Video Article

Determination of the Settling Rate of Clay/Cyanobacterial Floccules

Tiffany Playter[1], Kurt Konhauser[1], George W. Owttrim[2], Denise S. Whitford[2], Tyler Warchola[1], Cheryl Hodgson[1,3], Aleksandra M. Mloszewska[4], Bruce Sutherland[1], J.-P. Zonneveld[1], S. George Pemberton[1], Murray K. Gingras[1]

[1]Department of Earth and Atmospheric Sciences, University of Alberta

[2]Department of Biological Sciences, University of Alberta

[3]Department of Earth Sciences, Simon Fraser University

[4]Earth Sciences Department, University of Toronto

Correspondence to: Tiffany Playter at tdavies@ualberta.ca

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Abstract

The mechanisms underpinning the deposition of fine-grained, organic-rich sediments are still largely debated. Specifically, the impact of the interaction of clay particles with reactive, planktonic cyanobacterial cells to the sedimentary record is under studied. This interaction is a potentially major contributor to shale depositional models. Within a lab setting, the flocculation and sedimentation rates of these materials can be examined and measured in a controlled environment. Here, we detail a protocol for measuring the sedimentation rate of cyanobacterial/clay mixtures. This methodology is demonstrated through the description of two sample experiments: the first uses kaolin (a dehydrated form of kaolinite) and *Synechococcus* sp. PCC 7002 (a marine coccoid cyanobacteria), and the second uses kaolin and *Synechocystis* sp. PCC 6803 (a freshwater coccoid cyanobacteria). Cyanobacterial cultures are mixed with varying amounts of clay within a specially designed tank apparatus optimized to allow continuous, real-time video and photographic recording. The sampling procedures are detailed as well as a post-collection protocol for precise measurement of chlorophyll *a* from which the concentration of cyanobacterial cells remaining in suspension can be determined. Through experimental replication, a profile is constructed that displays sedimentation rate.

Video Link

The video component of this article can be found at https://www.jove.com/video/57176/

Introduction

Using present environmental conditions and processes to infer past depositional mechanisms has long been an underpinning of sedimentology. While modern depositional analogues, such as the Black Sea, have been used to understand the deposition of organic-rich, fine-grained deposits, laboratory experiments have the potential to shed additional light on the origin of shale deposits. One area of inquiry in the genesis of black shales is the deposition rate and mechanism of original formation. Traditionally, it has been hypothesized that black shales formed in environments where the sedimentation rate, primary productivity, and organic matter respiration rates promote the preservation of organic matter in the sediment[1,2,3]. However, the role of cyanobacterial and clay flocculation has largely remained unconsidered. This mechanism of flocculation would allow for rapid deposition of organic-rich, fine-grained sediments to occur, and does not necessitate low-oxygen. Considering this premise, this protocol has two goals: 1) measure the sedimentation rate of cyanobacterial/clay floccules, and 2) visualize the sedimentation process in real time. This methodology, in addition to geochemical analysis, has been used to demonstrate that cyanobacterial/clay flocculation may in fact be an important mechanism for shale formation[1]. While originally intended for modelling shale deposition, this method is applicable to other disciplines such as biology and environmental remediation where the influence of clay input on bacterial metabolism and population need to be measured.

Numerous studies have been conducted to observe the flocculation of cyanobacteria and clay, for mitigating harmful algal blooms[2,3,4,5,6,7,8,9,10,11,12]. However, while measuring cell concentration over time, these studies have not applied cyanobacteria/clay flocculation to modelling the deposition of the rock record. As such, these studies lack a visual component, which can be critical when modelling past sedimentological processes. Additionally, the majority of studies utilize cell-counting (*e.g.,* Pan *et al.*[11]), which can be laborious. Our method, with recent advances in measuring cyanobacterial flocculation, determines the changes in cyanobacterial cell concentration by measuring chlorophyll *a* (Chl *a*) at discrete time intervals. Pairing Chl *a* measurement with visual data is a new approach, which can be used to infer depositional conditions. The images generated can also be used to calculate sedimentation rate after the work from Du *et al.*[13]. The combination of visual and numerical data strengthens the reliability of the results. Furthermore, we outline additional protocols allowing for the sedimentation of dead biomass and clay to also be observed. This is important when considering past sedimentological environments, where live and dead biomass

may have co-occurred. Differences in the behavior of dead biomass during the flocculation (for example, decrease in flocculation rate) would potentially have sedimentological implications.

Protocol

1. Preparing Cyanobacterial Cultures

1. **Preparing inoculation cultures using solid media**
    1. Obtain axenic cyanobacterial cells from the American Type Culture Collection or Pasteur Culture Collection. For example, the unicellular, marine *Synechococcus* sp. PCC 7002 was obtained from the Pasteur Culture Collection, it will be referred to hereafter as *Synechococcus*.
    2. Maintain *Synechococcus* cells on plates containing solid media (A+ liquid media[14] and 1.5% agar[15]). These will be referred to hereafter as inoculation cultures.
    3. Incubate the plates at 32 ± 2 °C with continuous light provided at 30–50 µmol photons m^{-2} s^{-1}[1,15,18].
    4. Repeat steps 1.1–1.3 and replace A+ media with BG-11[16] in step 1.1.2 for freshwater cyanobacterial species, such as *Synechocystis* sp. PCC 6803[16,17], referred to hereafter as *Synechocystis*. Refer to **Tables 1–4** for the composition of A+ and BG-11 media.

2. **Preparing liquid cultures: Each tank experiment requires one 400 mL cyanobacterial culture.**
    1. Collect one 250 mL and two 1 L heat-resistant Erlenmeyer flask.
    2. Rinse each flask with a 4 M hydrochloric acid (HCl) solution (30 mL of HCl solution for each flask), followed by distilled water. Caution: Hydrochloric acid is corrosive and toxic. Ensure that a lab coat, safety goggles, and acid-resistant gloves are worn while using the 4 M HCl solution. Perform this step in a laboratory sink and follow local regulations regarding the disposal of the 4 M HCl solution
    3. Fill the 250 mL flask with 50 mL of liquid media (A+ or BG-11). Fill one 1 L flask with 400 mL of liquid media (A+ or BG-11) and the other 1 L flask with 600 mL of media (A+ or BG-11).
    4. Seal each flask with a gas permeable foam stopper and cover the foam stopper and flask neck with tinfoil.
    5. Label and autoclave the flasks at 121 °C and 100 kPa for 25 min. Allow the flasks to cool to room temperature.
    6. Prepare a laminar flow hood by sterilizing the surface with 70% alcohol.
    7. Collect a wire inoculation loop, a Bunsen burner, the cooled 50 mL liquid media flask (from step 1.2.5), and the inoculation culture (from step 1.1). Place these items in the sterilized flow hood.
    8. Sterilize the wire inoculation loop briefly using the flame of a Bunsen burner and allow it to cool down.
    9. Drag the cool loop along the surface of the inoculation culture to collect cyanobacterial cells.
    10. Remove the foam stopper and foil cap from the 250 mL flask (without touching the foam stopper) and sterilize the rim of the Erlenmeyer flask by passing it briefly through the flame.
    11. Tilt the flask so the media is near the neck of the flask and insert the inoculation loop coated with cells into the flask. Once the loop is submerged into the media, stir the inoculation loop to remove the cells.
    12. Remove the inoculation loop and re-sterilize the lip of the flask using the Bunsen burner flame.
    13. Replace the foam stopper and foil cap onto the Erlenmeyer flask (without touching the foam stopper).
    14. Sterilize the inoculation loop briefly within the Bunsen burner flame.
    15. Prepare a growth room (referred to as the growth chamber) where the temperature is maintained at 30 °C[15,18] and light intensity is 30–50 µmol photons m^{-2} s^{-1}[15,18]. Humidity is not actively controlled.
    16. Allow the inoculated 50 mL culture to incubate by shaking at 150 rpm in the growth chamber, with a maintained temperature of 30 °C. Keep the mouth of the flask covered during the agitation to control evaporative losses and prevent contamination.
    17. Allow the cyanobacterial culture(s) to grow for 96 h. A successful culture should appear bright green and have an optical density (OD) at 750 nm of 0.4–0.6.
    18. Once the culture has grown sufficiently, place the 50 mL culture and the 1 L flask containing 400 mL of liquid media (from step 1.2.5) in the laminar flow hood. Ensure the flow hood is sterilized following step 1.2.6.
    19. Repeat step 1.2.10 for both flasks within a laminar flow hood.
    20. Pour the 50 mL culture into the 400 mL of media in the 1 L flask and re-sterilize the lip of the 1 L flask using the Bunsen burner flame.
    21. In the place of the foam stopper and foil cap, attach a sterile bubbling apparatus consisting of a foam stopper, pipette, plastic tubing, in-line filter, and foil cover to the mouth of the flask.
    22. Agitate the 1 L flask at 150 rpm at 30 °C with the bubbler apparatus attached to an air pump, which circulates a humidified air mixture through the solution[15,18] at 300 mL/min.
    23. Allow the culture(s) to grow within the growth chamber at 30 °C for 120–168 h. A successful culture should appear bright green and have an $OD_{750\ nm}$ of 0.4–0.6.
    24. Repeat step 1.2 to produce a control culture, which will not be mixed with the clay.
    25. Experiments using dead biomass require an additional step. Autoclave the 1 L flask culture for 60 min at 121 °C and allow it to cool to room temperature.

2. Experimental Set Up

1. Place a rectangular acrylic tank (length of 20 cm, width of 5.1 cm, and height of 30 cm) in front of a bank of fluorescent lights (nine T8 bulbs, at least 2,600 lumens; **Figure 1**)[19].
2. Place a translucent, white plastic sheet between the bank of lights and the tank to diffuse the light, which shines through the tank. This set-up was adapted from Sutherland *et al.*[19]
3. Mark the acrylic tank at a height of 10 cm, measured vertically from the base. All measurements should be done at this height in the water column to ensure the consistency of all sampling.

4. Place a camera on a tripod, 1 m in front of the tank to record each experiment. A video camera is recommended as images can be extracted from video files, and by using mathematical modeling software, video files can be used to model settling dynamics[19].
5. Place a black cloth over the light bank, tank, and camera to shield the experiment from outside light sources (**Figure 1**).

3. Flocculation Experimental Protocol

1. Collect the 400 mL culture (step 1.2), a large graduated cylinder, and 600 mL of extra growth media in a 1 L flask (step 1.2.5).
2. Dilute the 400 mL culture (initial cell concentration of 4–6 mg/mL) with additional growth media (A+ or BG-11) to final volume of 1 L using the graduated cylinder. Add this solution to the acrylic tank.
3. Prepare 2 mL microfuge tubes by labeling and placing them in a convenient location near the acrylic tank. Measure 50 g of clay for the experiment.
4. Begin video recording. In the case of multiple experiments, a sign with the experiment identifier can be temporarily held before the camera.
5. Using a pipette, take a 1 mL sample and place it in a labeled microfuge tube. Use this sample to determine the initial cyanobacterial cell count by measuring the $OD_{750\ nm}$ values. Take all samples in triplicate to ensure accurate results.
6. Quickly pour the clay (50 g) into the tank with vigorous agitation using a stirring stick for 10–15 s.
7. Start the timer and take the initial 1 mL sample for Chl *a* determination using a P1000 pipette (sample $Time_0$).
8. Take additional samples at appropriate time intervals (*e.g.*, 1 min, 2 min, 3 min, 4 min, 5 min, 10 min, 15 min, 30 min, 60 min, 180 min, and 240 min).
9. Once the experiment is finished, save the video file, turn off the video camera, and process all samples for Chl *a* determination (step 4).
10. Autoclave the remaining clay/cyanobacterial solution. Depending on cyanobacterial species, local regulations, and lab policy, dispose of the solution using appropriate methods. Clean the tank with soap and water, rinse it with distilled water, and air dry it.
11. Prepare a control experiment with no clay added. Take samples at the same time points. This data can be compared to the other experimental data to confirm that settling rates are enhanced due to the flocculation with clay, not a result of natural settling.
12. If dead biomass is used during the experiment, use the same experimental procedure. However, the disposal procedure of the solution does not require autoclaving.

4. Sample Processing and Evaluation

1. Determine Chl *a* concentration in each cell sample using a procedure modified from Owttrim[16]. Once collected, pellet the cells from each sample for 3 min at 13,000 x *g* using a microcentrifuge at room temperature.
2. Remove excess media using a P1000 pipette and add 1 mL of 100% methanol (20 °C). Vortex the sample at full speed for 1 min to resuspend the cell pellet.
   CAUTION: Methanol is toxic and flammable. Wear gloves and safety glasses, and keep methanol away from ignition sources.
3. Incubate the samples at -20 °C for 24 h to facilitate the extraction of Chl *a*.
4. After incubation, pellet the cellular debris as described in 4.1, transfer the green supernatant to a cuvette using a pipette, and place the cuvette into the spectrophotometer. Allow the sample to rest in the spectrophotometer for 1 min to ensure that any remaining clay within the sample settles and does not interfere with the measurement.
5. Determine Chl *a* concentration spectrophotometrically by measuring absorbance at 665 nm and 652 nm, and OD at 750 nm. The Chl *a* concentration is determined using the formula Chl $a = 16.29 \times (A_{665} - OD_{750}) - 8.54 \times (A_{652} - OD_{750})$ [20]. Chl *a* concentration is used as a proxy for cell concentration. Additionally, a conversion factor of 7.4×10^{10} cells/L = 10g/L[21] can be used to calculate the cell concentration in grams of some cyanobacterial species.
6. Plot calculated Chl *a* values versus time.

Representative Results

When exposed to clay, cyanobacterial cells are brought out of suspension[22]. This is demonstrated in the representative results given here. To determine the effect of clay on cyanobacterial populations and to observe the sedimentation rates, two experiments were conducted during which *Synechococcus* and *Synechocystis* were exposed to 50 g/L kaolin clay (**Table 5–6**, **Figure 2–3**). Cyanobacterial cultures were grown as described in step 1. Subsequently, after setting up the tank (step 2), the diluted *Synechococcus* culture was poured into the tank and mixed with 50 g of kaolin clay following step 3. This was repeated for the *Synechocystis* culture. All samples were collected from the same position in the tank. The samples were processed and measurements of OD_{652} and OD_{665} as well as the calculated Chl *a* value are given for *Synechococcus* (**Table 5**) and *Synechocystis* (**Table 6**). These results were plotted graphically in line plots and compared to the results of the standards to determine if the settling rate of cyanobacterial/clay mixtures was higher than the natural settling rate of cyanobacteria only. These results demonstrate that the cyanobacterial populations were brought out of suspension within 10 min of clay exposure (**Figure 2–3**). The marine *Synechococcus* showed a more rapid sedimentation rate than the fresh water *Synechocystis,* which is consistent with the hypothesis that trivalent cations (prevalent in the growth medium, which mimics the salinity of salt water) act as the bridging agent between the clay particles and bacterial cells, facilitating flocculation and, therefore, sedimentation[1].

It is important to note that in **Figure 3**, there are some anomalously high results, specifically at data point 2. This is an example of sample processing during which step 4.4 was not sufficiently followed and the sample became turbid during the measurement. This produces an artificially high result (**Figure 2**, data point 2). An example of images in time series, extracted from a video are provided in **Figure 4**, adapted from Playter *et al.*[22]. It is important to note that kaolinite (not kaolin) was used in Playter *et al.*[22].

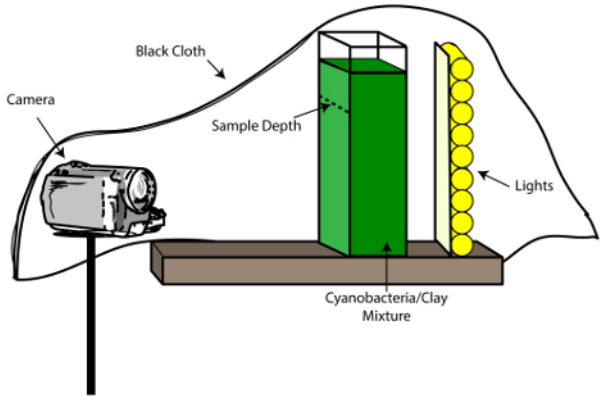

Figure 1: Explanatory diagram illustrating the experimental setup. A video camera is set up at least 1 m from the tank apparatus. The tank is filled with 1 L of cyanobacteria and liquid media. Lights illuminate the tank from behind and the light is dispersed by a translucent film. This entire set up is draped with a black cloth to eliminate additional light sources. This figure has been modified from Sutherland *et al.*[19]. Please click here to view a larger version of this figure.

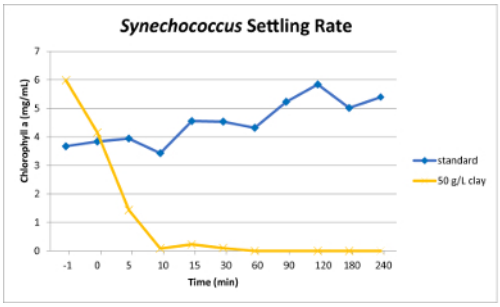

Figure 2: Chl *a* concentrations calculated from the OD values given in Table 1. These values are from a *Synechococcus*/kaolin mixture (yellow). Comparison with Chl *a* concentrations for *Synechococcus* only (blue) shows an increased sedimentation rate for the cyanobacterial/clay mixture. This figure has been modified from Playter *et al.*[22]. Please click here to view a larger version of this figure.

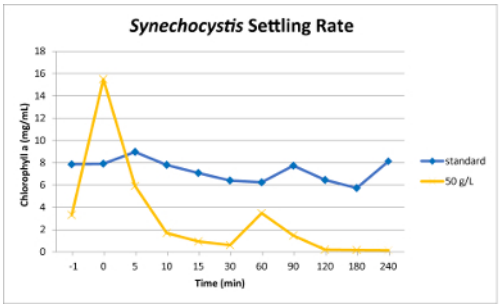

Figure 3: **Chl *a* concentrations calculated from the OD values given in Table 2.** These values are from *Synechocystis*/kaolin mixture (yellow). Comparison with Chl *a* concentrations for *Synechocystis* only (blue) shows an increased sedimentation rate for the cyanobacterial/clay mixture. This figure has been modified from Playter *et al.*[22]. Please click here to view a larger version of this figure.

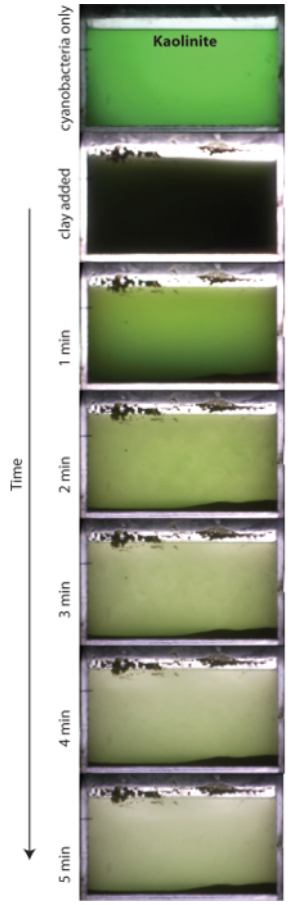

Figure 4: Representative time course of *Synechococcus*-kaolinite deposition. These snapshots are extracted from the recorded video at discrete time intervals (1 min). This figure has been modified from Playter *et al.*[22]. Please click here to view a larger version of this figure.

For 1 L
18 g NaCl
0.6 g KCl
1 g $NaNO_3$
5 g $MgSO_4 \cdot 7H_2O$
1 mL KH_2PO_4
7.2 mL $CaCl_2$
161 µL Na_2EDTA
1 mL $FeCl_3 \cdot 6H_2O$
10 mL Tris HCl pH 8.2
1 mL P1 metals stock*

Table 1: Recipe for A+ media[14]. Refer to **Table 2** for the composition of P1 metals stock. This recipe produces 1 L of media.

For 1 L P1 metal stock
34.26 g H_3BO_3
4.32 g $MnCl_2 \cdot H_2O$
0.315 g ZnCl
0.03 g MoO_3 (85%)
0.003 g $CuSO_4 \cdot 5H_2O$
0.01215 g $CoCl_2 \cdot 6H_2O$

Table 2: Recipe for P1 metals stock required for A+ media.

For 1 L
10 mL $NaNO_3$
1 mL K_2HPO_4
1 mL $MgSO_47H_2O$
1 mL $CaCl_2$ 2 H_2O
1 mL Citric Acid H_2O
1 mL Ferric Ammonium Citrate
1 mL DiNaEDTA
1 mL Na_2CO_3
1 mL A5 Microelements*

Table 3: Recipe for BG-11 media[16]. Refer to **Table 4** for the composition of A5 Microelements. This recipe produces 1 L of media.

For 1 L of stock solution
2.86 g H_3BO_3
1.81 g $MnCl_2$ 4 H_2O
0.222 g $ZnSO_4$ 7 H_2O
0.390 g Na_2MoO_4 2 H_2O
0.079 g $CuSO_4$ 5 H_2O
0.40 g $CoCl_2$ 6 H_2O

Table 4: Recipe for A5 Microelement stock required for BG-11 media.

Sample time (min)	OD 665 nm	OD 652 nm	Chl *a* (mg/mL)
-1	0.563	0.373	5.986
0	0.428	0.33	4.154
5	0.112	0.046	1.432
10	0.024	0.036	0.084
15	0.027	0.025	0.226
30	0.007	0.002	0.097
60	0	0.061	0.000
120	0.007	0.061	0.000
180	0.005	0.012	0.000
240	0.005	0.012	0.000

Table 5: OD_{665} and OD_{652} measurements for *Synechococcus* in the presence of 50 g/L kaolin clay. Chl *a* values are calculated using the formula in step 4.4. Note that samples t6 and t7 are missing. This is an example of not keeping a consistent sample interval as per the cell sampling protocol. Data for the standard is taken from Playter, *et al*. (2017)[2].

Sample time (min)	OD 665 nm	OD 652 nm	Chl *a* (mg/mL)
-1	0.384	0.345	3.309
0	1.863	1.739	15.497
5	0.64	0.528	5.916
10	0.217	0.216	1.690
15	0.104	0.089	0.934
30	0.126	0.169	0.609
60	0.424	0.402	3.474
90	0.115	0.048	1.463
120	0.016	0.007	0.201
180	0.012	0.004	0.161
240	0.011	0.005	0.136

Table 6: OD_{665} and OD_{652} measurements for *Synechocystis* in the presence of 50 g/L kaolin clay. Chl *a* values are calculated using the formula provided in step 4.4.

Discussion

Flocculation catalyzed by cyanobacterial cell-clay interaction has attracted a lot of interest in the fields of ecology and engineering[2,3,4,5,6,7,8,9,10,11,12], however, the investigation of these interactions with the intent of modelling the deposition of sedimentary deposits (such as shales) is relatively new. The visualization of this process, in this case for sedimentological applications, has not been reported. In past flocculation studies, these interactions have been investigated by mixing clay and cyanobacteria in jars, test tubes, or beakers, and sampling at discrete time intervals[9,11,12,26] to determine the changes in cell concentration through time. These studies have measured the concentration of cyanobacterial cells in different ways. For example, the sampled cyanobacterial cell populations have been measured by counting using a microscope[9,11,12,26]. When compared to the initial cell concentration, the removal efficiency can be calculated[9,11,12,26]. In a separate study, after allowing the clay/cell mixture to settle, the fluid remaining above the settled cell/clay sediment was sampled and analyzed for fluorescence to estimate the number of remaining cells in suspension[3]. Measurements of Chl *a* directly from water column samples have also been used to calculate cell concentration[8,10].

In contrast, our methodology measures the cells in suspension over time, building on the methodology of Sengco, *et al.*[4] by taking measurements at select times throughout the process, and pairing these time intervals with real-time video imaging. When compared with other experimental protocols involving the settling of clay in the presence of anionic flocculants, using either biological (algae or cyanobacteria) or non-biological (synthetic flocculent agents) flocculants, our analysis differs on many counts. First, our study involves the use of a marine coccoid species of cyanobacteria, while many other studies involve fresh water species or species which produce extracellular polysaccharide substances[5,23,24,25]. Additionally, our study involves the use of a standard tank, within which the solution remains static. This contrasts with studies done using test tubes or cylinders[3,12,14] or flume tanks, where the fluid flow is a variable of interest[6,7]. The static nature of our experimental method allows for the measurement of baseline sedimentation rates, where floccules are not kept in suspension by turbulent flow. Additionally, using a tank instead of a test tube, jar, or beaker, allows for better visualization of the resultant deposits because of the flat tank sides; the video images show no distortion due to curving and the light is evenly dispersed. Third, the methodology described herein measures flocculation rates over both the short (2 min intervals) and long (hour intervals) term; additionally, images can be compiled at scales of seconds to minutes, allowing both visualization of the process, and an additional measurement of the settling rate. Most studies measure flocculation rates over half to full hour intervals[5,6,23,25] or simply measure the final number of cells left in suspension after a single time point (2–2.5 h)[9,24], although some studies[9] have measured changes in cell or Chl *a* concentration over 2 minute intervals. The sample interval chosen is critical, as flocculation can occur rapidly (within 5–10 min)[22]. Finally, a major strength of our methodology is that it produces real-time images of the flocculation process, not merely of the produced aggregates (*e.g.*, Avnimelech *et al.*, 1982)[24]. Having two lines of data, cell concentration from sampling and visual evidence, strengthens the reliability of the results and supports robust conclusions.

While suitable for measuring flocculation rates of clay and cyanobacteria, our protocol requires diligence with regards to the sampling location within the tank. The same area of the tank (x, y, z) must be sampled each time or the sample results can become skewed. Care also must be taken to allow all particulates to settle before OD measurements are made. Critical steps include: i) using an appropriate sampling interval which remains consistent for all experiments, and ii) ensuring that the clay/cyanobacterial pellet is sufficiently broken after the addition of methanol to ensure the adequate extraction of chlorophyll from the cells.

The technique described here is also limited to modelling flocculation under fully oxygenated conditions. The experimental apparatus and procedure would need to be modified to model a stratified water column with anoxic bottom waters.

Within the sedimentological context, this method has the potential to be applied to modelling deposits of different clay ratios or biological materials in order to understand aspects such as changes in organic matter and salinity from riverine input. Furthermore, the sediments produced using this method can be used to investigate the impact of potential storm reworking on floc-coherence by remixing or agitation. By pairing direct measurement of cyanobacterial cell concentration with visual images, this protocol transcends the traditional applications of cyanobacteria/clay flocculation processes and allows for these processes to be applied to sedimentological modelling of the rock record.

Disclosures

The authors have nothing to disclose.

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