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Effect of tertiary sewage effluent additions on *Prymnesium parvum* cell toxicity and stable isotope ratios

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ABSTRACT

We investigated the ability of the ichthyotoxic haptophyte *Prymnesium parvum* to use sewage-originated nutrients applying stable carbon (C) and nitrogen (N) isotope techniques. *P. parvum* was cultured under N and phosphorus (P) sufficient and deficient conditions in either sewage effluent-based medium or in a nitrate- and phosphate-based control. Cell densities and toxicities were monitored and stable carbon N isotopes signatures ($\delta^{13}C$ and $\delta^{15}N$) of *P. parvum* and the sewage effluent analysed. Nitrogen and P sufficient cultures achieved the highest biomass followed by P and N deficient cultures, regardless of sewage effluent additions. The P deficient cultures with sewage effluent had higher toxicity, estimated as haemolytic activity ($9.4 \pm 0 \times 10^{-5}$ mg Saponin equiv. cell^{-1}) compared to the P deficient control and to all N deficient and NP sufficient cultures. Nutrient deficient conditions had no effect on the cell $\delta^{15}N$, but a decreasing effect on $\delta^{13}C$ in the inorganic N deficient treatment. Growth in sewage-based media was followed by a substantial increase in the cell $\delta^{15}N$ (10.4–16.1‰) compared to the control treatments (2.4–4.9‰), showing that *P. parvum* is capable of direct use of sewage-originated N, inorganic as well as organic. Uptake of terrestrial derived C in the sewage treatments was confirmed by a decrease in cell $\delta^{13}C$, implying that *P. parvum* is able to utilize organic nutrients in sewage effluent.

1. Introduction

Prymnesium parvum (Carter), Haptophyceae, is a eurohaline flagellate known from fresh, brackish and marine waters. The species is ichthyotoxic and responsible for massive fish-killing blooms resulting in great ecological and economical losses worldwide (Guo et al., 1996; Edvardsen and Paasche, 1998; Lindholm et al., 1999). The capacity to form blooms in coastal waters might in part be explained by the mixotrophic behaviour of *P. parvum*, i.e. the ability to utilize both organic and inorganic nutrients. Ingestion of organic particles, phagotrophy, is a well documented in Prymnesiophyceae, particularly in the genera of *Prymnesium* and *Chrysochromulina* (Nygaard and Tobiesen, 1993; Tillmann, 1998, 2003; Skovgaard and Hansen, 2003). Using its haemolytic toxin prymnesin *P. parvum* can immobilise and ingest various preys, from bacteria to large dinoflagellates (Tillmann, 1998, 2003; Skovgaard and Hansen, 2003; Stoecker et al., 2006). Further, the toxin can be used to kill or inhibit growth of competitors under nutrient limited conditions (Granéli and Johansson, 2003; Fistarol et al., 2003, 2004).

The ability to take up dissolved organic matter (DOM), osmotrophy, has been confirmed in *P. parvum* but is far from well assessed (Carlsson and Granéli, 1998; Palenik and Morel, 1991). Osmotrophic harmful algae can take advantage of the organic part of anthropogenic nutrient sources in coastal waters, e.g. the dinoflagellate *Prorocentrum minimum*, capable of using humic acids and DOM for growth and toxin production (Granéli et al., 1985; Granéli and Moreira, 1990; Carlsson et al., 1999; Glibert et al., 2001; Heil, 2005).

Harmful algal blooms (HABs) have increased worldwide, both in size and frequency (Hallegraeff, 1993) and a strong correlation has been shown between increased eutrophication and the increase in HABs (Paerl, 1997; Glibert et al., 2001; Anderson et al., 2002; Glibert and Burkholder, 2006). Anthropogenic nutrient sources nursing HABs are, among others, municipal sewage, atmospheric deposition and runoff from agricultural areas (reviewed by Anderson et al., 2002). These increasing inputs of nutrients alter the natural ratios between nitrogen (N) and phosphorous (P), and silica (Si) and P, leading to an altered species succession, favouring HABs (Hodgkiss and Ho, 1997; Zingone and Enevoldsen, 2000; Glibert and Burkholder, 2006).

The $\delta^{13}C$ (the ratio of ^{13}C and ^{12}C compared to a standard) of primary producers is to a large extent determined by composition of the carbon (C) source used during photosynthesis (Chanton and

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Lewis, 1999). Further, as $\delta^{13}C$ is carried through the food chain with very little change per trophic level (DeNiro and Epstein, 1978; Fry and Sherr, 1984) it has been used successfully to trace the origin of organic matter in marine systems (Thornton and McManus, 1994; Lee, 2000; Grey and Jones, 1999). Terrestrial plants mainly use atmospheric CO_2 (−8‰), giving them lower $\delta^{13}C$ signatures than marine phytoplankton using marine dissolved inorganic C (DIC) (0‰) (Peterson and Fry, 1987; Boschker and Middelburg, 2002). This difference in $\delta^{13}C$ can be used to trace terrestrial C from riverine detritus, sewage discharge and wastewater into different parts of the marine ecosystem, such as aquatic organisms, DOM and sediments (Hellings et al., 1999; Heikoop et al., 2000; Waldron et al., 2001; Herczeg et al., 2001; Rosenmeier et al., 2004). Further, the $\delta^{13}C$ can be affected by fractionation, i.e. the process where phytoplankton discriminates against the natural occurring ^{13}C and by doing so affect the $\delta^{13}C$. Several factors can influence fractionation in algae, e.g. cell size (Popp et al., 1998) growth rate and concentration of the C source use (Burkhardt et al., 1999).

Marine dissolved inorganic N (DIN) has a $\delta^{15}N$ value of 4–6‰, $\delta^{15}N$ being the ratio between N stable isotopes ^{15}N and ^{14}N compared to a standard (Owens, 1987; Peterson and Fry, 1987). Sewage and wastewater represent a higher $\delta^{15}N$ value than marine originated N, a fact used in numerous field studies tracing sewage nutrients in seaweeds (e.g. Cabana and Rasmussen, 1996; McClelland and Valiela, 1998; Costanzo et al., 2001; Rosenmeier et al., 2004; Savage and Elmgren, 2004). The high $\delta^{15}N$ signal in sewage can be traced through the food web, from primary producers to secondary consumers (Cabana and Rasmussen, 1996; Hansson et al., 1997; Jones et al., 2001; Savage and Elmgren, 2004). The enrichment of ^{15}N in sewage and wastewater ($\delta^{15}N$ of 28–38‰) is due to bacterial degradation of urea resulting in a ^{15}N-enriched product, as ^{14}N dominates the NH_4^+ that is being released into the atmosphere (Heaton, 1986; Savage, 2005). Tertiary-treated sewage effluent can substantially increase eutrophication on a local scale with mainly inorganic N but also dissolved organic N (DON) (Savage, 2005).

The experiment performed here aimed to investigate (1) if *P. parvum* is able to utilize organic as well as inorganic nutrients in sewage effluent; (2) if uptake of sewage-derived nutrients is detectable by stable C and N isotope analyses; (3) if cell density and toxicity of *P. parvum* is affected by cultivation with sewage-originated nutrients, compared to a nitrate (NO_3^-) and phosphate (PO_4^{3-})-based control both under N and P sufficient and deficient conditions.

2. Methods

2.1. Cultivation

P. parvum KAC 39 (Kalmar Algae Collection, Kalmar University, Sweden) was cultivated as non-axenic 500 ml batch cultures under NP sufficiency (N:P 16:1, [N] = 160 μM, [P] = 10 μM), and N (N:P 1.6:1, [N] = 16 μM, [P] = 10 μM), and P (N:P 160:1, [N] = 160 μM, [P] = 1 μM) deficiency, all in four replicates. Medium was prepared from filtered (1.2 μm, Munktell glass fibre filters) autoclaved Baltic Sea water with addition of trace mineral and vitamins according to Guillard (1975). Two sets of cultures were made, one inorganic control with addition of NO_3^- and PO_4^{3-} and one with tertiary-treated sewage effluent from the Kalmar sewage treatment plant (collected in 2004). Both culture sets were adjusted to give equivalent inorganic N and P concentrations according to additions in Table 1. Prior to use, particulate nutrients in the sewage effluent were removed by 0.2 μm filtration using Sartorius syringe filters. The treatment plant had in 2004 removal efficiency at 93.9 and 66.9% of incoming P and N, respectively. Thus, in 2004, 1.1 ton N of the incoming 3.2 ton N was discharged and released into the Kalmar sound (Andersson et al., 2004).

The experiment was performed under controlled laboratory conditions regarding temperature (15 °C), salinity (7‰) and photon flux density (100 μE s^{-1} m^{-2} in a 16:8-h light:dark cycle). Cell densities were monitored daily by flow cytometry (Becton Dickinson, FACSCalibur). Fluorescent reference beads (Truecount (Tubes, Becton Dickinson) were used to calibrate the instrument and manual cell counts on parallel samples were made for correlation. Specific growth rate was calculated according to $\mu = (\ln N_2 - \ln N_1)/(t_2 - t_1)$ where N_2 and N_1 are cells ml^{-1} at respective time, t_2 or t_1.

Samples for manual cell counts were preserved in 2% acid Lugol's solution and kept in the dark at 8 °C until counted in an inverted microscope (Nikon Diaphot T300). When the cultures entered the stationary growth phase samples were taken for analyses of DIN (NO_3^-, NO_2^-, NH_4^+) and dissolved inorganic P (DIP) (PO_4^{3-}), particulate C (POC), N (PON) and P (POP), stable C and N isotope signatures, chlorophyll *a*, toxicity analyses and bacterial counts. The treatments entered stationary growth at different times, thus N deficient cultures were sampled at day 11, P deficient cultures at day 13 and NP sufficient at day 15. Chlorophyll *a* was analysed by filtration of 5 ml culture onto Whatman GF/C filters, ethanol extraction over night and measured spectrophotometrically according to Jespersen and Christoffersen (1987). Samples for bacterial counts were preserved with 1.5% formaldehyde and counted with flow cytometry (Becton Dickinson, FACSCalibur) using SYTO 13 staining of nucleic acids according to Troussellier et al. (1999).

2.2. Nutrient analyses

Inorganic nutrients were measured in the sewage effluent prior to the experiment and in the cultures at the beginning and at the end according to Valderrama (1995). Total N and P concentration (inorganic and organic) of the sewage effluent was also measured according to Valderrama (1995). For POC, PON and POP analyses 25 and 15 ml of culture for POC–PON and POP, respectively, were filtered onto pre-combusted (450 °C, 2 h) 25 mm Whatman GF/C filters. The filters were dried at 60 °C for 24 h before stored in

Table 1
Intended N:P ratios and additions of inorganic (NO_3^- and PO_4^{3-}) and sewage effluent nutrients in each *Prymnesium parvum* culture, to be cultivated under NP sufficient (NP suf), N and P deficient (N and P def) conditions with addition of sewage effluent or as an inorganic control

	Intended N:P ratio (atomic)	Addition NO_3^- (μM)	Addition PO_4^{3-} (μM)	Addition sewage (ml)	Addition sewage-DIN (μM)	Addition sewage-DON (μM)	Addition sewage-DIP (μM)
NP suf control	16	160	10	–	–	–	–
N def control	1.6	16	10	–	–	–	–
P def control	160	160	1	–	–	–	–
NP suf sewage	16	–	0	151	160	17	1.0
N def sewage	1.6	–	9.9	15	16	1.7	0.1
P def sewage	160	–	0	151	160	17	1.0