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Temporary cyst formation in phytoplankton: a response to allelopathic competitors?

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Summary

Competition among phytoplankton for limiting resources may involve direct or indirect interactions. A direct interaction of competitors is the release of chemicals that inhibit other species, a process known as allelopathy. Here, we investigated the allelopathic effect of three toxic microalgae species (*Alexandrium tamarense*, *Karenia mikimotoi* and *Chrysochromulina polylepis*) on a natural population of the dinoflagellate *Scrippsiella trochoidea*. Our major findings were that in addition to causing death of *S. trochoidea* cells, the allelopathic species also induced the formation of temporary cysts in *S. trochoidea*. Because cysts were not lysed, encystment may act as a defence mechanism for *S. trochoidea* to resist allelochemicals, especially when the allelopathic effect is moderate. By forming temporary cysts, *S. trochoidea* may be able to overcome the effect of allelochemicals, and thereby have an adaptive advantage over other organisms unable to do so.

Introduction

Phytoplankton compete for the same limiting resources, including nutrients and light. The exploitation of a common resource has led to a number of adaptations, in which various species have evolved different abilities to better exploit a resource, and thereby improve their competitive ability. This is referred to as indirect competition (Lampert and Sommer, 1997). However, some species can have a direct effect on their competitors, for example through the release of chemicals, known as allelopathy (Lampert and Sommer, 1997). Chemically mediated interactions have been reported for aquatic environments, especially pred-

ator–prey interactions (Van Donk *et al.*, 1999 and references within; Wolfe, 2000), but also allelopathy (Pratt, 1966; Keating, 1977; Vardi *et al.*, 2002; Fistarol *et al.*, 2003). Allelopathy is the release of organic compounds by plants or microorganisms that affect their potential competitors for resources (Rice, 1984). It is a chemically elicited interaction mediated by many types of compounds with different sites and modes of action (Seigler, 1996).

In allelopathic interactions, the species that are not killed by allelochemicals must possess some strategy to survive such conditions, such as avoidance or tolerance mechanisms. To our knowledge, the only report of a survival strategy is the formation of what was considered to be temporary cysts by the dinoflagellate *Heterocapsa circularisquama*, to avoid being killed by another dinoflagellate and diatoms (Uchida *et al.*, 1996; 1999). Because the mechanisms of how phytoplankton could resist chemical attack have not been reported, it is unknown whether phytoplankton could, for example, release enzymes which would destroy/neutralize the allelochemicals. Nevertheless, it is likely that a behavioural strategy could be used as a mechanism to tolerate allelochemicals. Such a response could be that the target species would temporarily avoid the allelopathic competitor. This type of avoidance strategy has been detected in some phytoplankton species in order to avoid ingestion by grazers. For example, the raphidophyte *Gonyostomum semen* and the dinoflagellate *Peridinium aciculiferum* avoid grazers by not germinating/migrating to the water column in the presence of zooplankton (Hansson, 1996; Rengefors *et al.*, 1998), and instead remain as resting cysts on the sediment.

Dinoflagellates are a group of eukaryotic, flagellated phytoplankton (Taylor, 1987) with many members that can form cysts. Cyst formation allows them to alternatively inhabit the water column as motile cells, or the benthos as cysts, and is usually explained as an adaptation to reduce mortality during periods of unfavourable environmental conditions (Dale, 1983; Fryxell, 1983; Pfiester and Anderson, 1987). Dinoflagellates can form two basic types of cysts: (i) resting (hypnozygotic) cysts, a product of sexual reproduction; and (ii) temporary (pellicular) cysts, also referred to as thin walled cysts (Pfiester and Anderson, 1987). Induction of sexuality and formation of resting cysts appear to be induced by stressful environmental conditions, such as turbulence or nutrient limitation. However, endogenous processes are also involved

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in encystment (von Stosch, 1973; Pfister, 1975; Anderson and Keafer, 1987). Temporary cysts are also formed under adverse conditions. Though, temporary cysts, as opposed to resting cysts, can quickly re-establish a vegetative, motile existence, when conditions become favourable again, thus allowing cells to withstand short-term environmental fluctuations (Anderson, 1998). Both resting and temporary cysts show high chemical resistance (Fensome *et al.*, 1996; Kokinos *et al.*, 1998).

As temporary cysts are believed to be an escape from unfavourable conditions, and because of their high chemical resistance, they may act as a defence mechanism, not only to abiotic stress factors, but also to organic chemical compounds. Given the widespread occurrence of predation, phagotrophy, parasitism, allelopathy, and the presence of sexual interactions in dinoflagellates, it is likely that most, if not all of them, may respond to chemical clues released by other aquatic organisms.

We tested the response of diverse organisms from a natural plankton community to allelochemicals released by three toxic microalgae species (the dinoflagellates *Alexandrium tamarense* (Lebour) Balech KAC 02, and *Karenia mikimotoi* Gert Hansen and Moestrup Tinduff 95, and the prymnesiophyte *Chrysochromulina polylepis* Manton and Parke strains CCMP 289 and K-0259). Here we report the effects on the dominant dinoflagellate (Fistarol *et al.*, 2004) in that community, *Scrippsiella trochoidea* (Stein) Loeblich III, as it showed a different response from all the other members of the plankton community. The allelochemicals caused death of *S. trochoidea* and also induced formation of temporary cysts. After observing these two responses, we tested whether the formation of temporary cysts showed any correlation with the mortality caused by the allelochemicals. We found that algal filtrates causing higher mortality induced less formation of temporary cysts, probably because cell membrane damage occurred before cysts could be formed. The two *C. polylepis* strains will, hereafter, be referred only as CCMP 289 and K-0259, their strains names.

Results

Scrippsiella trochoidea was the dominant dinoflagellate

on the plankton community from Trondheimsfjord, Norway, during the period between September 3–9 (Fistarol *et al.*, 2004). Two distinct effects were observed on *S. trochoidea* after exposure to cell-free filtrates of the three toxic microalgae: (i) death of *S. trochoidea*, which was shown by the decrease of *S. trochoidea* cell numbers in the filtrate treatment, and by the observation of lysed cells and empty thecae during the cell counts, and (ii) formation of temporary cysts. Both cell death and cyst formation were considered to be a result of allelopathy, as both were the effect of one microorganism upon another caused by released compounds.

A decrease in *S. trochoidea* intact cell numbers was observed after three days of exposure to filtrate from the toxic algae (Table 1). At the end of the experiment, the number of intact *S. trochoidea* cells in the treatments that received filtrates from *A. tamarense*, *K. mikimotoi* and CCMP 289 were significantly lower than in the controls (Student's *t*-test, $P < 0.0001$, $P < 0.02$, $P < 0.012$, respectively) (Table 1). Compared to the beginning of the experiment, *S. trochoidea* cell numbers decreased in the treatment that received filtrate from K-0259, whereas the cell numbers increased in the control. Despite this, the difference in the *S. trochoidea* cell numbers between treatment with filtrate from K-0259 and the control was not significant (Student's *t*-test, $P > 0.10$).

The number of *S. trochoidea* temporary cysts increased after exposure to algal filtrates and was significantly higher in all filtrate treatments than in the respective control on day 3 (Student's *t*-test, $P < 0.05$, for all treatments) (Fig. 1). Even though K-0259 filtrate did not cause a significant decrease in the number of intact *S. trochoidea* cells, this filtrate significantly induced the formation of temporary cysts (Student's *t*-test, $P < 0.002$). The percentage of the total affected cells (see *Experimental procedures*) that formed temporary cysts varied among the filtrate treatments (Fig. 2). Filtrate from K-0259, the one causing the lowest mortality, induced the highest formation of temporary cysts (84% of the affected cells formed cysts).

The percentage of dead *S. trochoidea* cells also varied depending on the origin of the filtrate it was exposed to. *Alexandrium tamarense* filtrate caused the highest cell

Table 1. *Scrippsiella trochoidea* intact cell numbers, before and after exposure to filtrate from the different toxic microalgae ($n = 3$, mean \pm SD).

Toxic microalgae	<i>Scrippsiella trochoidea</i> (cell l ⁻¹)		
	Start of experiment	After 3 days of exposure	
		filtrate	control
<i>Alexandrium tamarense</i>	9570 \pm 853	2420 \pm 151	10333 \pm 718
<i>Karenia mikimotoi</i>	9570 \pm 853	7440 \pm 746	10597 \pm 1254
<i>Chrysochromulina polylepis</i> CCMP 289	10600 \pm 212	6390 \pm 1043	11285 \pm 1635
<i>Chrysochromulina polylepis</i> K-0259	10600 \pm 212	9240 \pm 531	11285 \pm 1635

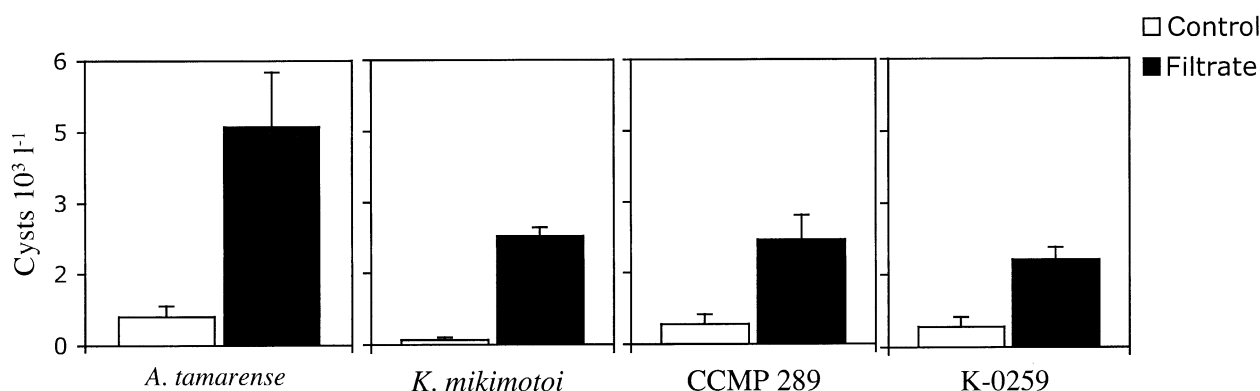


Fig. 1. Concentration of *Scrippsiella trochoidea* temporary cysts in the treatments that received filtrate from the toxic algae and in the controls at the end of the experiment (day 3) ($n = 3$, mean \pm SD).

death ($PD = 39\%$), whereas K-0259 caused the lowest ($PD = 3\%$) (Fig. 2). A negative correlation was found between the percentage of dead *S. trochoidea* cells and the percentage of cells that became temporary cysts ($R^2 = 0.69$; significance of the regression = t -test, $P < 0.001$), i.e. the stronger the effect of the filtrate, the fewer cysts were formed.

Discussion

We found that the dinoflagellate *S. trochoidea* form temporary cyst when exposed to allelochemicals, which are released by competing microalgae. To our knowledge, this is among the first evidence available of a behavioural defence to allelopathy among phytoplankton. *Scrippsiella trochoidea* was the only organism in the plankton community from Trondheimsfjord to show this response. The other phytoplankton organisms, when exposed to the same filtrates, died or decreased their growth rate (Fistarol *et al.*, 2004; G. O. Fistarol, unpublished).

Scrippsiella trochoidea occurs in the Trondheimsfjord almost through the year, from April to October. *Alexandrium* and *Chrysochromulina* have been reported to co-occur with *S. trochoidea* in April (Chuaychan, 1998), but they probably also co-occur during the whole year, as Chuaychan reports the presence of dinoflagellates and flagellates, which are grouped as a class, during the whole year. *Karenia* occurs in April and then again from July to October.

Cell death and encystment

All toxic microalgae tested induced temporary cyst formation in some *S. trochoidea* cells, while other cells were killed by lysis. We have observed that strong allelopathic effects cause the cells to lyse quickly (within 1–24 h, depending on the allelopathic species and the target organisms). Based on this observation, and as lysed cells

did not form cysts, we hypothesized that a stronger allelopathic effect allows fewer temporary cysts to be formed, as the cell membrane is probably permanently damaged before cysts are formed. Thus, strong allelopathic effects would cause a higher mortality, while mild allelopathic effects could cause other, non-lethal, effects, e.g. cyst formation. Our hypothesis was supported by the negative correlation obtained between the percentage of dead cells and the percentage of cysts formed.

The fact that some *S. trochoidea* cells responded to allelochemicals by forming temporary cysts indicates that they must have receptors to respond to chemical cues. This notion is supported by a study of Balzer (1996), who showed that addition of melatonin induces formation of

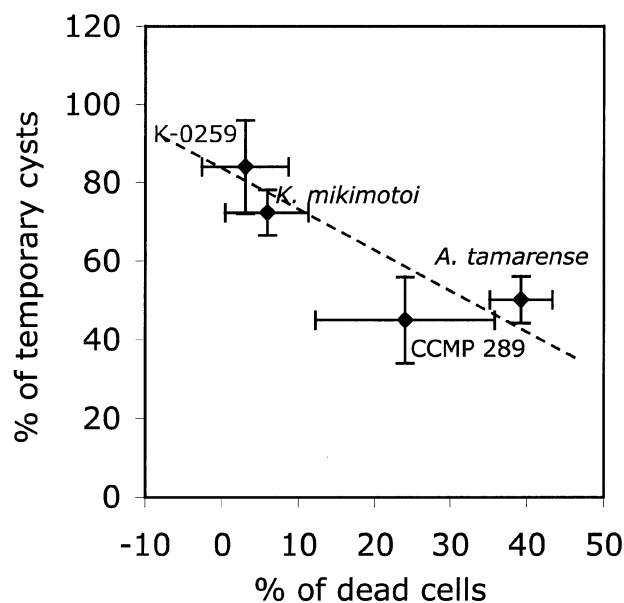


Fig. 2. Negative correlation between the percentage of dead cells (PD) and the percentage of temporary cysts (PC), in each of the filtrate treatments (ANOVA, $P < 0.01$, and $R^2 = 0.69$).

asexual cysts (temporary) in the dinoflagellate *Gonyaulax polyedra*. The differential response of *S. trochoidea* cells, i.e. cyst formation versus lysis, can be the result of differences in the physiological status of the cells within the *S. trochoidea* population. Within a population, there are cells that are growing, dividing (Gisselson *et al.*, 1999), dying, and also cells with, e.g. different cellular nutrient status (Gisselson *et al.*, 2001; Rengefors *et al.*, 2003). It seems plausible that cells in different growth phases and with different physiological status would respond differently to stress conditions, as, for example, to allelochemicals. Similarly, not all dinoflagellate cells within a given population form resting cysts under stress conditions, and the percentage of encystment can range from 2 to 40% (Anderson *et al.*, 1985; Lirdwitayaprasit *et al.*, 1990; Kremp and Heiskanen, 1999).

Inducible defence

Microorganisms have complex and sophisticated sensory and behavioural adaptations to respond to changes and chemical signals that occur in their environment (Wolfe, 2000). Their responses to stimuli can be by developmental changes, attachment or endocytosis (uptake of extracellular material by invagination of the plasma membrane), or movement. The developmental changes include mating, aggregation or colony formation, and encystment (Wolfe, 2000). Some of the responses triggered by chemical cues will act as defence mechanisms. Inducible defences are phenotypic responses induced directly by cues associated with biotic agents (e.g. phytoplankton allelochemicals; chemicals released by predators) that can reduce the effects of subsequent attacks by these agents (Harvell and Tollrian, 1999). As no lysis of *S. trochoidea* cysts was observed, we suggest that temporary encystment of *S. trochoidea* may act as a defence mechanism induced by allelochemicals released into the medium, and be used by *S. trochoidea* to survive the effect of these compounds. Further investigation is necessary to determine if and when excystment occurs after the allelochemical effect ceases, for example, after the cyst sinks or after the allelochemicals are degraded.

Inducible defences (morphological – colony forming, spines; and palatability) have been observed in phytoplankton, as reviewed by Van Donk *et al.* (1999). Nevertheless, they only describe response to chemicals released by predators (zooplankton), and encystment was not one of the defence mechanisms observed. Induction of encystment in dinoflagellates caused by other phytoplankton has been reported only for the dinoflagellate *Heterocapsa circularisquama* (Uchida *et al.*, 1996; 1999). These authors reported that *H. circularisquama*, when grown with the dinoflagellate *Gymnodinium mikimotoi*

(now *Karenia mikimotoi*) (Uchida *et al.*, 1999) and the diatoms *Chaetoceros didymus*, *Stephanopix palmeriana* and *Licmophora* sp. (Uchida *et al.*, 1996), ceased movement and became round or elliptical in shape, which the authors considered to be temporary cysts. These cysts recover to the motile form again when isolated and cultured in fresh medium. However, Uchida *et al.* (1999) claimed that cell contact is necessary for *G. mikimotoi* to induce cyst formation on *H. circularisquama*. The effect of the diatoms was also caused when diatoms cells were mixed with *H. circularisquama*, but it was not tested if cell contact was necessary for this to occur. We show here that the exposure of *S. trochoidea* just to the cell-free filtrate of the toxic algae tested induced formation of temporary cysts.

Implications for community ecology

Interactions between organisms may cause them to co-evolve. It is believed that during the course of evolution, when chemical-mediated interactions are involved, the organisms that were able to take advantage of, tolerate, or avoid external metabolites from their neighbours, would be selected. The organisms that failed to tolerate, or avoid external metabolites must have become extinct (Lucas, 1947). Because most allelopathic studies report receptor organisms with no tolerance strategy, Lewis (1986) argued that the lack of a defence against a particular allelochemical is inconsistent with natural selection principles. Lewis (1986) claimed that the above could only be expected if allelopathic interactions were sporadic, e.g. during bloom events. Lewis' view was justified by the lack of examples of resistant strategies against allelochemicals. However, here we provide an example of a tolerance/avoidance mechanism to overcome the effect of allelochemicals, which indicates that the selective pressure for a resistant form may occur.

There are examples of allelopathic effects among several phytoplankton species, some lethal, and some inhibitory (Pratt, 1966; Arzul *et al.*, 1999; Rengefors and Legrand, 2001; Fistarol *et al.*, 2003; Granéli and Johansson, 2003; Legrand *et al.*, 2003 and references within). The presence of inhibitory but non-lethal effects, indicates that those species that can survive, albeit growth-inhibited, under such allelopathic attacks, may have some tolerance to allelochemicals. Because there is solid evidence that allelopathy among phytoplankton occurs in the environment (Keating, 1977; Vardi *et al.*, 2002), we may expect to find more examples of tolerance or avoidance mechanisms to allelochemicals. These examples would provide further evidence that allelopathic interactions do cause selective pressure on the target phytoplankton, as toxic algal compounds do on herbivores (Hairston *et al.*, 2001).

Therefore, the ability to form temporary cysts might be an adaptive advantage for *S. trochoidea*. We do not claim that the ability of *S. trochoidea* to form temporary cysts has evolved as a result of allelopathic interactions. However, because this organism has the ability to encyst, *S. trochoidea* might be able to survive the effects of allelochemicals, while other organisms that do not have any avoidance/tolerance mechanism might not. Phytoplankton species that have a tolerance mechanism may, therefore coexist with other allelopathic algae. *Scrippsiella trochoidea* often dominates dinoflagellate communities, and the capacity to form cysts, together with other characteristics of this species, such as the ability to tolerate a wide variety of environmental conditions (Kim and Han, 2000), may explain *S. trochoidea*'s success.

We found that, besides cell death (Fig. 3,a), temporary cyst formation (Fig. 3,b) occurred in *S. trochoidea* as a response to allelochemicals, and we suggest that this mechanism may be used by this species as a strategy to avoid chemical interaction. Our results showed that the proportion of cells that died and that became cysts, depended on the strength of the allelochemicals, and probably on differences in the physiological status of the cells within *S. trochoidea* population. As no lysis of cysts was observed (Fig. 3,c), temporary encystment might help *S. trochoidea* survive chemical mediated interactions, such as allelopathy, especially when exposed to weak allelochemicals. Temporary cyst formation may therefore give *S. trochoidea* a competitive advantage over other species of microalgae that cannot tolerate allelochemicals. Further investigations are needed on the fate of the cysts when released from the exposure to the allelochemicals (Fig. 3,d). Temporary cyst formation is

one of the possible tolerance mechanisms that we may find among phytoplankton species. Because of the widespread occurrence of chemical interactions in the aquatic environment, we may expect to find more examples of such mechanisms. The lack of observations until now may be because of the few studies on algal defence mechanisms triggered by other phytoplankton. The increase in observations of other defence mechanisms would support the hypothesis that co-evolution and selection will favour allelochemical resistant populations/species.

Experimental procedures

The allelopathic effect of the toxic dinoflagellates *Alexandrium tamarense* (KAC 02), and *Karenia mikimotoi* (Tinduff 95), and the prymnesiophyte *Chrysochromulina polylepis* (strains CCMP 289 and K-0259) was tested on *S. trochoidea* within a natural plankton community. *S. trochoidea* was the dominant dinoflagellate from the plankton community in Hopavågen Bay (Trondheimsfjord, Norway) during the period from 3 to 9 September (Fistarol *et al.*, 2004). The three toxic microalgae also occur in Trondheimsfjord (Norway) and co-occur with *S. trochoidea* (Chuaychan, 1998).

Sampling of the plankton community

Scrippsiella trochoidea was part of a natural plankton community from Trondheimsfjord, Norway. The natural plankton communities were collected on two occasions in late summer 2001: on September 3, for the experiments with *Alexandrium tamarense* and *Karenia mikimotoi*, and on September 9, for the experiments with the two *Chrysochromulina polylepis* strains. The water temperature was 13.6°C and salinity 31.8‰ on September 3, and 14°C and 31.5‰ on September 9. The plankton community was sampled from 2 to 6 m depth

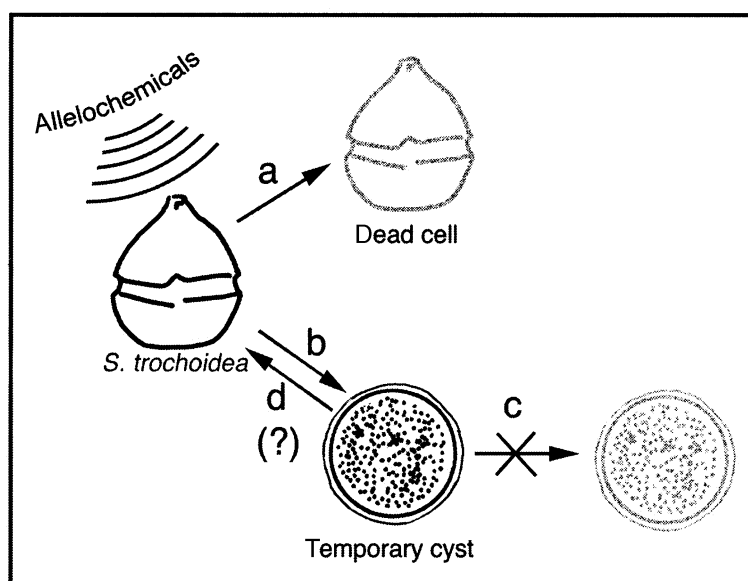


Fig. 3. Schematic model of the effect of allelochemicals on *Scrippsiella trochoidea*. Allelochemicals released from *Alexandrium tamarense*, *Karenia mikimotoi* and *Chrysochromulina polylepis* caused (a) death of the *S. trochoidea* cells, and (b) induced formation of temporary cysts. (c) No lysis of *S. trochoidea* temporary cysts was observed. (d) Further investigations will determine if the cysts return to the vegetative state.

using a Niskin-bottle (a non-reversing water sampler bottle). At the laboratory, the samples were subsequently filtered through a 150 µm mesh-size nylon net, in order to remove the mesozooplankton.

Experimental set-up

The toxic microalgae were grown in f/2 medium (Guillard, 1975) at 20°C, 32‰, and a light:dark cycle of 16 : 8 h. The experiments were performed in triplicate by adding cell-free filtrate of the three toxic microalgae, separately, to the natural plankton community. The cultures were in exponential growth when the filtrates were obtained. Filtrates (150 ml) from each of the toxic microalgae were added to triplicate tissue culture flasks (750 ml) containing 350 ml of the plankton community. Cell-free filtrates were obtained by gentle filtration (a pressure lower than -2 kPa was used to create initial vacuum for the filtration) of the algal cultures through GF/F glass fibre filters. Controls were made by adding the corresponding amount of f/2 medium, instead of filtrate, with nitrate and phosphate concentrations adjusted to the same levels found in the filtrates from each toxic algal culture, to avoid discrepancies caused by different nutrient conditions. Controls were made to observe the plankton community without the interference of the algal filtrates. For each filtrate treatment, one control (also in triplicate) was made, except for the two *C. polylepis* strains that had the same control. The additions of algal filtrate and medium used as control, to the plankton community, were made daily for three days. Each day, 150 ml was removed from the test bottles, and replaced with fresh filtrate or medium used as control. The bottles were incubated at 14°C.

Samples for cell counts were taken at the beginning and at the end of the experiment. During the microscopical observations, we counted the number of intact *S. trochoidea* cells and the number of temporary cysts. The total difference between the number of intact cells in the control (*Nlcont*) and in the respective filtrate treatment (*Nlfilt*) represents the total affected cells (TA) (dead cells plus cells that formed cysts) (Equation 1).

$$Nlcont - Nlfilt = TA \quad (1)$$

By subtracting the number of cysts (*C*) counted from *TA*, we could determine how many cells died (*D*) (Equation 2).

$$TA - C = D \quad (2)$$

The allelopathic effect was considered to be stronger when it resulted in higher mortality. The control represents how much *S. trochoidea* would have grown if there was no interference of allelochemicals. Thus, by knowing the number of cells that died, we could calculate the percentage of dead cells (*PD*) relative to the control (Equation 3). In this equation, *Nlcont* is the intact cell concentration in the control on the third day of the experiment, and *D* is the number of dead *S. trochoidea* cells in the filtrate treatment on the corresponding day. Through these calculations, we could assess which algal filtrate caused the strongest effect (i.e. highest mortality).

$$PD = \frac{(D \times 100)}{Nlcont} \quad (3)$$

Given that two effects (mortality and cyst formation) were observed, we also assessed, based on the total affected cells (*TA*), the percentage of cells that formed temporary cysts (*PC*, percentage of cysts) (Equation 4). Equation 4 represents the percentage of temporary cysts in relation to total affected cells.

$$PC = \frac{C \times 100}{TA} \quad (4)$$

Analytical procedure

Cell counts of *S. trochoidea* were made in samples preserved with Lugol's solution. Samples were settled and counted with an inverted microscope using the method described by Utermöhl (1958). At least 100–250 intact cells and 100–250 cysts were counted per sample.

Statistical analysis

Statistical analyses were performed using the software SPSS 10 for Macintosh. Student's *t*-test was used to compare differences between means: differences in *S. trochoidea* cell numbers and cysts, at the end of the experiment, between each filtrate treatment and the respective control. A linear regression was used to correlate mortality (percentage of dead cells) with the number of temporary cysts formed in each of the filtrate treatment. Student's *t*-test was used to verify if the regression was significant. The data were tested for normality and homogeneity of variance.

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