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Separation and concentration of living dinoflagellate resting cysts from marine sediments via density-gradient centrifugation

Abstract—A method for separating and concentrating resting cysts of dinoflagellates from marine sediments via centrifugation in a nontoxic, isosmotic density gradient has been developed and tested. The density-gradient medium is an aqueous suspension of colloidal silica (Nalco 1060) made isosmotic with seawater of salinity 32‰ using sucrose. The density of the medium, which is isosmotic throughout a density range of 1.086–1.405 g cm^{-3}, may be adjusted by varying the proportion of sucrose solution mixed with the colloidal silica. Unlike other methods, there is no problem with jelling of the silica in seawater with this method, and Nalco 1060 is not highly toxic to aquatic organisms as are some other commonly used formulations. Cysts of *Scrippsiella trochoidea* and *Alexandrium fundyense* were extracted quantitatively from a muddy silt marine sediment and showed no sign of differential mortality related to the centrifugation procedure. Cultures of *S. trochoidea* were successfully initiated with centrifuged cysts.

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Sediment resting cysts, or hypnozygotes, are important stages in the life cycles of many dinoflagellate species, including several associated with toxicity in economically important marine fishes and shellfishes (*see* Dale 1983). The distribution of living cysts in natural sediments has been used extensively in studies of the ecology and bloom dynamics of numerous species (e.g. Dale 1976; Anderson and Wall 1978; Tyler et al. 1982). Living cysts are usually identified, isolated, or enumerated microscopically after sonication and sieving of whole sediment (e.g. Wall and Dale 1968; Dale 1979; Matsuoka et al. 1989). Because sieved material consists mostly of sediment and de-

tritus, only small volumes can be examined and unless cyst concentrations are high, enumeration and isolation is very time consuming. In addition, it is difficult to state with reasonable certainty that any given area of bottom sediment is free of cysts because present methods limit the amount of sediment examined to substantially <1 ml.

Recently, paralytic shellfish poisoning (PSP) toxins in mussels have been reported from several locations around Newfoundland subsequent to the initial incidence in 1982 (White and White 1985). Because of the growing economic importance of shellfish in the region, a project was initiated to determine the causes of the apparent recent spread of PSP and presumably of *Alexandrium fundyense*, the organism responsible, into the region. Sediment cysts provide a useful record of previous blooms of *A. fundyense*. Cysts are well preserved in sediment, especially anoxic sediment, for long periods; they are easy to identify and culture to assess viability and precise taxonomy; and they do not have to be collected during a bloom as is required of phytoplankton surveys. If sampling locations are selected carefully, cysts provide proof positive of the existence of *A. fundyense* in a general area at some time in the past. It is equally important, however, to be able to verify that no cysts were encountered in the sediments of an area or that the concentrations were extremely low. Cysts from volumes of sediment $\gg 1$ ml must be concentrated for these purposes.

The specific gravities of cysts of different dinoflagellate species vary widely. Even within a single species, densities can range from 1.05 to 1.39 g cm^{-3}, as for example in *Scrippsiella trochoidea* (Anderson et al. 1985). Metrizamide gradients have been used to show that cysts of *A. fundyense* (formerly *Gonyaulax tamarensis*) range in density from ~ 1.15 to 1.30, with a mode near 1.2 g cm^{-3} (Anderson et al. 1985).

The Percoll-seawater density gradient procedure, which has been used to separate live meiofauna and microfauna from sediments (Schwinghamer 1981), is not suitable for dinoflagellate cyst fractionation because the maximum density of the medium is ~ 1.15 g cm^{-3}. Although cysts are routinely encountered in samples that have been separated from sediment with this medium, the method is not quantitative because the heavier cysts remain in the sediment plug. Ludox TM (du Pont Ind. Chem. Div.), with a density of 1.4 g cm^{-3}, has been used as a gradient medium to separate cysts from marine sediments (Blanco 1986), but it is toxic to aquatic organisms and gels on contact with seawater. The metrizamide gradient method used by Anderson et al. (1985) has the correct range of densities for dinoflagellate cysts but is prohibitively expensive for large-scale, repetitive studies. The method described here was developed to examine relatively large volumes of sediment and to overcome problems with existing density-gradient methods.

Sediment was collected from 16-m water depth in Harbour Grace, Conception Bay, Newfoundland, with a 15- × 15-cm Eckman grab. Duplicate 5.5-cm^2 subcores were taken from the surface 3 cm of the muddy silt with a cutoff, disposable 50-ml syringe. Subcore contents were placed in 120-ml, screwcap specimen cups and held at 2°–4°C in the dark before analysis. Sediment was collected from the southern Gulf of Maine (43°00′00.2″N, 70°18′56.6″W) for some of the *A. fundyense* studies.

Before centrifugation 5 ml of sediment were transferred to a 50-ml flat-bottomed, polyethylene cylinder, and 20 ml of filtered seawater were added. The resulting slurry was sonicated for 45 s with a Branson model 250 sonifier, with power set at 4. It was then rinsed sequentially through 80- and 20-μm Nitex nylon sieves with filtered seawater. The material remaining on the 20-μm sieve was washed into a graduated 50-ml centrifuge tube and the volume restored to 5 ml with filtered seawater. This procedure was also used to disaggregate and "clean" cysts (uncentrifuged cysts) for viability tests in comparison with cysts that were subjected to the following additional treatment.

The 5-ml slurry was underlain in the centrifuge tube with a 40-ml, linear density gradient using a 100-ml gradient mixer. For the purpose of this work, gradients were generated individually and delivered to the centrifuge tubes via a single-channel peristaltic pump. The light solution in the mix-

Table 1. Density at 20°C of buffered Nalco 1060/sucrose (Nalco 1060 with 11.23% wt/wt sucrose, 0.025 M Tris/Tris-HCl buffer) mixed in differing proportions with a similarly buffered 22.46% aqueous solution of sucrose.

Proportion of Nalco 1060/sucrose (by volume)	Density (g cm^{-3})
0	1.068
20	1.15
36	1.20
50	1.247
51	1.25
67	1.30
82.5	1.35
100	1.405

ing chamber (20 ml) was 22.46% (wt/vol) sucrose in distilled water, buffered to pH 8.1 with 0.0125 M Tris plus 0.0125 M Tris-HCl (final concn.). The dense mixture in the reservoir chamber (20 ml) was Nalco 1060, a 50% (wt/wt) aqueous suspension of colloidal silica (Nalco Chem. Co.), plus sucrose to a final concentration of 11.23% (wt/wt), buffered to pH 8.1 with 0.0125 M Tris and 0.0125 M Tris-HCl. The gradient produced was therefore isosmotic with seawater of salinity 32‰ (Handbook Chem. Phys. 1968), the salinity that prevails in the mixed, upper water column of Conception Bay to depths well in excess of 16 m. Alternatively, step gradients can be made by mixing the dense mixture with the light solution in various proportions (Table 1).

The filled centrifuge tubes were balanced by adding or removing liquid at the surface with a 50-ml syringe with a canula. Centrifugation was at 3,000 rpm (~1,600 × *g* midway along the tube) for 30 min at 4°C.

Centrifuged material was withdrawn in 5.0-ml aliquots with a threaded conical cap, machined from acrylic fitted to the top of the tube (Fig. 1). The cap was machined with an angle of 50° at the apex. Other dimensions of the cap depend on the type of centrifuge tube used. They are critical only insofar as they allow for tight fit of tubing and threaded joint with the centrifuge tube. The cap was tightly screwed onto the centrifuge tube, taking care not to disturb the gradient. The two sections of flexible tubing for introducing the dense mixture and collecting the gradient fractions were then pushed into their respective openings in the

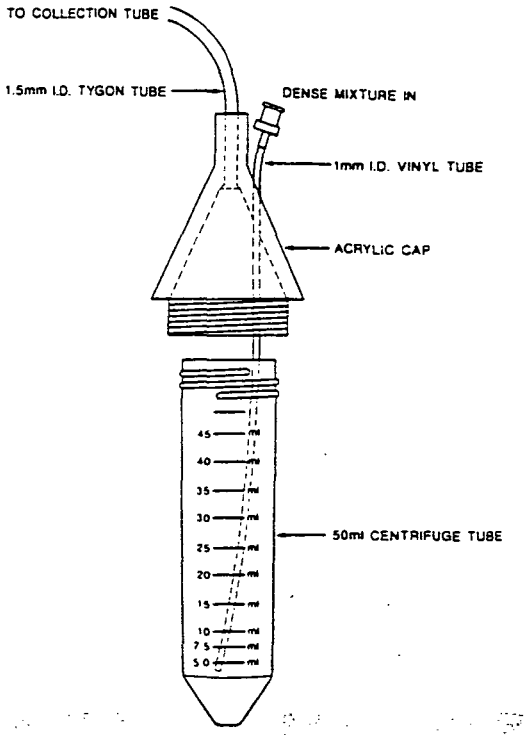

Fig. 1. Conical acrylic cap used to withdraw layers from a density gradient via upward displacement as described in the text. The cap is shown with a 50-ml centrifuge tube.

cap (Fig. 1). The fit of the tubing in the openings in the cap was friction tight for easy emplacement and removal and to prevent leakage.

Each layer was displaced, in turn, upward through the cap and into a length of clear, flexible tubing to a 6.5-ml collection tube by introducing 5.0 ml of the dense mixture at the base of the gradient through canula tubing. The collection tube was then tightly capped. Shear along the side of the centrifuge tube, which would mix material from different density layers, was not noticeable if the displacement mixture was introduced slowly. The fit between the rim of the tube and the conical cap was critical; it had to be tight all around to avoid turbulent mixing as liquid entered the cone. The density of each 5-ml layer was measured gravimetrically with subsamples of precise volume to ensure that the gradients were consistently linear.

Table 2. Numbers of cysts of *Alexandrium fundyense* and *Scrippsiella trochoidea* recovered from different densities after centrifugation in a linear gradient of Nalco 1060 and sucrose as described in the text. Numbers are averages and standard deviations of counts per lengthwise transect of a 0.5-ml Sedgwick-Rafter counting cell under a 25× objective.

Density (g cm $^{-3}$)	*A. fundyense* ($n \pm$ SD)	*S. trochoidea* ($n \pm$ SD)
1.086	0	0
1.131	0.4±0.7	1.4±1.3
1.177	15.8±4.9	28.6±2.5
1.222	23.2±2.6	64.8±9.7
1.268	3.3±2.0	22.0±3.5
1.313	0	0
1.358	0	0
1.404	0	0

Material from each layer was then thoroughly rinsed with filtered seawater onto a 20-μm Nitex sieve. The remaining particles were examined microscopically in Sedgwick-Rafter counting cells of 0.5 or 1.0-ml volume. Uncentrifuged sediment that had been sonicated and sieved was examined in the same way. Cysts of *S. trochoidea* were isolated from the counting cell via a glass micropipette to assess the effects of the procedure on cyst viability. Cysts of *A. fundyense* were too rare in these sediments to allow viability testing. The cysts were placed in 120 μl of f/2 medium (Guillard 1975) in 96-well, half-area, styrene culture plates and incubated at 15°C under ~150 μmol quanta m^{-2} s^{-1} "cool-white" fluorescent illumination on a 14 : 10 L/D cycle. Viability was assessed by observing the percentage of intact cysts remaining at the bottom of the wells after 7 d; viable cysts of these species should have excysted in that time. Differential mortality caused by the centrifugation procedure was determined by comparing the viability of centrifuged cysts with that of uncentrifuged cysts.

After centrifugation, distinct bands of material were observed in some of the density gradients but not in others. Microscopic examination revealed that, although each layer consisted of a mixture of different types of particles, each type occurred unimodally at a particular density. Layers of equal volume were withdrawn from the gradients used in this experiment rather than trying to withdraw distinct bands of material because the latter process would have resulted in layers of variable thickness with no guarantee of clear definition of particle type associated with a particular density.

Table 3. Viability of cysts of *Scrippsiella trochoidea* after 7 d of incubation in f/2 medium at 15°C. Germination of cysts was assumed to have occurred if cysts originally placed in wells were empty, not seen (living or dead), or if vegetative cells were observed in wells after incubation. There is no significant difference ($P > 0.05$) via the binomial test in the fraction germinated.

Treatment	Cysts germinated	Cysts not germinated	Cysts dead
Uncentrifuged	57	4	1
	91.9%	6.5%	1.6%
Centrifuged	73	4	1
	93.6%	5.1%	1.3%

Clear modes of the distribution of cysts of *A. fundyense* and *S. trochoidea* within the gradients occur at a density of 1.222 g cm^{-3}, with lesser numbers of cysts being found in a density range of 1.131–1.268 g cm^{-3} (Table 2). In subsequent separations, a step gradient was used to bracket this range and obviate the need for a linear gradient. We made a step gradient of 1.15, 1.20, 1.25, and 1.30 g cm^{-3} and were able to recover most of the cysts in the 1.20 layer. Detrital material also settled in the density range in which the cysts were found, but mineral sediment sank to the bottom of the gradient. Pipetting the cysts from the centrifuged material into culture plates was simpler than from uncentrifuged, sieved sediment because of the much lower concentration of detritus and silt in the former. This is especially significant since the material in the Sedgwick-Rafter cell represented 5–10 ml of sediment in the case of centrifuged material and only 0.25–0.5 ml of sediment in the case of uncentrifuged material.

There was no indication of differential mortality of cysts of *S. trochoidea* attributable to centrifugation or contact with the Nalco 1060 during separation. Cysts were >90% viable whether they were pipetted directly from sonicated, sieved sediment or subjected to the centrifugation procedure (Table 3). Unlike Blanco's (1986) method with the toxic colloidal silica, Ludox TM, in distilled water, our method controls toxic and osmotic effects of the density gradient

separation on cysts and also on other organisms living in the sediment. Yet it allows separation of particles with densities considerably greater than 1.15 g cm^{-3}—the maximum that Schwinghamer's (1981) Percoll method will efficiently separate. In addition, Nalco 1060 is a relatively inexpensive chemical; Percoll, like metrizamide, is too expensive to use routinely for extensive surveys.

The proposed method is simple, there is no noticeable mortality, the materials required are relatively inexpensive, and the time required to examine sediments, especially those with low cyst concentrations or high silt content, is less than with other methods. Our experience leads us to recommend that the proposed step-gradient method replace the standard methods of enumerating cysts in sonicated, sieved sediment (Anderson et al. 1982; Tyler et al. 1982). Furthermore, this method allows processing of samples in batches with increased efficiency using an inverted microscope and plankton counting chambers rather than a Sedgwick-Rafter slide.

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