

Phosphorus uptake by resting cysts of the marine dinoflagellate *Scrippsiella trochoidea*

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Abstract. Resting cysts of the marine dinoflagellate *Scrippsiella trochoidea* were produced under phosphorus (P)-deficient conditions, separated from vegetative cells, and incubated for 28 days in darkness at 4 and 20°C in P-enriched and P-deplete medium. The P content of cysts incubated in the P-replete medium was significantly higher than that of cysts in P-deplete medium. As the P content of the cysts increased through time, dissolved inorganic phosphate was depleted in the medium. This decrease cannot be attributed to free-living bacterial uptake, since there was no corresponding increase in bacterial particulate P. Disappearance of P from the medium can, therefore, only be explained by uptake associated with the cysts. This could be either direct cyst uptake, uptake by bacteria closely associated with the cysts, or adsorption of P on the cyst wall. Evidence is strongest that the cysts incorporated phosphate during the resting stages of dormancy and quiescence, despite the fact that these are periods of significantly reduced metabolism. Accumulation of P during these benthic resting stages would increase the survival of newly excysted vegetative cells as they re-enter the water column after germination, providing a competitive advantage over other phytoplankton. Freshwater and marine sediments provide a P-rich environment which may serve as a potential nutrient pool for dinoflagellate resting cysts. Mobilization of nutrients to and from the sediments via cysts must now be evaluated to ascertain whether this could be a significant term in nutrient budgets.

Introduction

Many freshwater and marine dinoflagellates produce resting cysts during their life histories. These resistant cells allow species to survive unfavorable environmental conditions such as winter temperatures, they provide the inoculum for bloom initiation, facilitate species dispersal through long-distance transport and make adaptive benefits possible through genetic recombination (Wall, 1971).

One important factor in cyst/motile cell dynamics is the role of nutrients in the formation of cysts and in the resulting survival of the emerging germlings. Many studies suggest that cells must be nutrient starved for sexuality and encystment to begin (von Stosch, 1973; Pfiester, 1975, 1976; Anderson *et al.*, 1984; Anderson and Lindquist, 1985). Nevertheless, after a prolonged resting state with a low but functional metabolism (Binder and Anderson, 1990; Lirdwitayaprasit *et al.*, 1990), cysts must still possess sufficient nutrient reserves for germination and survival of the excysted cell. Anderson and Lindquist (1985) and Anderson *et al.* (1985a) inferred that planozygotes (the swimming stage that follows gamete fusion and precedes cyst formation) need to accumulate sufficient nitrogen (N) and phosphorus (P) for cyst formation to occur. This conclusion was based on the large numbers of planozygotes that never completed the transition to resting cysts in nutrient-limited batch cultures.

One possible strategy for nutrient acquisition during the dinoflagellate encystment/excystment cycle that has not been considered in the past is for nutrients to be incorporated during the resting stage. This is analogous to the suggestion that

certain limnic cyanobacteria accumulate P while resting on the benthos (Barbiero and Welch, 1992; Istvánovics *et al.*, 1993; Pettersson *et al.*, 1993). The benthic environment provides an excellent opportunity for algae to incorporate P due to the relatively high phosphate concentrations in sediment pore water (Pettersson *et al.*, 1993). Pore water concentrations are usually ~10–100 times higher than those of the water column [e.g. interstitial water was 0.3–60 μM , while dissolved reactive P in overlying lake water ranged from 0.06 to 1.2 μM according to Holdren *et al.* (1977)]. P uptake by resting cysts would allow for germination and subsequent cell divisions before uptake from the water would be necessary, thereby providing the cyst-forming species with a competitive advantage over non-meroplanktonic species.

Here, we test the concept that dinoflagellate cysts are able to take up P during dormancy and quiescence. Dormancy is defined as a period of internal maturation during which the cyst cannot germinate, even under optimum growth conditions. Quiescence, on the other hand, is when the cyst is mature, but is prevented from germination by an unfavorable external environment (Pfiester and Anderson, 1987).

Method

Culture

The dinoflagellate used for the experiment was a non-axenic clonal culture of *Scrippsiella trochoidea* (Stein) designated SA2, originally isolated from Perch Pond (Falmouth, MA). The culture was maintained in *f/2*-enriched Vineyard Sound seawater (Guillard and Ryther, 1962) at 20°C under a 14:10 h light:dark (LD) cycle (cool white fluorescent lamps, ~250 $\mu\text{E m}^{-2} \text{s}^{-1}$). The medium was modified by adding copper and selenium to final concentrations of 10^{-8} M as in K-medium (Keller *et al.*, 1987).

Cyst production

Cyst production was induced by inoculating SA2 into *f/2* medium with a 1/40 concentration of phosphate (1.8 μM = 58 $\mu\text{g P l}^{-1}$). The new culture was grown at 20°C on a 14:10 h LD cycle. Cysts were harvested 10 days later when cyst number had peaked at ~10% of the total cell concentration. Cysts were collected by aspirating away the overlying medium and motile cells, scraping the material at the bottom with a Teflon spatula, and then resuspending it in nutrient-deplete Sargasso seawater. In the next separation step, cysts were separated from vegetative cells by density centrifugation using a sterile Percoll (Pharmacia Fine Chemicals) –sorbitol solution with a density of 1.15 g ml^{-1} (Price *et al.*, 1978; Anderson *et al.*, 1985b; Binder and Anderson, 1987). The supernatant was aspirated away and the pellet of cysts in the Percoll was resuspended in Sargasso seawater. Any remaining vegetative cells were removed by filtering through a 25 μm sieve and washing the cysts with Sargasso seawater. A maximum of 2% contamination by vegetative cells resulted. The cysts were resuspended in Sargasso seawater, counted and immediately transferred to the experimental tubes.

Nutrient uptake

Cysts were incubated in tubes containing 10 ml of f/2 medium which was either P replete (with 1.8 μM of added phosphate) or in medium which was phosphate depleted ($<0.5 \mu\text{M}$). The tubes were placed in the dark at 4 and 20°C, and three tubes were removed on Days 0, 1, 7, 15, 21 and 28. Tubes were exposed to light during sampling. Aliquots were taken from each of the three replicate tubes for the determination of soluble reactive phosphorus (SRP) in the medium after filtration, particulate P (for cysts and for bacteria), carbon and nitrogen content (CHN) (material from the three replicate tubes was combined) and cell counts. Three milliliters of the cyst suspension were filtered and collected onto 10 μm Nuclepore filters which were then rinsed with 5 ml of 0.2 μm -filtered Sargasso seawater to remove bacteria. The filtrate was passed through a 0.2 μm filter which was used to determine bacterial particulate P. The filtrate from that step was collected to measure SRP in order to calculate the phosphate concentration in the medium. SRP and particulate P (after persulfate digestion) were analyzed according to the method of Murphy and Riley (1962). The remaining cyst suspensions from each of the three replicates were combined and the cysts (and bacteria) were collected on pre-combusted GF/F Whatman filters and washed with Sargasso seawater. The filters with cysts were analyzed for carbon and nitrogen using a Perkin Elmer CHN analyzer. Triplicate cell counts were made for each replicate by enumerating at least 400 cysts in a 1 ml Sedgewick-Rafter slide.

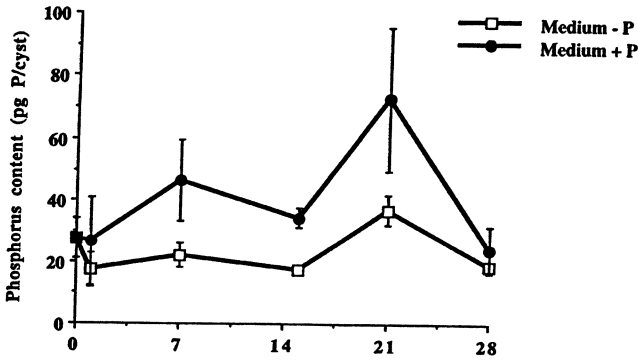
The data were analyzed with a two-factor ANOVA test using the *Super-ANOVA*TM program.

Results

Cysts incubated in P-replete medium showed a higher particulate-P content than those incubated in P-deplete medium. At 4°C in P-replete medium, cyst P content more than doubled from 28 pg cyst⁻¹ at the start of the experiment to 72 pg cyst⁻¹ by Day 21, while in the medium with no added P, the quota remained more or less stable over the same interval (Figure 1A). The difference between the cysts in P-deplete and P-replete medium was highly significant ($P = 0.004$) throughout the 28 day period when tested with a two-factor ANOVA. At 20°C, the same pattern was observed. The P content increased to a maximum of 59 pg P cyst⁻¹ on Day 21, while the P content in the P-deplete medium stayed low at <38 pg P cyst⁻¹ (Figure 1B). Once again, the difference between the cysts in the control and in P-replete medium was highly significant ($P = 0.0001$). No significant difference was found between the temperature treatments when comparing 4°C with 20°C P-replete treatments ($P = 0.558$). At both 4°C and 20°C, there seemed to be a tendency of increasing cyst P content with time up until Day 21, when maxima were reached in the P-replete medium (Figures 1 and 2). The difference between Day 1 and Day 21 was significant at the 1% level (one-factor ANOVA) at both temperatures. On Day 28, P content per cyst fell in both temperature treatments.

SRP decreased in the P-replete medium at both temperatures (regression significant at the 1% level, *t*-test). During the 28 day period at 4°C, the

A Temperature + 4°C



B Temperature + 20°C

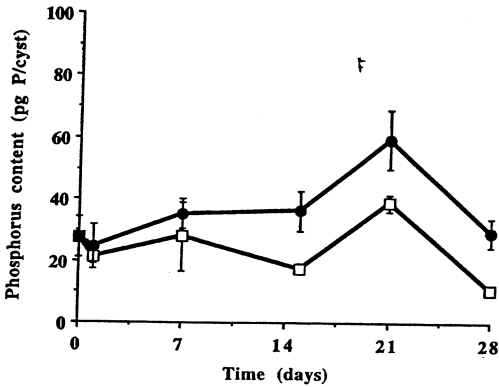


Fig. 1. Phosphorus content of *S.trochoidea* cysts in pg P cyst⁻¹ during incubation at 4°C (A) and 20°C (B), and incubated in P-deplete (-P) and in P-replete (+P) medium.

concentration fell from 1.8 to 0.88 μM (Figure 3A), and from 1.8 to 0.16 μM at 20°C during the 28 day incubation (Figure 3B). SRP in tubes with P-deplete medium was $\sim 0.56 \mu\text{M}$ on Day 0, fell quickly to below 0.16 μM on Day 1, and stayed low thereafter.

Free-living bacterial P did not appear to increase through time in P-replete medium at either temperature. At 4°C and 20°C, there was no significant difference between the P-deplete and the P-replete treatments ($P = 0.57$ and $P = 0.18$) (Figure 4). Bacterial P was always lower than the P content of cysts ($P = 0.0001$) (Figure 5). In the P-replete tubes, bacterial P was fairly constant between 0.3 and 1.2 μM , whereas cyst P was 5–10 times higher. Note that the pattern of cyst P content is different in Figure 5 (compared to Figure 1B) since P content has been

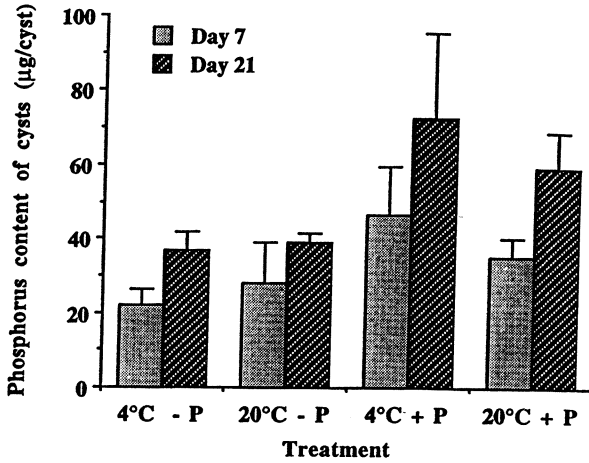


Fig. 2. Phosphorus content of *Strochoidea* cysts in pg P cyst⁻¹ comparing the content at 7 and 21 days of incubation at 4 and 20°C in P-deplete (-P) and P-replete (+P) medium.

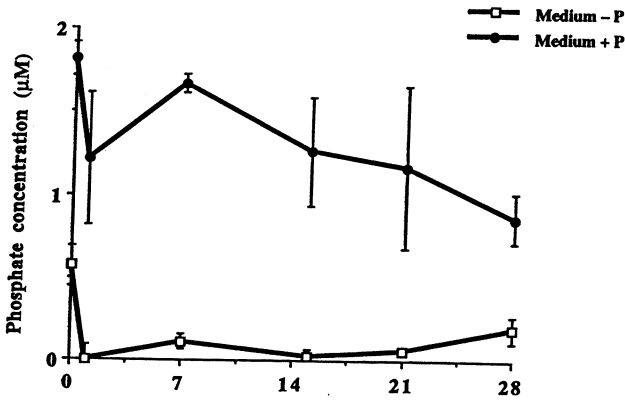
converted to P concentration in the medium (µM) in order to be able to compare cyst P, bacterial P and medium P.

The carbon (C) content of the cysts did not change according to any apparent trend during the incubation period, although it ranged between 4.2 and 11.9 ng C cyst⁻¹ (Table I). The carbon content of cysts tended to be higher in the 4°C treatment ($P = 0.0013$) than at 20°C. However, there was no difference in C content between P treatments. Nitrogen per cyst varied between 0.3 and 0.68 ng N cyst⁻¹. A trend of increasing N content through time was evident in the 4°C treatment. The N content was significantly higher ($P = 0.018$) at 4°C than at 20°C. No difference in cyst N was seen between the different P treatments. The bacterial contribution to total amounts of C and N on the filters was estimated to be of minor importance (<1.5% for C and <3.5% for N) when calculated from bacterial counts and using typical ratios for C:N:P (Fenchel and Blackburn, 1979).

Table I. Change in the carbon and nitrogen content of cysts through time when incubated at 4 and 20°C in medium with and without phosphorus (P)

| Temperature (°C) | Treatment | Initial (day 0) | Final (day 28) | Change = final - initial |
|------------------|------------|-----------------|----------------|--------------------------|
| Carbon | | | | |
| 4 | medium - P | 8.5 | 11.9 | 3.3 |
| | medium + P | 8.5 | 7.7 | -0.8 |
| 20 | medium - P | 8.5 | 4.2 | -4.3 |
| | medium + P | 8.5 | 7.5 | -1.0 |
| Nitrogen | | | | |
| 4 | medium - P | 0.32 | 0.60 | 0.29 |
| | medium + P | 0.32 | 0.61 | 0.29 |
| 20 | medium - P | 0.32 | 0.29 | -0.03 |
| | medium + P | 0.32 | 0.39 | 0.07 |

A Temperature + 4 °C



B Temperature + 20 °C

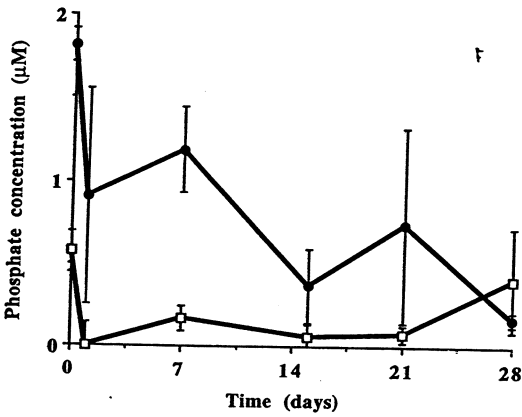
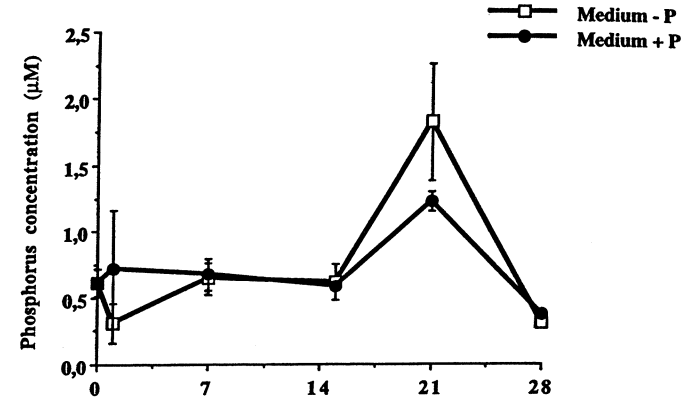


Fig. 3. Change in phosphate concentration in the medium with time, after removing cysts and bacteria by filtering. Concentrations are given in micromolar at 4°C (A) and 20°C (B) for P-deplete (-P) and P-replete (+P) medium.

N:P, C:P and C:N ratios (by weight) were calculated for the cysts (Table II). Initial quotas were taken on Day 0, and averages were calculated for Days 15–28. The N:P ratios increased with time in treatments without P. No N:P changes occurred in P-treated cysts. The C:P ratios of cysts decreased in the P treatments, and remained close to initial ratios when no P was added to the medium. C:N ratios decreased equally with time in both treatments.

The percentage of vegetative cells reached a maximum on Day 7 in all treatments. Tubes in the 20°C P-replete treatment showed the largest number of vegetative cells, a maximum of 8% of the total number of cysts plus vegetative cells. There was no significant correlation between the particulate P fraction and



B Temperature + 20°C

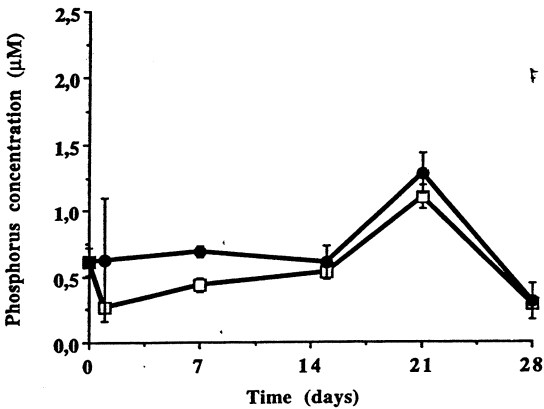


Fig. 4. Phosphorus content of bacteria (and other particles) collected on 0.2 µm filters after removing cysts. Bacterial content is converted to micromolar P in the medium. Incubations are at 4°C (A) and 20°C (B) in P-deplete (-P) and P-replete (+P) medium.

the percentage of vegetative cells (Pearson's correlation test, $r = -0.136$), showing that vegetative cells were not responsible for phosphate uptake. No temporary cysts were observed.

Discussion

The experiments described here demonstrate for the first time that dinoflagellate cysts are able to accumulate P during the resting states of dormancy and quiescence. This is a surprising result, since cysts have a significantly reduced metabolism (Binder and Anderson, 1990), in which many proteins and other macromolecules are presumably catabolized or non-functional. Furthermore,

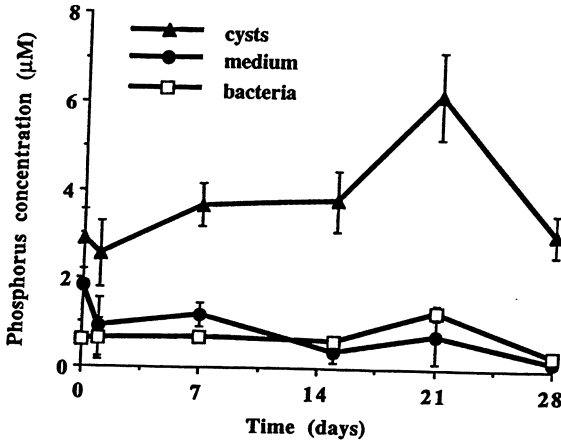


Fig. 5. Comparison of the contribution of the cyst fraction (10 µm filters), bacterial fraction (0.2 µm) and medium when converted to micromolar P. The diagram shows the situation at 20°C and in P-replete medium, serving as an example of similar trends in the other treatments.

cysts are highly resistant cells with outer walls that can withstand environmental extremes, including digestion (D.M.Anderson, unpublished data). Maintenance of active nutrient-uptake systems seems inconsistent with the nature of the cyst wall.

These results have several important ecological implications. The first is that the acquisition of nutrients during the resting state in the sediments allows cells to take advantage of a high-nutrient environment and to obtain nutrients that can maximize the survival of newly germinated cells. This consideration is significant in light of the nutrient limitation which presumably triggers sexuality and cyst formation. A second consideration is that the germination of cysts and migration of motile cells into surface waters can be a mechanism to mobilize sediment nutrients to the water column. These and other issues highlighted by this study are discussed below.

Phosphorus uptake

Scrippsiella cysts have a dormancy period of 25 days (Binder and Anderson, 1987). The cysts used in this study were thus in true dormancy at the start of the experiment, as they were at most 10 days old. After 2 weeks of incubation, some

Table II. N:P, C:P and C:N ratios, by weight, are calculated for cysts. Initial ratios are calculated for Day 0 and an average is calculated for Days 15–28. Ratios are given for 4 and 20°C, and for cysts incubated in P-deplete (-P) and P-replete (+P) medium

| Time | Treatment | N:P | C:P | C:N |
|-----------|-----------|------|-------|------|
| Initial | | 11:1 | 309:1 | 27:1 |
| Average | 4°C - P | 23:1 | 403:1 | 17:1 |
| day 15–28 | 4°C + P | 16:1 | 217:1 | 15:1 |
| | 20°C - P | 21:1 | 313:1 | 15:1 |
| | 20°C + P | 10:1 | 170:1 | 16:1 |

cysts may have entered quiescence, remaining in the resting state because they require light to initiate germination (Binder and Anderson, 1986).

Cysts incubated in P-replete medium had a significantly higher P content than those incubated in P-deplete medium (Figure 1). This is a strong indication that P uptake actually occurs in dormant and/or quiescent cysts formed under P limitation. Dissolved inorganic phosphate was depleted in the medium through time, providing further evidence for P uptake. This decrease cannot be accounted for by free-living bacterial uptake since there was no significant increase in bacterial particulate P through time. Disappearance of P from the medium can therefore only be explained by uptake associated with the cysts, either through direct cyst uptake, uptake by bacteria closely associated with the cysts, or adsorption of P on the cyst wall.

The increase in cyst P content is not likely due to adsorption since cysts were washed with low-P Sargasso seawater to remove bacteria and precipitates. Furthermore, if the increased P content were due to adsorption, we would expect a rapid increase in the cyst P quota, followed by a plateau. Instead, the increase was gradual until Day 21 when P content peaked. Phosphorus precipitation is most unlikely at the low levels of phosphate used in the experiment.

There are several arguments to suggest that P uptake by attached bacteria was not the cause of the increase in cyst P content. The first is that there was no significant uptake of P by free-living bacteria over the same interval (Figure 4). One would expect that the uptake behavior of attached bacteria would be similar to that of the free-living forms since both have phosphate available in the P-enriched medium. Being attached to a cyst would not provide a better phosphate environment. Another argument against P uptake by attached bacteria is that the P content should have been higher at 20°C, since bacterial growth is strongly temperature dependent. There was, however, no difference in the particulate P of cysts at 4 and 20°C. We conclude, therefore, that the observed increase in cyst P was due to uptake by the cysts themselves.

An interesting aspect of the observed variation in P quota through time is that the P content in the cysts peaked on Day 21 and decreased on Day 28 at both temperatures. This decrease may be due to experimental error, since mass balance (excluding dissolved organic phosphorus) calculations were low in both P treatments for Day 28 (e.g. some P could not be accounted for). The standard deviation around the mean was 15–30%.

In a study of *S. trochoidea* cysts by Lirdwitayaprasit *et al.* (1990), P remained constant throughout the resting stage. That incubation lasted only for 7 days, however, compared to our 28. By Day 7 in our experiment, no increase had yet occurred in the cysts exposed to high P.

Ecological considerations

There are several ecological/physiological implications of nutrient uptake during dormancy and quiescence. Encystment of both marine and freshwater dinoflagellates has been repeatedly linked to nutrient depletion (Anderson and Lindquist, 1985; Anderson *et al.*, 1985a; Hickel, 1988; Chapman and Pfister, 1995).

Vegetative cells that are depleted of nutrients form gametes which fuse to become planozygotes and then cysts, and those cysts must contain sufficient nutrient reserves for dormancy, prolonged quiescence and eventual germination. Uptake of nutrients during the planozygote stage preceding encystment, as proposed by Anderson and Lindquist (1985), Anderson *et al.* (1985a) and Lirdwitayaprasit *et al.* (1990), would help to alleviate this shortage, but continued uptake as a cyst would be an additional supplement. Our results indicate that P may be taken up during the resting stage when the cysts are in high-nutrient, benthic environments.

Previous studies on *S.trochoidea* have shown that ATP is utilized during dormancy, but nevertheless increases immediately prior to germination (Lirdwitayaprasit *et al.*, 1990). Those authors suggest that future efforts should be directed to studies of the chemical form of stored P and its role in regulating cyst germination. It has been shown in higher plants that, prior to germination, seeds utilize phosphate bound in phytin when phosphate may be limiting seed germination (Mayer and Shain, 1974).

Phosphorus uptake by dinoflagellate cysts in surface sediments makes sense chemically, since available P is much higher in the sediments than in the overlying water (Löfgren, 1987). Pore water concentrations may range between 0.3 and 60 μM , which is much higher than the 1.8 μM in our P-replete medium. Furthermore, Ohle (1964) showed a positive relationship between primary production and P concentrations in pore water, illustrating the importance of sediment P to the pelagic system. Simultaneously, the P content in the sediments is determined by the organic P load from the pelagic zone. By storing P in cyst form, the cells can ensure that they contain enough P for several cell divisions as they migrate to surface waters. This might be a significant advantage to cysts deposited in deeper waters, where it may take days of swimming upwards to reach depths where light levels are sufficient for photosynthesis.

On a broader perspective, cysts can be viewed as carriers of nutrients in both downward (encystment) and upward (excystment) directions. Heaney *et al.* (1986) and Pollinger *et al.* (1993) discuss the importance of freshwater *Ceratium* in bringing accumulated P to the sediments as cysts settle in the fall. If planozygotes do increase their nutrient content before encystment, the result is a transport of nutrients from the epilimnion to the sediments. In a similar manner, if cysts accumulate nutrients during their resting state in the sediments, motile cells will eventually transport P back to the epilimnion after germination. One ecological and environmental implication of this is that dinoflagellates may contribute to the internal loading of P in lakes, marine estuaries or bays. This is a likely scenario since dinoflagellates can form large blooms in both freshwater and marine environments.

Carbon and nitrogen

The data from this study also suggest that *Scrippsiella* cysts may be capable of N uptake. The N content was higher at 4°C than at 20°C, and apparently increased with time. This occurred despite N-replete conditions prior to encystment, as sexuality was induced by P limitation. Marine dinoflagellates are known to accumulate and store excess N during their vegetative stage (Bhovichitra and Swift, 1977;

Dortch *et al.*, 1984). There are also indications that freshwater dinoflagellates can store N when it is abundant, and then utilize the surplus when ambient nutrients decrease (Chapman and Pfiester, 1995). This strategy would be useful in competition with smaller flagellates, which have low half-saturation constants and high growth rates (Pollinger, 1988). Luxury storage of N as well as P by larger cells weakens the selection for small, fast-growing species (Grover, 1990). The same arguments and implications as discussed regarding cyst uptake of P can be used for N, although uptake of this important nutrient during dormancy and quiescence must be more thoroughly studied in a similar experiment.

Carbon quotas of the cysts differed between temperature treatments. The lower and possibly decreasing C content at 20°C suggests higher respiration of carbohydrates at the higher temperature, consistent with a higher respiration rate at 20°C as shown by Binder and Anderson (1990).

The unchanged N:P ratios in the P-treated cysts are most probably an effect of the cysts' uptake of N and P. The increasing N:P ratios (with time) seen in cysts incubated in P-deplete medium also suggest an uptake of N. An uptake of N, or possibly a reduced N metabolism at 4°C, explains the higher N:P ratios relative to 20°C. The low C:P ratios found in P-treated cysts are also a result of P uptake. C:N ratios decrease in both treatments since C is metabolized faster than N (Lirditayaprasit *et al.*, 1990).

In summary, this study reports the uptake of P by resting cysts of a marine dinoflagellate. This type of uptake is unexpected and, thus far, unprecedented. Future studies are needed to not only confirm these results with *S.trochoidea* and other cyst-forming species, but to identify the uptake mechanisms. Uptake of N by cysts may also have been occurring, although this needs verification in studies of P and N uptake by N-limited cysts (instead of P-limited cysts, as used here). If our results are confirmed in other dinoflagellate species, cysts must be considered an important source and sink for nutrients in nutrient budgets of freshwater and marine systems. The germination of cysts, along with the subsequent migration of cells to the overlying waters, may be a significant transfer mechanism in internal nutrient loading.

Acknowledgements

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