell morphology were controlled by salinity. Extracellular toxicity was higher at 7 PSU in all strains, but no correlation was found between intra- and extracellular toxicity. The variation of extracellular toxicity in response to salinity was much greater than that of intracellular toxicity, which indicates that *P. parvum* may be producing a variety of substances contributing to its various types of 'toxicity'.

KEY WORDS: Allelopathy · Extracellular toxicity · Harmful algal species · Intracellular toxicity · *Prymnesium parvum* · Salinity · Strain

INTRODUCTION

Prymnesium parvum Carter, the so-called 'golden algae', is a harmful and toxic microalga, which causes severe damage to coastal marine ecosystems and fish farming through massive fish kills (Igarashi et al. 1996, Edvardsen & Paasche 1998, Edvardsen & Imai 2006, Oikonomou et al. 2012). *P. parvum* possesses mechanisms that are suspected to support bloom development (Smayda 1997), including (1) the production of allelochemicals, (2) antipredatory defense mechanisms and (3) its tendency for mixotrophic nutrition. The chemical substances enabling mechanisms (1), (2), partly (3) and toxicity to fish are insufficiently described; still, they are generally attributed to a collection of compounds known as prymnesins (Manning & LaClaire 2010). However, recent work (Henrikson et al. 2010, Schug et al. 2010) suggested that *P. parvum* toxins responsible for organismal toxicity may not be the classic prymnesins described by Igarashi et al. (1996). It is not yet clear if the toxins stored intracellularly, causing mortality of *P. parvum* grazers due to ingestion (mechanism 2) (Tillmann 2003, Barreiro et al. 2005), are similar to the toxins released extracellularly, which may function as competitive mechanisms or aid in immobilization of prey cells for mixotrophic nutrition (mechanisms 1 and 3) (Skovgaard & Hansen 2003, Tillmann 2003). *P. parvum* is a euryhaline species that can tolerate a wide range of temperatures. Studies of different strains suggest optimal growth at salinity 8 to 34 and a temperature of 15 to 30°C (Larsen & Bryant 1998). However, *P. parvum* blooms often occur below salinity 8 and at colder water temperatures (Amsinck et al. 2005, Baker et al. 2009).

Production of toxins may be the key mechanism by which *Prymnesium parvum* gains a selective advantage over other phytoplankton (Fistarol et al. 2003, Granéli & Johansson 2003), and lower salinity might be a stress factor increasing toxin production in *P.*

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parvum (Reigosa et al. 1999, Granéli et al. 2012). Baker at al. (2007) reported that *P. parvum* toxicity was higher in suboptimal salinity conditions for growth. However, the literature is contradictory on the response of *P. parvum* toxicity towards salinity changes; previous studies observed an inverse relation between toxicity and salinity (Shilo & Rosenberger 1960), found maximum toxicity in *P. parvum* at intermediate salinities and observed toxicity decreases at low or high salinities (Padilla 1970, Hagström 2006) or found no clear trend (Larsen & Bryant 1998).

Toxicity in *Prymnesium parvum* is evaluated using a number of tests and bioassays, of which hemolytic tests are among the most common (Brooks et al. 2010). As hemolytic assays are often not sensitive enough to determine *P. parvum* exotoxin production (Uronen et al. 2005), bioassays with organisms such as the cryptophyte *Rhodomonas salina* (Fistarol et al. 2003, Tillmann 2003), the brine shrimp *Artemia* (Meldahl et al. 1994) or fish (Reich & Parnas 1962, Schug et al. 2010) are used as an alternative. Since intra- and extracellular toxicity are not necessarily linked, both need to be measured to evaluate *P. parvum* toxicity (Parnas 1963, Manning & La Claire 2010). However, these measurements are seldom done concurrently, and protocols often vary from one laboratory to another. While the effect of environmental conditions on *P. parvum* toxicity has been largely studied, the sensitivity of target cells to *P. parvum* lytic activity in response to environmental conditions has barely been examined (Fistarol et al. 2005).

Most studies investigated *Prymnesium parvum* growth and toxicity responses to changed environmental conditions based on single strains. However, the few studies using several strains showed a large intraspecific variability that affected growth and toxicity more than environmental factors (Larsen et al. 1993, Larsen & Bryant 1998). Further, toxicity varies with the growth stage of *P. parvum* (Manning & La Claire 2010). Under natural conditions, phytoplankton do not always grow at maximum rates, and blooms of *P. parvum* have been shown to increase toxicity during stationary phase in the field (Schwierzke et al. 2010). Therefore, we tested the toxicity of *P. parvum* in exponential and stationary phases and at the stage of maximum disturbance (and nutrient depletion), the senescent phase. The present study aimed to identify patterns in physiology as well as intracellular and extracellular toxicity in response to salinity changes, by considering strain variability. This was achieved by incubating 4 *P. parvum* strains in 2 different salinities. Hemolytic activity (HA) of *P. parvum* cell extracts and the *Rhodomonas* bioassay were used as proxies for intra- and extracellular toxicity, respectively.

MATERIALS AND METHODS

Culturing conditions

Four *Prymnesium parvum* strains — CCAP 946/1D (Strain A), CCAP 946/6 (Strain B), KAC 39 (Strain C) and CCAP 946/1B (Strain D) — were obtained from the Culture Collection of Algae and Protozoa (CCAP), UK, and the Kalmar Algal Collection (KAC), Sweden. All strains were isolated from different geographical areas (Table 1) and were maintained at either 7 or 26 PSU for 3 yr before the present study.

The strains were cultured at 2 salinities (7 and 26 PSU) in triplicate batch cultures at the Linnaeus University in Kalmar, Sweden. Strains were grown in modified f/10 medium (160 µMol nitrate; Guillard 1975) at 15°C, 90 µmol photons m^{-2} s^{-1} and a day: night cycle of 14 h light:10 h dark. Baltic seawater (7 PSU) was used as a base for culture media in both studies. The salinity of 26 PSU was achieved by the addition of NaCl to Baltic seawater. The cryptophyte *Rhodomonas salina* (KAC 30) was cultured under the same conditions as *Prymnesium parvum*.

Experimental setup

In Trial 1, growth of the 4 strains of *Prymnesium parvum* at both salinities was monitored for 36 d. Subsamples for cell counts and chlorophyll *a* (chl *a*) were taken daily when cultures were growing exponentially and every 2 to 4 d during stationary and senescent phases. Cellular carbon, nitrogen and phosphorus were measured during exponential, stationary and senescent growth of the algae. Samples were taken at 3 and 4 occasions at 7 and 26 PSU, respectively (see Fig. 1). Hemolytic and lytic activities were measured at the same days (and 2 d later in exponential phase at 7 PSU) using a red blood cell lysis test modified from Stolte et al. (2002) and a *Rhodomonas* bioassay (Tillmann 2003).

Table 1. *Prymnesium parvum*. Geographical origin of the 4 strains used in Trials 1 and 2

Strain	Strain code	Origin	Ecozone
A	CCAP 946 1/D	Fishponds; Israel	Brackish
B	CCAP 946/6	Pier; Scotland	Marine
C	KAC 39	Fjord; Norway	Brackish
D	CCAP 946/1B	River; England	Brackish

In Trial 2, the effect of salinity on the susceptibility of the target organism *Rhodomonas salina* to extracellular toxins (allelochemicals) was tested by incubating the target organism, cultured at 7 and 26 PSU, with both filtrate from *Prymnesium parvum* cultured at 7 PSU and filtrate from *P. parvum* cultured at 26 PSU.

Determination of *Prymnesium parvum* growth rates

Aliquots of the cultures were fixed with 2% Lugol's solution and transferred into a Palmer Maloney chamber (0.1 ml). Cells were counted using an Olympus CKX 41 microscope. At least 200 *Prymnesium parvum* cells were counted in each sample. Growth rate was calculated separately for each replicate by calculating the slope of the linear regression of the natural logarithm of *P. parvum* cell abundance vs. time during exponential growth. The time span of exponential growth was determined as the linear part of the curve that was generated by plotting the ln-transformed *P. parvum* cell number versus time (at least 5 growth points were used for the calculation).

Chlorophyll *a*

Culture samples (3 to 4.5 ml) were filtered onto Gelman A/E glass fiber filters and frozen at −20°C prior to chl *a* extraction for 8 h (dark) using 96% ethanol (Jespersen & Christoffersen 1987). Concentrations were measured using a Turner Trilogy fluorometer.

Cellular nutrients

Prymnesium parvum cultures (25 to 50 ml) were filtered onto precombusted (450°C, 2 h) Whatman glass fiber carbon (GF/C) filters, dried at 60°C for 8 h and stored in a desiccator until further analysis. The analysis of particulate organic carbon and nitrogen was performed with a HEREAUS CHN analyzer. Organic carbon was measured after removal of carbonate with 2 N HCl. Phosphorus was measured in accordance with Solorzano & Sharp (1980).

Intracellular toxicity

Intracellular toxicity of *Prymnesium parvum* was measured using horse red blood cells (Igarashi et al. 1998). Saponin was used to produce a standard hemolytic curve for reference. In Trial 1, the method was modified after Stolte et al. (2002). Samples of algal cultures were filtered on a Whatman GF/C filter and stored frozen until further analysis. Cells retained on the filters were extracted in 2 ml 100% methanol and incubated with 2.5 to 5% horse blood suspension at room temperature in the dark for 90 min, until the degree of hemolysis was measured in a microplate reader (BMG Labtech FLUOstar).

EC_{50} values, i.e. *Prymnesium parvum* cell concentration causing lysis of 50% of the red blood cells, were calculated using the sigmoidal curve fit function in the mathematical program STATISTICA with the following formula:

$$y = b_0/[1 + (x/b_2) \cdot b_1] \quad (1)$$

where b_2 is the EC_{50} (cells ml^{-1}), b_0 is the expected response at saturation (100%), and b_1 determines the slope of the function. The saponin (sap.) concentration causing 50% lysis of horse red blood cells was calculated in the same way. Intracellular toxicity is presented as pg sap. eq. $cell^{-1}$ and was calculated by dividing the saponin concentration causing 50% hemolysis of the red blood cells by the EC_{50}.

Extracellular toxicity

To exclude predation (*Prymnesium parvum* is a mixotroph) and direct cell contact in the *Rhodomonas* bioassay, the *P. parvum* culture was filtered prior to the extracellular toxicity test using a 3 µm polycarbonate filter (Osmonics). A dilution series was made using volumes of *P. parvum* filtrate corresponding to *P. parvum* cell densities of 15 to 800 × 10^3 cells ml^{-1}. Six different dilutions of *P. parvum* filtrate were used for each test. The volumes of filtrate added differed for each test as the cell concentrations varied over the growth period (see Table A1 in the appendix). The bioassay was incubated in triplicates and supplemented with culture medium up to 4 ml (total volume of the bioassay). An aliquot (4 ml) of pure culture medium served as a control. *Rhodomonas salina* was added to all samples to a final density of ~12 × 10^3 *R. salina* cells ml^{-1}. After incubation for 6 h, samples were fixed with Lugol's solution (2% final concentration v/v), and intact *R. salina* cells were counted. Values of the EC_{50} were calculated using Eq. (1). Allelopathic activity is presented as $1/EC_{50}$.

Statistical analyses

All statistical analyses were performed using the STATISTICA software package. As the experimental data were neither homogenous in variances nor normal distributed, non-parametric tests were used for statistical analyses. When measurements of growth or cellular content were repeated in time, the effect of time was tested by Friedmann analysis of variance (ANOVA). For comparisons of effects of the 2 tested salinities, Mann-Whitney *U*-tests were performed (2 groups). When the strains were compared to each other, Kruskal-Wallis ANOVAs were performed (multiple groups). When intra- and extracellular toxicity were investigated for differences, the confidence intervals of the EC_{50} values were compared to each other. If those did not overlap between strains or treatments, the EC_{50} values were regarded as significantly different.

Table 2. *Prymnesium parvum*. Growth rates (d^{-1}) at 7 and 26 PSU in Trial 1 (n = 3, mean ± SD)

Strain	7 PSU	26 PSU
A	0.28 ± 0.04	0.18 ± 0.02
B	0.35 ± 0.04	0.25 ± 0.02
C	0.44 ± 0.01	0.31 ± 0.02
D	0.44 ± 0.04	0.26 ± 0.06

RESULTS

Prymnesium parvum growth and cell yield

Growth rates ranged from 0.18 to 0.44 d^{-1} (Table 2). All strains grew faster at 7 PSU than at 26 PSU (Mann-Whitney *U*-test, $p < 0.05$). Growth rates were also significantly different among the strains (Kruskal-Wallis ANOVA, $p < 0.05$; Fig. 1), with growth rates of Strains A and B significantly lower than growth rates of Strains C and D in both salinities (factorial ANOVA, Fisher least significant difference post-hoc, $p < 0.05$).

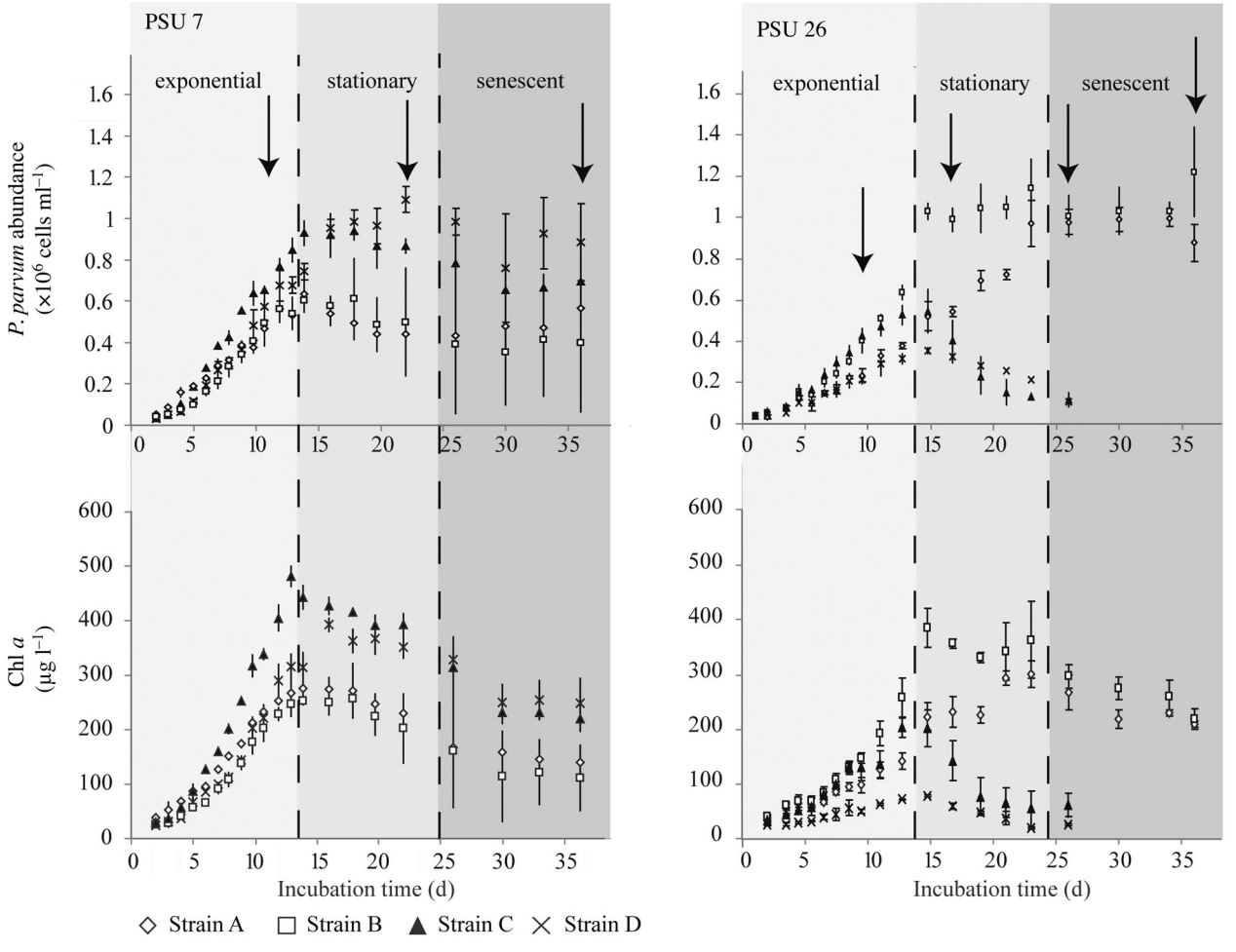

Fig. 1. *Prymnesium parvum* abundance and chl *a* concentration for Strains A, B, C and D in Trial 1 at 7 and 26 PSU. Arrows show sampling occasions for allelopathy, hemolysis and C, N and P measurements (n = 3, mean ± SD)

Cellular content of N, P, C and chl *a* in *Prymnesium parvum*

During exponential growth, cell contents of N, P and C as well as N:P, C:P and N:P ratios and chl *a* contents were similar among the strains (Kruskal-Wallis ANOVA, $p < 0.05$; Table 3, Fig. 1). As the condition of the strains largely influenced their C, N and P contents in later growth stages, the effect of salinity was only compared when cells grew exponentially.

Whereas N and P contents per cell did not differ between 7 and 26 PSU, C content per cell was higher at 26 PSU during exponential phase (Mann-Whitney *U*-test, $p < 0.05$), which also resulted in higher C:N ratios (for all strains) and higher C:P ratios (significant for 2 strains) at 26 PSU. The effect of salinity on chl *a*:C ratios differed among the strains: 2 of the strains had a higher chl *a*:C ratio at 26 PSU, whereas 1 strain had a lower chl *a*:C ratio, and 1 did not change (Mann-Whitney *U*-test, $p < 0.05$).

During stationary and senescent stages, strains reaching lower cell yields showed a higher nutrient content per cell and also accumulated more C compared to N and P. Extreme C:N:P ratios (1000:50:1) were obtained during the decline of Strains A and B at 26 PSU. Generally, N, P and C contents increased during the experimental incubation; however, this relation was only significant for C at both salinities. In comparison to N and P, more C was accumulated in *Prymnesium parvum* cells during growth. Therefore, C:N and C:P ratios also increased during the stationary and senescent phases (C:P significant at both salinities, C:N significant at 26 PSU), whereas chl *a*:C content decreased (significant at 26 PSU) (Friedman ANOVA, $p < 0.05$).

Intracellular toxicity and hemolytic activity

HA varied greatly among strains and growth phases at both salinities (Fig. 2). Intraspecific variation in HA was higher at 7 PSU than at 26 PSU in both trials (Levene's test; $p < 0.02$). Low salinity positively affected HA in 3 strains (B, C, D). At 7 PSU, HA was lowest in exponential compared to stationary and senescent phases (Trial 1). At 26 PSU, this difference disappeared.

Table 3. *Prymnesium parvum*. Cellular concentrations of particulate organic N, P and C (pg cell^{-1}), atomic N:P, C:N and C:P ratios and C:chl *a* ratio in cells cultivated at 7 and 26 PSU during exponential (Exp), stationary (Stat) and senescent (Sen) growth phases (n = 3, mean ± SD) in Trial 1

Strain	Phase	PON	POP	POC	N:P	C:N	C:P	C:chl *a*
7 PSU								
A	Exp	2 ± 0	0.49 ± 0.1	14 ± 1	10 ± 3	8 ± 0	79 ± 25	15 ± 2
	Stat	4 ± 0	0.51 ± 0	31 ± 1	15 ± 1	10 ± 0	152 ± 7	8 ± 1
	Sen	2 ± 0	0.38 ± 0.1	27 ± 6.6	13 ± 1	13 ± 1	166 ± 16	5 ± 2
B	Exp	2 ± 0	0.47 ± 0.1	14 ± 1	10 ± 2	8 ± 0	76 ± 12	12 ± 3
	Stat	3 ± 0	0.41 ± 0.1	22 ± 5	14 ± 1	10 ± 0	137 ± 9	11 ± 4
	Sen	3 ± 2	0.50 ± 0.4	34 ± 19	16 ± 1	12 ± 1	187 ± 26	5 ± 6
C	Exp	2 ± 0	0.35 ± 0	13 ± 1	12 ± 1	8 ± 0	95 ± 10	22 ± 1
	Stat	2 ± 0	0.26 ± 0	19 ± 2	15 ± 2	12 ± 1	186 ± 28	20 ± 3
	Sen	2 ± 0	0.30 ± 0.1	17 ± 1	13 ± 4	13 ± 1	177 ± 52	12 ± 3
D	Exp	1.8 ± 0.2	0.51 ± 0.1	13 ± 0	8 ± 1	8 ± 1	65 ± 11	13 ± 1
	Stat	1.5 ± 0.1	0.25 ± 0	17 ± 1	13 ± 2	13 ± 1	168 ± 11	22 ± 3
	Sen	1.4 ± 0.3	0.20 ± 0.1	17 ± 3	19 ± 4	14 ± 1	267 ± 71	15 ± 4
26 PSU								
A	Exp	2.1 ± 0	0.43 ± 0.1	18 ± 0	10 ± 1	10 ± 0	107 ± 12	21 ± 3
	Stat	2.2 ± 0.3	0.31 ± 0	20 ± 3	15 ± 1	10 ± 2	159 ± 32	21 ± 1
	Sen	1.7 ± 0	0.07 ± 0	22 ± 1	56 ± 1	15 ± 0	841 ± 4	12 ± 1
B	Exp	1.8 ± 0.2	0.39 ± 0	16 ± 2	10 ± 1	10 ± 0	103 ± 2	23 ± 2
	Stat	1.7 ± 0.2	0.20 ± 0	16 ± 2	19 ± 4	11 ± 1	213 ± 34	22 ± 2
	Sen	1.6 ± 0.1	0.06 ± 0	28 ± 3	54 ± 9	21 ± 1	1102 ± 139	11 ± 0
C	Exp	1.8 ± 0.2	0.4 ± 0.1	17 ± 2	9 ± 1	11 ± 0	106 ± 11	17 ± 3
	Stat	3 ± 0.4	0.42 ± 0.1	27 ± 3	15 ± 2	11 ± 0	164 ± 15	13 ± 2
	Sen	7.6 ± 0.2	1.05 ± 0.3	63 ± 1	15 ± 2	10 ± 1	154 ± 20	7 ± 2
D	Exp	1.5 ± 0.1	0.35 ± 0.1	17 ± 4	9 ± 0	15 ± 1	126 ± 8	13 ± 1
	Stat	1.8 ± 0.1	0.42 ± 0.1	21 ± 1	9 ± 1	14 ± 0	129 ± 17	9 ± 1
	Sen	3.7 ± 0.4	0.97 ± 0.1	38 ± 1	9 ± 1	12 ± 1	99 ± 8	5 ± 1

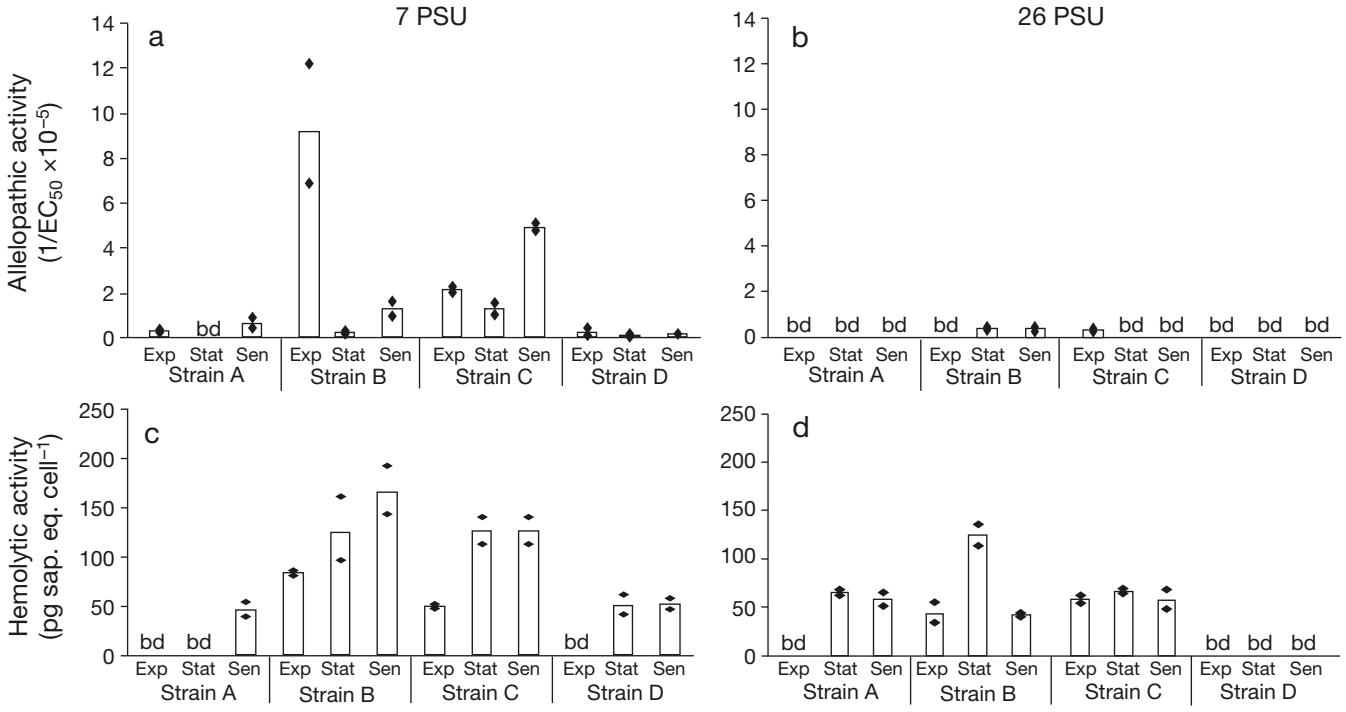

Fig. 2. *Prymnesium parvum*. (a,b) Allelopathic activity ($1/EC_{50}$) and (c,d) hemolytic activity (in pg saponin equivalent $cell^{-1}$) during Trials 1 and 2 in exponential (Exp), stationary (Stat) and senescent (Sen) phases (Trial 2 only measured in exponential phase). 95% CI and detection limits for each test are found in Table 4. ♦: upper and lower CIs; bd = below detection

Extracellular toxicity and lytic activity

The extracellular toxicity of *Prymnesium parvum* varied among strains and growth stages (Fig. 2, Table 4). In general, extracellular toxicity was higher at 7 PSU compared to 26 PSU (Mann-Whitney *U*-test; Fig. 2a,b). Intraspecific variation of the extracellular toxicity was large at 7 PSU. No trend was found in the levels of lytic activity in relation to growth phases (Fig. 2c,d).

Table 4. *Prymnesium parvum*. Allelopathic effect (EC_{50} and 95% confidence interval in *P. parvum*, $\times 10^3$ cells ml^{-1}) of filtrate from Strains A to D, incubated at 7 and 26 PSU, on the target species *Rhodomonas salina* during Trial 1. Significance of non-linear regression model for all treatments is $p < 0.001$, and *t*-values are shown. bd: below detection

Strain	Phase	7 PSU	*t*-value	26 PSU	*t*-value
A	Exp	368 (317–426)	179	bd	
	Stat	bd		bd	
	Sen	163 (113–234)	66	bd	
B	Exp	11 (8–14)	64	bd	
	Stat	526 (386–716)	33	265 (235–291)	196
	Sen	79 (61–103)	87	266 (204–354)	67
C	Exp	46 (43–50)	308	309 (264–353)	177
	Stat	79 (63–100)	100	bd	
	Sen	20 (19–21)	534	bd	
D	Exp	512 (230–1140)	34	bd	
	Stat	1770 (710–444)	33	bd	
	Sen	793 (687–915)	192	bd	

Allelopathic sensitivity of the target organism *Rhodomonas salina*

In Trial 2, *Rhodomonas salina* grown at 7 PSU was more sensitive than *R. salina* grown at 26 PSU to extracellular lytic compounds of *Prymnesium parvum* grown at the respective salinities. Further, both 7 and 26 PSU *R. salina* cultures were more sensitive to lytic compounds from 7 PSU *P. parvum* compared to 26 PSU *P. parvum* (Table 5, Fig. 3).

Table 5. *Prymnesium parvum*. Allelopathic effect (EC_{50} and 95% confidence interval in *P. parvum*, $\times 10^3$ cells ml^{-1}) of filtrate from Strain C, incubated at 7 and 26 PSU, on the target species *Rhodomonas salina*, cultivated at 7 and 26 PSU in Trial 2. Significance of non-linear regression model for all treatments is $p < 0.001$, and *t*-values are shown

Treatment (PSU)	EC_{50} in *P. parvum*	*t*-value
P. parvum (7) *R. salina* (7)	14 (12–16)	151
P. parvum (7) *R. salina* (26)	36 (32–41)	162
P. parvum (26) *R. salina* (7)	31 (27–37)	129
P. parvum (26) *R. salina* (26)	61 (52–72)	135

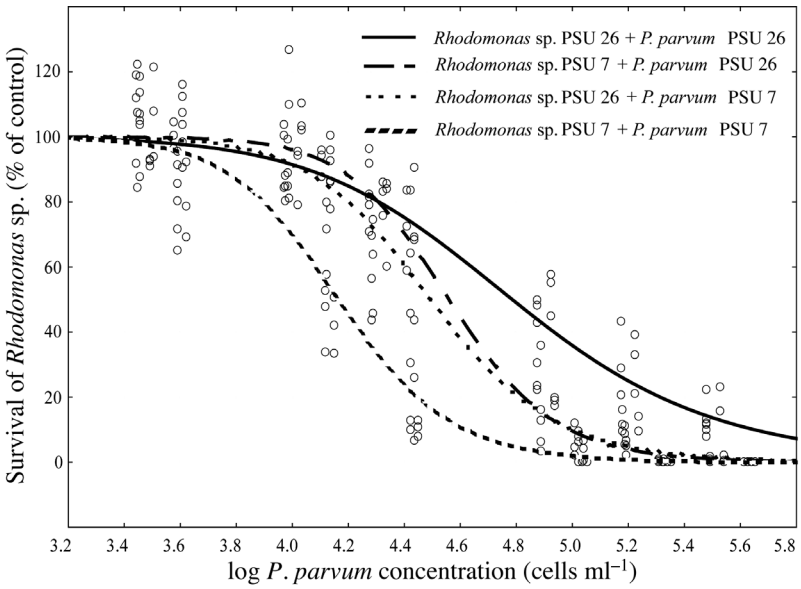

Fig. 3. *Prymnesium parvum*. Dose-response curve describing lytic capacity of filtrate cultured at 7 and 26 PSU, toward *Rhodomonas* sp., cultured at 7 and 26 PSU (Trial 2). Each curve represents a non-linear, sigmoidal curve fit of the mean concentration of *Rhodomonas* sp. after 6 h incubation (as % of control) as a function of log-transformed *P. parvum* concentration of 3 replicate cultures

DISCUSSION

Whereas physiological features among the strains were similar, intra- and extracellular toxicity and growth rates varied among the 4 strains. In general, low salinity affected intracellular and extracellular toxicity of *Prymnesium parvum* positively. The strength of this trend was stronger for extracellular toxicity (lytic activity), which was higher by one order of magnitude at 7 PSU compared to 26 PSU.

Growth, physiological status and intraspecific differences in *Prymnesium parvum*

Results from Trial 1 showed different cell yields and growth rates for the 2 salinities, 7 and 26 PSU. This suggests that some strains were better adapted to one of the salinities. For instance, strains reaching highest cell yields at 7 PSU showed lowest cell yields at 26 PSU. However, strains (B, C) isolated from marine/brackish waters did not grow better at 26 PSU and vice versa for strains (A,D) isolated from brackish/freshwater. *Prymnesium parvum* strains used in our study were maintained as laboratory cultures for several decades but were adapted to 7 and 26 PSU for 3 yr before the present study. Strain characteristics may have changed during their time in culture (Lakeman et al. 2009). To reduce this bias, studies investigating intraspecific variation should establish strain cultures directly prior to the trial. Nevertheless, our results stress the importance of repeatability of the data in *Prymnesium* studies.

The intracellular C:N:P ratio of phytoplankton groups varies around the common concentration of nutrients in seawater, the Redfield ratio (C:N:P = 116:16:1). The C:N:P ratios of *Prymnesium parvum*, grown in nutrient replete media, varied between 80 and 250 C to between 10 and 20 N to 1 P in laboratory studies (Johansson & Granéli 1999, Hagström 2006, Lindehoff et al. 2009) and averaged around 80:10:1 in prymnesiophytes during growth (Quigg et al. 2003). In our study, *P. parvum* cells grown at 7 PSU showed a lower C:N:P ratio during exponential stage compared to that at 26 PSU (80:8:1 vs. 100:10:1). This leads to 2 conclusions: (1) the Redfield ratio may not be appropriate to mirror the actual needs for C, N and P in *P. parvum* (Quigg et al. 2003), and (2) salinity influences intracellular C:N:P needs. As strains cultured at 26 PSU showed lower extracellular toxicity, an explanation for the higher C:N:P ratios at 7 PSU could be that cells are accumulating extra P via the uptake of organic material (e.g. bacteria) into the surrounding water. Bacterial abundance was not measured during the course of the present experiment, and more sampling points during exponential stage would be informative to confirm the stability of the measured C:N:P ratios, as for instance the C:P ratio of *P. parvum* cells has been found to vary between 50 and 150 during exponential growth (Skingel et al. 2010).

Intra- and extracellular toxicity

Previous reports claiming that toxicity in *Prymnesium* showed no clear trend in relation to salinity are partially supported by our study as intracellular toxicity (hemolysis activity) among the 4 strains exhibited little or no variation between 7 and 26 PSU. In contrast, extracellular toxicity (*Rhodomonas* test) was measured at 7 PSU, while it was not detected in 80% of the cases at 26 PSU. This result was consistent for all strains in both trials. This positive effect of low salinity on the extracellular toxicity of *P. parvum* was not coupled to suboptimal growth conditions, as

reported by Baker et al. (2007), who showed that suboptimal salinity and reduced growth increased extracellular toxicity of *P. parvum* to fish.

Intracellular toxicity measured as hemolytic activity can vary over 2 orders of magnitude from 0.050 to 4 ng sap. eq. cell^{-1} (Stolte et al. 2002, Uronen et al. 2005, Sopanen et al. 2006, present study), 5 to 12 ng sap. eq. cell^{-1} (Freitag et al. 2011) to 1–60 × 10^3 ng sap. eq. cell^{-1} (Legrand et al. 2001, Sengco et al. 2005, Lindehoff et al. 2009). Comparison with other studies of *Prymnesium parvum* is difficult as horse erythrocytes are not used, and results are not expressed in saponin equivalents. In contrast, extracellular toxicity in *P. parvum* measured with a bioassay varies less, e.g. with EC_{50} = 10 × 10^3 to 100 × 10^3 cells ml^{-1} (*Rhodomonas* test; Freitag et al. 2011, present study), ED_{50} = 100 × 10^3 to 500 × 10^3 ml^{-1} (fish test; Baker et al. 2007, Schug et al. 2010) and EC_{50} = 2.5 × 10^3 to 350 × 10^3 cells ml^{-1} (*Artemia* test; Edvardsen & Paasche 1992).

Large variations in toxicity, intracellular (Uronen et al. 2005, Hagström 2006) as well as extracellular (Skingel et al. 2010), have been reported during the different phases of *Prymnesium parvum* growth. Extracellular toxicity can show a maximum during late exponential growth (Skingel et al. 2010), but no particular growth phase was identified in our study. In contrast, we measured extracellular toxicity at only 3 to 4 occasions over 36 d, which could be limiting to describe a pattern. The mechanism of toxin exudation in *P. parvum* is unknown. Some authors suggest that toxins are released during the decay of *P. parvum* cells (Shilo & Aschner 1953), whereas measurements of extracellular toxicity during exponential growth indicate active exudation of *P. parvum* toxins (Skovgaard & Hansen 2003, present study). Filtration also might lead to the lysis of phytoplankton cells and induce the release of naturally intracellular material into the surrounding water (Goldman & Dennett 1985). Nevertheless, the uncoupling between intra- and extracellular toxicity during the different growth phases indicates an active release of the toxins. N and/or P depletion is a factor increasing toxicity in *P. parvum* (Granéli & Johansson 2003, Uronen et al. 2005). In Trial 1, although the nutrients in the culture medium were adjusted to the Redfield ratio, the intracellular C:N:P ratio was lower during exponential growth at both salinities, indicating that *P. parvum* cells were likely P limited during stationary and senescent growth. However, this P limitation only led to an increase in both intra- and extracellular toxicity during growth at 7 PSU, whereas no such trend was observed at 26 PSU.

Few studies have measured intra- and extracellular toxicity in *Prymnesium parvum* at the same time, and both toxicities may be related or not. Plotting intra- against extracellular toxicity data for all strains and both trials did not reveal a significant relationship between the 2 parameters. Furthermore, cultures (Strains A and D; Trial 1, exponential stage) with no intracellular toxicity showed an extracellular activity. Inconsistencies between intra- and extracellular activity are frequently observed (Guo et al. 1996, Schug et al. 2010) and may indicate that several chemical compounds with distinct toxic properties are involved in intra- or extracellular toxicity or both (Schug et al. 2010, Valenti et al. 2010). Recently, it has been found that the toxins responsible for fish kills in *P. parvum* are compounds other than the prymnesins isolated by Igarashi et al. (1996) (Henrikson et al. 2010). The utilization of different bioassays in identifying intra- and extracellular toxicity may therefore show the effect of different chemical compounds and can lead to an extreme variability in the results (Valenti et al. 2010). In the present study, different targets were used to determine intracellular (horse blood cells) and extracellular (*Rhodomonas*) toxicity. Nevertheless, as the lysis of a target cell due to toxicity (probably due to an increase in membrane conductance; Manning & La Claire 2010) was measured in both tests, we assume that the same cocktail of chemicals is involved in both toxicity measurements. The sensitivity of the target plays a role in the outcome of any bioassay, as revealed in Trial 2. The cryptophyte *R. salina* grown at 7 PSU was more sensitive to *P. parvum* extracellular phase than the one grown at 26 PSU; this could be due to the fact that membrane permeation is altered by salinity in some euryhaline species (Pick 2004). More studies with potential target algae of *P. parvum* should investigate if brackish species are generally more sensitive to extracellular toxins than marine species. This is relevant for estuaries, intermittently connected water bodies (e.g. saltmarshes; Miller et al. 2009), and during high water events when marine/brackish harmful blooms can reach freshwater bodies (e.g. wetlands). Mixotrophs, such as *P. parvum*, might capture and immobilize their prey more successfully in brackish water (Skovgaard & Hansen 2003) as both their extracellular toxins are more potent and/or present in higher levels and the prey (competitor or grazer) is more sensitive to the toxins. In addition, short-term environmental changes in salinity and temperature affect intra- and extracellular toxicity in a few hours (Freitag et al. 2011).

In dinoflagellates, toxicity is a relatively stable trait in a given clone (Bachvaroff et al. 2009, Tillmann et al. 2009). For *Prymnesium parvum*, toxicity is also strain-specific but seems to vary significantly in differing environmental conditions (Larsen & Bryant 1998, present study). However, we observed substantial differences in toxicity among strains, indicating that the environmental conditions do not completely mask the genetic variation among strains. As blooms of *P. parvum* can be genetically heterogeneous (Barreto et al. 2011), it would be informative to know to what extent functional differences among the different genotypes affect the yield and toxicity of the blooms.

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Appendix 1. *Prymnesium parvum* cell abundance before allelopathy tests

Table A1. *Prymnesium parvum* abundance (cells ml^{-1}; mean ± SD) in the cultures before the allelopathy tests were accomplished during Trial 1. Values correspond to the lowest dilution of *P. parvum* used in the test

Strain	Growth phase		
	Exponential	Stationary	Senescent
7 PSU			
A	510 369 ± 22 910	401 735 ± 33 628	515 482 ± 183 013
B	511 647 ± 59 622	453 709 ± 236 481	362 115 ± 303 629
C	698 669 ± 34 539	788 559 ± 31 797	633 489 ± 54 767
D	613 466 ± 66 936	993 048 ± 58 063	804 322 ± 169 766
26 PSU			
A	152 514 ± 21 851	345 074 ± 11 713	495 033 ± 23 715
B	221 955 ± 18 179	577 680 ± 31 383	900 602 ± 50 354
C	271 372 ± 23 989	480 122 ± 40 341	368 933 ± 86 986

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