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Particle tracking in a salinity gradient: A method for measuring sinking rate of individual phytoplankton in the laboratory

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Abstract

This paper presents a new method to measure the sinking rates of individual phytoplankton "particles" (cells, chains, colonies, and aggregates) in the laboratory. Conventional particle tracking and high resolution video imaging were used to measure particle sinking rates and particle size. The stabilizing force of a very mild linear salinity gradient (1 ppt over 15 cm) prevented the formation of convection currents in the laboratory settling chamber. Whereas bulk settling methods such as SETCOL provide a single value of sinking rate for a population, this method allows the measurement of sinking rate and particle size for a large number of individual particles or phytoplankton within a population. The method has applications where sinking rates vary within a population, or where sinking rate-size relationships are important. Preliminary data from experiments with both laboratory and field samples of marine phytoplankton are presented here to illustrate the use of the technique, its applications, and limitations. Whereas this paper deals only with sinking phytoplankton, the method is equally valid for positively buoyant species, as well as nonbiological particles.

Phytoplankton sinking and ascent rates can affect basic ecosystem dynamics and interspecies competition in both marine and freshwater systems (Harrison et al. 1986; Oliver and Ganf 2000; Huisman and Sommeijer 2002; O'Brien et al. 2003). However, these rates can vary both within and between species over short- and long-time scales (Smayda 1970; Ibelings et al. 1991; Brookes et al. 1999). Theoretical calculations of sinking and rising velocities of phytoplankton and sediment particles are generally impractical, due to variations in the size, shape, porosity, mucilage coatings, and density. The relationship between size and sinking (or ascent) rate can also vary widely for both phytoplankton and sediments, and this relationship is critical in the development of aggregation models (e.g., Jackson and Lochmann 1998). Hence, there is a need for accurate measurements of the distribution of individual sinking and ascent rates for populations of aquatic particles, including phytoplankton.

Bulk settling techniques such as MARS (Rothwell and Bienfang 1978) and SETCOL (Bienfang 1981) provide a good estimate of the mean settling rate for a population. The SETCOL technique has become most broadly used, due to its simplicity and practicality in the field and laboratory. Bulk settling measurements for a number of size distributions within a population of sediments can be determined using the LISTT-100 (Agrawal and Pottsmith 2000; Mikkelsen 2002). However, none of these methods directly measure sinking or rising rates of individual particles within the population.

Sinking and ascent rates of a small number of individual flocs and aggregates have been determined from visual tracking in situ by divers (Alldredge and Gotschalk 1988; Alldredge and Gotschalk 1989), and in laboratory observations of very large phytoplankton cells collected manually by divers (Villareal 1988). Interference caused by convection currents in settling chambers has been the major obstacle to using particle tracking to determine sinking/ascent rates of individual particles within large populations. Walsby and Holland (2006) used laser scanning to measure mean sinking rates for populations of phytoplankton and inert particles, suppressing convection with a vertical Percoll density gradient. However, the method was unable to resolve the sinking rates of individual particles

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within a population. Size-specific sinking rates can be generated for small particles, down to 4–5 μm, by coupling the SETCOL method with electronic particle counting (Waite et al. 1992, 1997). However, the well-quantified limitations of electronic particle counting mean that such methods cannot measure the size of even the smallest clusters.

This paper presents a non-invasive method for the measurement of size and sinking /rising velocities of phytoplankton and other aquatic particles. A mild linear salinity gradient (1 ppt over 15 cm) was used to stabilize the water column in a small laboratory "settling tank." This density gradient was sufficient to inhibit convection currents but too small to affect cell physiology or velocity over the duration of the experiments. Particles were videotaped as they sank through a vertical halogen light sheet in the settling tank. Images of the particles were captured on Sony Hi-8 videotapes, digitized, and analyzed using a particle tracking method to calculate individual particle velocity and size.

The method was applied to laboratory and field samples of marine diatoms. The results show the strength of this method in differentiating phytoplankton populations in the field with very different size/sinking characteristics. This method can be used to measure the sinking or ascent rate of cells, chains, colonies, aggregates, or nonbiological particles. For simplicity, all of these forms are referred to generically here as "particles" and negative buoyancy is assumed.

Materials and procedures

Laboratory methods—The experiments were conducted in a room kept at constant temperature. All water, samples, and equipment were kept at the same temperature overnight prior to the experiment to prevent small temperature variations from causing convection currents in the settling tank.

The camera and light source were assembled prior to filling the settling tank (Fig. 1). The camera was a SONY XC-8500CE Progressive Scan B/W CCD Camera Module (shutter speed = off) with a Mitutoyo M Plan Apo 20x microscope objective (FOV 1.5 mm). The light bank consisted of three 50W halogen bulbs. The light from these bulbs passed through two sequential parallel slots (5–10 mm in width) in aluminum faceplates, creating a thin, vertical light sheet in the settling tank. A cooling fan was attached to the light bank to prevent overheating of the lights, which could potentially create convection currents in the tanks. The calibration ruler was placed in the settling tank temporarily prior to filling, perpendicular to the camera lens. The light sheet was positioned to illuminate the increments on the ruler. This was the only time the light sheet was turned on prior to the start of filming. The camera was leveled on all planes using a spirit level. Throughout the experiment, the settling tank, lights, and camera were not moved from this initial position.

The two filler tanks were filled with media, filtered seawater, or sample. Salinity in the primary filler tank was diluted by 2 ppt with distilled water. Both filler tanks were placed on

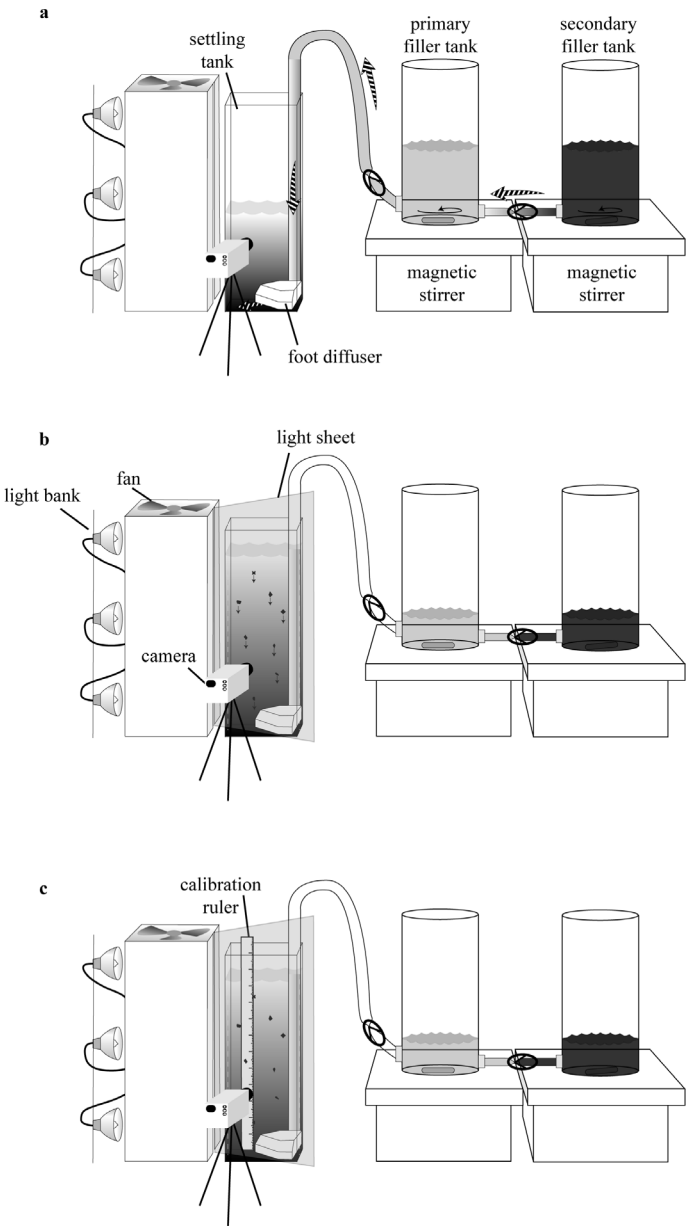

Fig. 1. (a) Filling the settling tank. A linear salinity gradient was set up in the settling tank (60 × 60 × 180 mm Perspex). Water entered the tank horizontally through a foot diffuser, which minimized any disturbance to the stratification. The primary filler tank was connected to the foot diffuser with plastic tubing (internal dia of 5 mm). The initial density of the water in the primary tank was approximately 2 ppt less than in the secondary tank. The filler tanks were connected by plastic tubing. Particles were either included in the filler tanks or added to the settling tank once it had been filled. (b) Capturing images. A video camera was used to capture images of particles sinking in a vertical light sheet. The salinity gradient in the settling rate tank prevented interference from convection currents. The light bank consisted of three 50W halogen lamps. Vertical slots in two sequential parallel faceplates channeled the light into a narrow vertical sheet. The lamps were cooled by a small rotary fan. (c) Calibration image. Once the experiment was complete, a ruler was inserted in the tank and filmed for calibration purposes.

magnetic stirrers at the same horizontal level (Fig. 1a). Filling of the settling tank then commenced, using the "two-tank" method (Hill 2002) to create a linear density gradient. The valve between the primary and secondary filler tanks was opened, so that as water flowed out of the primary tank, heavier water was drawn in from the secondary tank and mixed by the magnetic stirrer. This caused the density of water in the primary filler tank to increase over time and produced a linear salinity gradient in the settling tank, stabilizing the water column. Hence convection currents, which generally occur in small laboratory vessels as a result of small temperature fluctuations, were prevented.

Water entered the settling tank through a "foot diffuser" fixed to the bottom of the tank. This device forced the water to flow out horizontally and so minimized vertical mixing, which could potentially break down the stratification. The rate of filling was also controlled to prevent mixing. Typical filling times in these experiments were approximately 45 min. The final salinity difference in the settling tank was approximately 1 ppt over a depth of 15 cm.

Some trial and error was required to determine whether particles were added once the settling tank was filled or were included in the filler tanks prior to filling. For cases where the sample was quite dilute, e.g., laboratory or field samples of phytoplankton, best results were obtained when the sample was placed in the filler tanks. However, this was only feasible for slow-sinking particles. If the particles sank quickly compared to the filling time, the sample was added to the settling tank after it was filled. This was done using a syringe to place single drops of the particle sample on the back of a spoon or spatula at the water surface. The initial force of entry of the sample into the tank caused slight mixing at the surface. Positioning the video positioned 1 to 2 cm from the top of the settling tank avoided capturing any effects of this disturbance.

When the settling tank was full and contained particles, the room was darkened to increase the contrast between the particles and the background. The halogen lights were turned on and filming commenced (Fig. 1b). After 1 to 2 h filming, the calibration ruler was inserted in the light sheet (Fig. 1c). This was done only at the end of the experiment because it disrupted the stratification and affected particle motion.

Calculation of sinking rate and size of particles—Individual images were captured from the video footage using a frame grabber and "cleaned" to remove background noise. From the images of the ruler, the calibration coefficients (mm/pixel) were determined in the horizontal and vertical directions.

Up to 20 sequential images separated by a fixed time interval were then added together to enable particle tracking. The trajectories of individual particles were identified manually. In each experiment, the absence of convection was verified by checking that all particle trajectories were vertical. Any horizontal motion would indicate the presence of convection currents.

Each trajectory involved anywhere from 4 to 20 observations of the same particle. Each of these observations and the direction of travel were identified manually. A semi-automated program calculated the average surface area and velocity of the particle, converting from pixels to millimeters using the calibration coefficients. The velocity was determined from the distance traveled by the particle during the time interval between each observation. Surface area was converted to equivalent spherical diameter. This process was repeated using different time intervals to cover the range of sinking rates in the population of particles. While this particle-tracking process was semi-automated, it can be improved through greater automation (e.g., Neumann 2004).

Small changes of horizontal position within the light sheet, and reflections or glare can cause uncertainty in the measurement of particle surface area and position. Particle tracking allowed multiple observations of individual particles to reduce this error. The number of particles measured in each experiment depended on the concentration of particles in the sample, the range of sinking rates within the population, and image quality.

Assessment

Water column stability—The sinking rates of many phytoplankton species can be very low (e.g., 0.1 m d^{-1}; Waite et al. 1997), and hence even very small convection currents in the settling tank can prevent accurate measurement of sinking rate. The success of this method relies on the stabilization of the settling tank water column by density stratification. This stability was tested using dye.

A drop of food coloring was added to one of the filler tanks when the settling tank was half full. This did not affect the density but created a distinct front within the settling tank. The lights and fans were set up to recreate the exact experimental conditions, and the initial position of the front marked on the tank. The position of the front was checked every 30 min. After 4 h, no movement in the front was detected. Since the sinking rate experiments were up to 2 h in duration, this confirms that particle settling was free from the interference of advection during the experiments.

Impact of density gradient on sinking rates—There are two mechanisms by which the density gradient in the settling tank could potentially affect the sinking rate of phytoplankton: physiological changes and the effect of fluid density on sinking rate. First, the salinity difference of 1 ppt over 15 cm was too small for physiological processes to affect phytoplankton sinking rates during the experiments (Bienfang and Szyper 1982). Second, the salinity difference equated to a density difference of 0.8 kgm^{-3} over the depth of the settling tank (5-30°C, Fofonoff 1985) and 0.008 kgm^{-3}over the 1.5 mm field of view. Typical phytoplankton cells have a density at least 20 kgm^{-3} greater than the surrounding water, and hence their sinking rates will be unaffected by the density gradient in the settling tank (Reynolds 1984; Villareal 1988). However, the density of some species (such as buoyancy-regulating *Microcystis aeruginosa*) can be very close to that of surrounding water, and

hence sinking/ascent rates may be affected by even small density gradients. The density profile in the settling tank can be determined from the initial and final temperature and salinity of filler tanks (Fofonoff 1985; Hill 2002). The effect of the density gradient on the measured sinking/ascent rate can thus be determined for particles of near-neutral buoyancy. Furthermore, measurement of the sinking/ascent rates of phytoplankton at different depths in the settling tank (i.e., in two different, known densities) may be used to determine the density of individual phytoplankton particles.

Measurement of sinking rate in laboratory cultures and field samples—The purpose of these experiments was to assess the ability of particle tracking in the settling tank (VIDEO method) to provide size-sinking rate data. The VIDEO method was also compared with SETCOL, a commonly used method for determining bulk settling rate (Bienfang 1981). Both methods were applied to marine phytoplankton from laboratory culture and from field samples.

While the VIDEO method measured the sinking rate of individual phytoplankton "particles," the SETCOL method measured the average sinking rate of phytoplankton biomass. A homogeneous sample of phytoplankton was added to a SETCOL settling column, of known height, at time zero. The vertical distribution of phytoplankton was initially uniform. The change in this vertical distribution over a fixed time interval, as measured by the change in biomass at the bottom of the column, was used to determine an average sinking rate of the population.

Trial samples were taken from cultures of *Skeletonema costatum*, isolate CS-167, (isolated by J.L. Stauber, South Australia, 1983) from the CSIRO culture collection in Hobart Tasmania. *S. costatum* was grown on F/2 medium. When the culture was in log phase, particle size and sinking rate were measured with the VIDEO technique, and bulk settling rate was measured with SETCOL. Due to the relatively low concentration of chains and the low sinking rate, the culture was used to fill the settling tank, rather than added afterward.

The VIDEO and SETCOL methods were also applied to two sets of field samples collected as part of a large field study in the Gullmarfjord, Sweden (Waite et al. 2005). The first of these consisted of 250 mL surface water. Due to the relatively low concentration and low sinking rate of particles, the sample was used to fill the settling tank, rather than added afterward. The second sample type was a > 90 μm plankton net haul taken in the center of the fjord. The diatom population > 90 μm was composed almost entirely of the large diatoms *Coscinodiscus concinnus* and *C. radiatus* (200–500 μm). Although *Coscinodiscus* spp. were of relatively low concentration in the sample, the sample was added to the settling tank after it had been filled due to the rapid transit times of the cells.

The size versus sinking rate relationships measured by the VIDEO method were quantified for each of these populations using the R^2 value calculated in Microsoft Excel. The significance of this relationship was tested using ANOVA (p values),

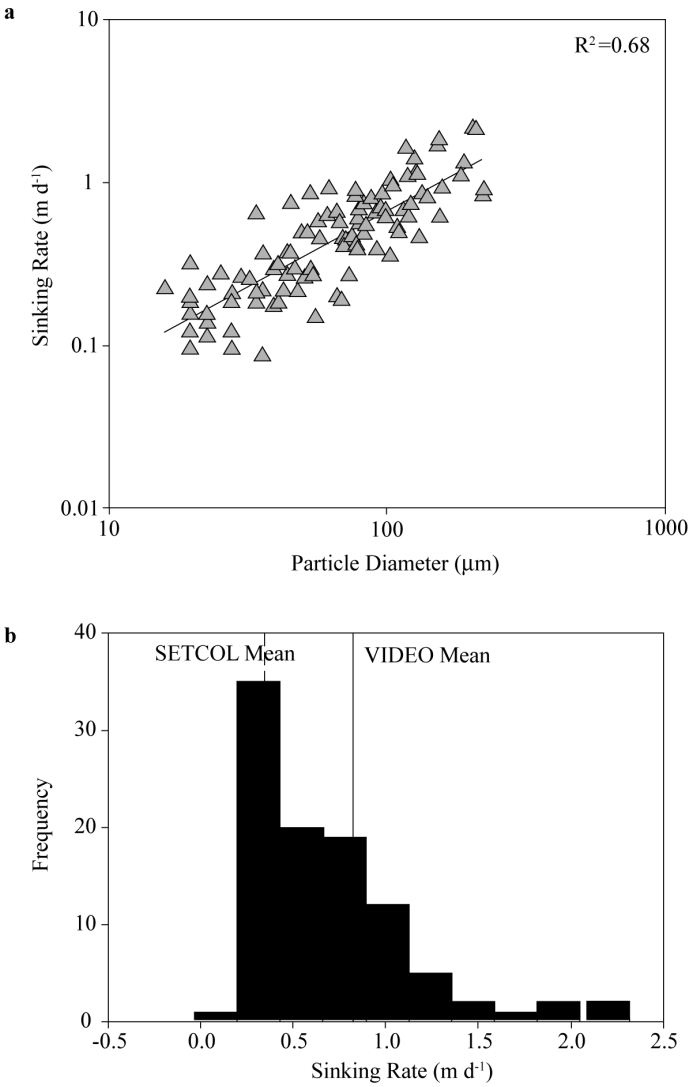

Fig. 2. (a) Size versus sinking rate data from VIDEO measurement of a uniform laboratory culture of *Skeletonema costatum*. Each point represents measurement of a single cell or (very short) chain. (b) Histogram of sinking rate measured using the VIDEO method, for the same laboratory culture of *S. costatum*. The mean sinking rates obtained by both the SETCOL and VIDEO methods are indicated.

also in Excel. The mean sinking rate measured by the VIDEO and SETCOL methods were compared using a t test.

Cells of the laboratory culture of *Skeletonema costatum* formed only very short chains in culture and averaged about 10 μm in diameter for an individual cell, such that the size of all the particles lay between 10 and 200 μm (Fig. 2a, 2b). There was almost always a highly significant relationship ($p < 0.001$) between *S. costatum* particle size and sinking rate (a typical VIDEO experimental outcome is shown in Fig. 2) and particle size described over 68% of the variance in particle sinking rate. This outcome is consistent with the assumption that particle shape, cell growth rate, and physiological state are all relatively constant under well-controlled laboratory conditions. It

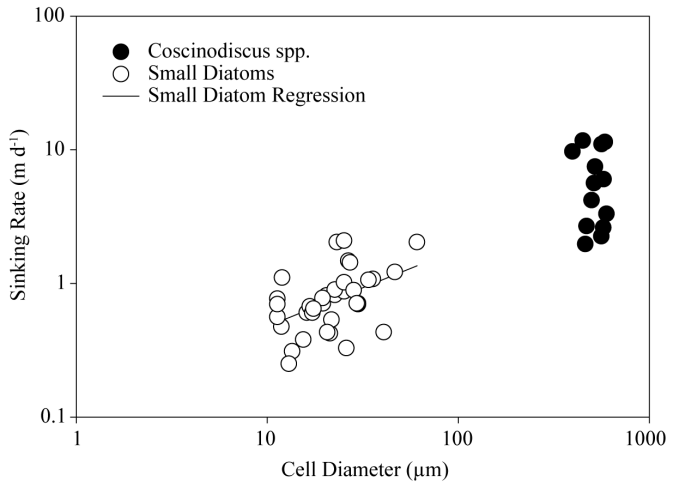

Fig. 3. Size versus sinking rate data from VIDEO measurement of a mixed assemblage of diatoms collected in the Gullmarfjord, Sweden: ○ small diatoms collected by Niskin bottle; ● population of large cohabiting *Coscinodiscus concinnus* and *Coscinodiscus centralis* cells collected via 90 μm net haul.

is also consistent with the hypothesis that the sinking rates of small diatom cells are highly dependent on cell size in comparison with the sinking rates of large cells (Waite et al. 1997).

The field samples illustrated the different relationships between size and sinking rates for different organisms. Particle size accounted for about 25% of the variance in sinking rate of the smaller (mostly *Chaetoceros* spp.) diatoms (Fig. 3; $p < 0.01$). In contrast, there was no significant relationship between cell size and cell sinking rate for the larger diatoms, *Coscinodiscus* spp. (Fig. 3, $p > 0.05$).

The average VIDEO sinking rate was higher than the SETCOL value for all cases, and this difference was statistically significant in two cases (Table 1). However, a simple comparison of means, in this case, can be misleading. The VIDEO mean is an average of velocities of individual particles, regardless of the biomass contained within individual particles. By contrast, the SETCOL sinking rate is a measure of the average sinking rate of biomass. Each of the methods is subject to different errors and limitations. The difference in the nature of these two measurements also makes the application of statistical tests problematic. For example, in Table 1, two SETCOL experiments are treated as a sample size of two, although the results of those experiments represent the pooling of the sinking rates of a large number of particles.

The SETCOL method can underestimate the sinking rates of the largest cells if there is wide variation in sinking rate of particles within the population. For example, the range of *Coscinodiscus* spp. sinking rates measured by the VIDEO method were simply not detectable in the SETCOL experiment. For the length of column used (0.5 m), the range of cell sinking rates is beyond the resolution of a single SETCOL run. Multiple experiments would have to be performed over varying lengths of time. A SETCOL experiment using a longer column or shorter timeframe would, in theory, have been able to measure the sinking rate of the very fastest particles. However, the small number of particles actually sinking at this rate might easily be missed. In addition, some prior knowledge of the sinking rates to be measured would be needed to determine the appropriate time frames for this experiment.

While the VIDEO method provides good information about the range of sinking rates within a population, an accurate population mean can only be determined if the distribution of sinking rates measured is truly representative of the population. For example, if the time steps used in the VIDEO analysis are biased toward the observation of faster particles, then the average sinking/ascent rate of the population will be overestimated. This could be rectified by a rigorous technique to ensure that the measurements are an accurate representation of the entire population. For sediment samples, this would be relatively straightforward, because particles can be added to the settling tank in high concentrations and velocity determined for a very large number of individual particles. However, the low density of phytoplankton samples, particularly field samples, means that only relatively small number of particles can be tracked (e.g., Table 1). In those cases, while the VIDEO method will provide information about the range of sinking rates and their relationship with size, the mean VIDEO sinking/ascent rate will not accurately represent the population mean.

Discussion

This study has shown that particle tracking in a tank with a mild salinity gradient can be used to determine size-sinking rate data for phytoplankton and potentially other particles. The mild salinity gradient suppressed convection and allowed measurement of very low sinking rates (~0.1 md^{-1}, i.e., 10^{-6} ms^{-1}). Sinking rates ranging across an order of magnitude were measured within a single population, and even greater ranges are within the scope of the VIDEO method. The method is also

Table 1. Summary of laboratory and field measurements using the new VIDEO method and bulk SETCOL measurements

Sample	SETCOL sinking rate (SD) md^{-1}	VIDEO sinking rate (SD) md^{-1}	Statistical significance of difference between SETCOL and VIDEO sinking rate
Skeletonema costatum laboratory culture	0.33 (0.24) n = 2 columns	0.57 (0.43) n = 99 particles	$p < 0.05$
Small diatoms Gullmarfjord	0.31 (0.35) n = 10 columns	0.85 (0.59) n = 12 particles	$p < 0.005$
Coscinodiscus spp. Gullmarfjord	1.90 (0.76) n = 10 columns	6.16 (3.72) n = 13 particles	Not significant

able to detect very large particles, such as aggregates, beyond the resolution of bulk settling measurements. It is impossible to gain the same data from bulk settling measurements.

An accurate estimate of mean settling rate is difficult to obtain from the VIDEO method alone, however. The most accurate way to determine mean sinking rate for a population would be to use the laser scanning method of Walsby and Holland (2006). In the absence of such specialized equipment, the VIDEO method could be used to determine the range of sinking rates of the population, and this information could be used to design multiple SETCOL runs to determine a bulk settling rate.

The information generated by the VIDEO method has a number of important applications. A few very large aggregates or colonies can contain a large proportion of a population's biomass and move at very high speeds relative to the rest of the population (e.g., O'Brien et al. 2004). Failure to identify such particles can lead to significant errors in estimates of export fluxes and in predictions of ecosystem dynamics (Waite et al. 2005). This method provides unprecedented levels of information about sinking rate-size relationship. These relationships are critical in the development of accurate models of aggregation (e.g., Jackson and Lochmann 1998). This method also provides detailed information about the distribution of sinking/ascent rates within a population, which affects the vertical distribution of phytoplankton in the water column (O'Brien 2002), as well as collision rates for aggregation processes (e.g., Jackson and Lochmann 1998).

The still water sinking/ascent rates measured here capture the fundamental gravitational settling/rising processes, which will affect phytoplankton velocity in all environments, including the turbulent conditions to which phytoplankton are routinely exposed. Theoretical analysis suggests that the intrinsic sinking/ascent rates of phytoplankton will unaffected by turbulent mixing at the intensities experienced in lakes, estuaries, and open ocean (Maxey 1987; Wang and Stock 1993; O'Brien 2002). In that case, the velocity of phytoplankton particles in any environment will be the sum of the intrinsic sinking/ascent rate, and the instantaneous velocity of the surrounding fluid. In contrast, Ruiz et al. (2004) found that the sinking/ascent rates of small particles increased in laboratory-generated turbulence. However, Ruiz et al. (2004) were unable to establish a quantitative prediction for particle velocity as a function of turbulence characteristics. Regardless of the effect of turbulence, the measured still-water sinking/ascent rate remains a fundamental parameter for quantifying the movement of phytoplankton in all environments.

Laboratory measurements of the sinking rates of large flocs, such as marine snow, can overestimate their true sinking rates in situ (Alldredge and Gotschalk 1988). This is because large aggregates can be very fragile and may be damaged in the process of collection and addition to the settling tank (e.g., O'Brien et al. 2004). Collection and handling can also affect the porosity and density of such particles. Hence, the velocities measured by the VIDEO method may not accurately represent sinking rates in situ for fragile particles. The effect of particle capture and handling on size may be quantified by comparing particle size measured before collection (e.g., using underwater cameras) with the particle size measured in the settling tank.

Comments and recommendations

Where many small cells must be quantified in a uniform culture, bulk methods such as SETCOL can yield good measurements more quickly and at lower cost than the VIDEO method. However, if significant variability is present within the population, the higher resolution data yielded by our technique becomes important. In particular, any aggregation or heterogeneity that increases the maximum sinking/ascent rate of the population of interest makes the VIDEO method preferable.

Whereas the VIDEO method has been applied here only to negatively buoyant marine phytoplankton, it could be applied to positively buoyant or freshwater phytoplankton, or other particles, such as sediments. Variations to the experimental procedure can accommodate these cases. For example, if using freshwater phytoplankton, the density gradient will be created by adding 2 ppt salt to the secondary filler tank, rather than diluting the primary tank. The settling tank can be filled from the surface rather than the bottom by attaching foam to the diffuser foot and placing the heavier water fraction in the primary tank rather than the secondary tank. Positively buoyant particles can be added either through the filler tanks or injected horizontally at the base of the settling tank. Since small deviations in methodology could result in convection currents in the settling tank, the absence of such currents should be verified in all applications of this method.

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