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PRIMARY RESEARCH PAPER

Effects of episodic turbulence on diatom mortality and physiology, with a protocol for the use of Evans Blue stain for live–dead determinations

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Abstract Short-lived, high-intensity turbulence in aquatic environments—or episodic turbulence—has been shown to cause mortality in zooplankton, but its effects on marine phytoplankton have rarely been investigated. Episodic turbulence derives from anthropogenic and natural causes such as boat propellers, strong winds, and breaking waves. This study focused on the effects of episodic turbulence on two diatoms: *Thalassiosira weissflogii* and *Skeletonema costatum*. 45 s exposure to turbulence intensities above 2.5 $cm^2\ s^{-3}$ reduced diatom abundance by up to 32% and increased the number of intact dead cells by 22%. After exposure to 4.0 $cm^2\ s^{-3}$, photosynthetic efficiency decreased by 25 and 9% in *T. weissflogii* and *S. costatum*, respectively. Turbulence also caused extracellular release of optically reactive DOM and biologically important trace metals such as iron. The turbulence levels tested are comparable to those under breaking surface waves and are substantially lower than those generated by boat propellers. An improved technique using the Evans Blue stain was developed to enable visual live/dead plankton cell determinations. When used in conjunction with preservation and flow cytometry, this staining method provides a way to study phytoplankton mortality due to turbulence and other environmental stresses.

Keywords Turbulence · Plankton mortality · Diatom · Evans Blue · Staining

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Introduction

Phytoplankton mortality in the marine environment is largely attributed to zooplankton grazing (Cullen et al., 1992; Lenz et al., 1992) and viral lysis (Suttle et al., 1990; Brussaard, 2004). Besides biological factors, the physical environment also imparts a variety of stresses on phytoplankton. Phytoplankton mortality due to fluctuations in nutrient availability, UV radiation, and temperature has been documented, but only in instances where fluctuations are rapid and acute (Llabrés & Agustí, 2006).

Turbulence in the environment spans many orders of magnitude, with measured energy dissipation rates (ε) ranging from 10^{-4} to 10^4 $cm^2\ s^{-3}$ (Osborn, 1980; MacKenzie & Leggett, 1991; Terray et al., 1996).

Even weak turbulence alters phytoplankton assemblage composition and physiology (Margalef, 1978). For example, chain-forming diatoms produce thicker tests and more flexible mucous filaments when exposed to bubble-derived turbulence with a relatively weak energy dissipation rate of 10^{-1} cm^2 s^{-3} (Clarson et al., 2009). With increasing turbulence, diatoms become dominant relative to dinoflagellates and chlorophytes (Thomas & Gibson, 1990; Estrada & Berdalet, 1997; Sullivan & Swift, 2003). Episodic turbulence is a short-lived (often on the order of seconds to minutes), strong disturbance produced by causes such as storms, boat propellers, and breaking waves. The main distinguishing feature of episodic turbulence is that it is ephemeral and orders of magnitude more intense than "ambient" turbulence, and has the potential to cause mortality and impart physical stress on phytoplankton. The frequency of the turbulence depends on both the environment and the source of turbulence, and episodic turbulence events typically occur hours or days apart with the exception of breaking waves.

Models of turbulent energy have traditionally considered the air-sea interface as an inverted wall model (Terray et al., 1996), but observations of turbulent energy using acoustic instrumentation have revealed energy dissipation rates up to 70 times higher than inverted wall model output (Melville, 1994), indicating that turbulence in surface waters has been under-estimated (Peters & Marrasé, 2000). Observed dissipation energy under wind-driven, breaking surface waves can reach 10^2 cm^2 s^{-3}, and those for waves breaking on shore 10^4 cm^2 s^{-3} (Agrawal et al., 1992), and intermittently stormy conditions 10 cm^2 s^{-3} (Gargett, 1989). The intermittency of episodic turbulence may also enhance its effects on phytoplankton. Phytoplankton exposed to intermittent turbulence experienced decreased growth rates compared to those exposed to constant turbulence (Gibson & Thomas, 1995), and dinoflagellate growth rate has been negatively correlated with the occurrence of waves above an ambient turbulence threshold (Tynan, 1993).

Besides natural events, intense episodic turbulence can be generated by boat activities. Damage to marine megafauna due to recreational boat propellers has been well documented (Beck et al., 1982; Cannon, 1998). A single outboard motor propeller may produce turbulence in excess of 3×10^2 cm^2 s^{-3} (Loberto, 2007). Propeller turbulence generated by shipping vessels can be up to 5×10^4 cm^2 s^{-3} (Killgore et al., 1987), well above natural turbulence intensities. Larval perch experimentally exposed to a turbulence dissipation shear of 35 N m^{-2} experienced 38% mortality after just 1 min (Morgan et al., 1976). Temporary alterations in the flow regime caused by shipping vessels also affect fish behavior and riverine migration patterns (Odeh et al., 2002). Recent studies have shown that boat turbulence may also impact even small marine plankton. Episodic turbulence may cause mortality in excess of 30% in natural copepod populations exposed to high volume boat traffic (Bickel et al., 2011). Zooplankton carcasses are more prevalent inside boat wakes than immediately outside of them, and increased turbulence intensity is correlated with increased percentage of copepod carcasses (Bickel et al., 2011).

Phytoplankton cell size is an important factor in predicting the effects of turbulence (Kiørboe, 1993). Intense turbulence produces shear forces that may be destructive, but only if the turbulence is strong enough to produce eddies on a scale comparable to the phytoplankton's cell length. The Kolmogorov microscale (L_K) is defined as:

$$L_K = \left(\frac{\nu^3}{\varepsilon}\right)^{1/4}$$

where ν is kinematic viscosity of water (10^{-2} cm s^{-1}) and ε is the turbulent kinetic energy dissipation rate. L_K describes the scale at which inertial forces dominate over viscous forces; it becomes smaller as turbulence intensity increases (Tennekes & Lumley, 1972). Phytoplankton generally do not experience damaging velocity gradients from the shear forces generated by turbulence unless L_K is at or below their cell length.

The Batchelor microscale (L_B) describes the smallest distance across which a gradient in substrate can exist before diffusive molecular forces dominate and diminish the gradient, and it is determined in part by turbulence energy dissipation rate as:

$$L_B = \left(\frac{\nu D^2}{\varepsilon}\right)^{1/4},$$

where D is mass diffusivity of a given scalar (for transition metals in 19°C water, $5.8 \pm 0.1 \times 10^{-6}$ cm^2 s^{-1}; Li & Gregory, 1974), and ε is the

turbulent energy dissipation rate. The Batchelor microscale is typically smaller than L_K and may affect organisms even when $L_K >$ cell size.

It is important to also consider the sub-lethal effects of turbulence on a phytoplankton cell. Even shear forces that do not kill the cell may still produce stress responses such as decreased photosynthetic efficiency (Thomas et al., 1995) and altered frustule shape and size (Clarson et al., 2009). Combined, these lethal and sub-lethal effects have important ramifications for phytoplankton composition and functions. For example, if larger cells are affected by a certain turbulence intensity threshold, smaller cells may benefit from the reduced competition and nutrients extruded by the larger cells. This mechanism is one of many that may affect phytoplankton composition following episodic turbulence events. Laboratory studies are suited for investigating turbulence effects on phytoplankton because turbulence exposure (intensity and duration) can be manipulated, and the specific responses of the cells can be observed under controlled conditions.

We conducted experiments to study both the lethal and sublethal effects of intense, episodic turbulence on two diatom species of different sizes, *Thalassiosira weissflogii* (20 μm) and *Skeletonema costatum* (9–12 μm solitary, 36–48 μm chains). The species were chosen based on their different size characteristics but otherwise similar physiologies (both diatoms grown in the same temperature, light regime, and medium). Also, detailed literature on the trace metal content of *T. weissflogii* is available for comparison. For sublethal effects, we measured cellular extrusion of dissolved organic material (DOM) and trace metals, as well as photosynthetic efficiency (F_v/F_m), before and after turbulence exposure.

The study of lethal effect presented a challenge because, unlike unarmored and motile cells, dead but intact diatoms may appear the same as live diatoms. It was therefore our goal to first develop a simple and reliable method to distinguish between live and dead diatoms. Staining is a simple, cost-effective way to make visual live/dead determinations. Staining protocols have been successfully developed for marine and freshwater zooplankton (Bickel et al., 2009; Elliott & Tang, 2009). Evans Blue (EB) is a mortal stain that is effective in making live/dead determinations in vascular plant cells, and has been tested on marine phytoplankton (Crippen & Perrier, 1974). The stain penetrates cells with compromised plasma membranes and binds to the cytoplasm, producing a clear, deep blue color in dead cells that is visible under light microscopy. In a preliminary trial, EB was compared with the vital stain Neutral Red, but the former proved more effective in distinguishing between live and dead phytoplankton.

This study consisted of two parts. In the first part, we assessed the efficacy of EB stain for determining live/dead phytoplankton cells, and developed a simple staining protocol for measuring diatom mortality in our turbulence experiments. We also tested the stain in conjunction with flow cytometry and preservation method to further expand its application for future work. In the second part, we conducted laboratory experiments in which we exposed the diatoms to different levels of turbulence and measured both lethal and sublethal effects.

Materials and methods

Evans Blue stain

Preparation of stain stock solution

EB powder (Sigma Aldrich 206334) was added to deionized (DI) water to make a 1% w:v stock solution. The mixture was stirred overnight to ensure complete dissolution. The stock solution was stored in a borosilicate amber glass bottle at room temperature until use. EB stock remained usable for at least 5 months under these conditions (Crippen & Perrier, 1974). The stain was tested on artificially killed phytoplankton obtained from live culture. Stained samples were visually counted, counted with flow cytometry, and subjected to various preservation methods.

Algal cultures

Four species of marine phytoplankton were used in EB stain trials. They included two diatoms [*T. weissflogii* (CCMP 1336) and *S. costatum* (CCMP 1332)], one cryptomonad [*Rhodomonas salina* (CCMP 1319)], and one chlorophyte [*Dunaliella tertiolecta* (CCMP 1320)]. Diatoms were grown in f/2+Si medium, *R. salina* in L1, and *D. tertiolecta* in f/2. New cultures were started with fresh media every 7 days. All cultures were grown at 19° C on a 12 h:12 h light–

dark cycle. Cells were taken from cultures in log phase for testing EB stain.

For the testing, it was necessary to produce dead cells by artificially killing a subsample of the cultures. Four killing methods were used: (1) heat: 1 ml samples from phytoplankton cultures were incubated in a 30°C convection oven for 15 min; (2) salinity shock: 1 ml samples from phytoplankton cultures grown at ~20 psu were gently concentrated on nylon mesh, and then rinsed into artificial sea water (ASW) of 27 psu. Tests were conducted with cells stained before and after concentrating to ensure that no significant amount of cells were killed by the handling; (3) UV radiation: 1 ml samples were exposed to intense UV A/B radiation by placing a UV lamp directly above sample cuvettes for 25 min; (4) biocide: 0.975 mg ml^{-1} sodium azide was added to 1 ml phytoplankton samples for a final concentration of 15 mmol l^{-1} (Pett, 1989).

In each of the tests, live and dead cells were mixed in ratios of 100:0, 75:25, 50:50, 25:75, and 0:100. The mixtures were treated with the stain, and the observed percentage of live (unstained) cells was compared to the expected percentage of live cells per sample.

Staining and counting

An aliquot of 15 ml was gently pipetted out from each of the live/dead mixtures, and EB stock solution was added in the amount of 0.02 ml EB per ml sample. The sample was stained for at least 15 min. Three 1 ml subsamples were transferred from the stained sample to Sedgewick-Rafter counting slides. Each subsample was allowed to settle for 2 min prior to microscopy. Cells were counted at 200× using a Nikon TS100 inverted microscope. About 600 cells per sample were randomly selected and counted on the Sedgewick-Rafter slide. Live cells appeared light green or colorless, whereas dead cells appeared deep blue due to EB uptake (Supplementary figure).

Flow cytometry

EB fluoresces at ~680 nm when excited at 620 nm (Saria & Lundberg, 1983). The stain protocol was therefore tested to determine if it would enable a flow cytometer to make live-dead differentiations under red excitation. Flow cytometry was tested on samples of *T. weissflogii* and *S. costatum.* Triplicate 1.5 ml samples of live diatom culture were analyzed without staining by an Accuri C6 Flow Cytometer. The flow cytometer was set to run at high speed for 2 min, consuming a volume of 131 µl. Afterward, triplicate samples were stained and analyzed by the flow cytometer for comparison. In another trial, 1.5 ml of a diatom culture was analyzed; then, half of the remaining sample was killed by either heat or biocide. The killed subsample was mixed with the untreated subsample to create a 1:1 live/dead mixture and analyzed by the flow cytometer. Afterward, the mixture was stained with EB and three replicates re-analyzed. Lastly, 1.5 ml of diatom culture was killed by either heat or biocide and analyzed before and after staining; three replicates per killing method were evaluated. Differences in fluorescence and side scatter were evaluated using the Batch Analysis tool in the BD Biosciences C6 software.

Preservation method

To expand the future usability of the staining technique, EB was tested in conjunction with refrigeration or formalin preservation. Heat- or azide-killed phytoplankton cells were mixed with live cells in triplicate, and then stained with EB for 20 min. The stained samples were counted immediately, then stored at room temperature, or refrigerated without preservative, or preserved in 5% formalin. The samples were re-counted after 24 and 48 h. The formalin-preserved samples were also counted after 7 days. Cell counts at each time point were compared using ANOVA.

Turbulence experiments

Experimental unit construction

The experimental unit consists of a rectangular 37.8 l glass aquarium, a turbulence generation device, and a Nortek Acoustic Doppler Velocimeter (ADV). The turbulence generation device was constructed using a 6-speed motor, non-toxic marine epoxy, a hollow styrene tube, and a 5 in. diameter, 4-blade plastic propeller. The device was positioned such that the motor was outside the aquarium and the shaft of the propeller extended downward into the aquarium, rendering the propeller midway down the height of the tank and parallel to the bottom of the tank. The

ADV was positioned at the opposite end of the aquarium such that there was a 15-cm space on all sides of the ADV and a 19-cm space between the ADV and the bottom of the tank.

Experimental procedure

The aquarium, propeller, and sample bottles were washed with HCl and DI water prior to each experiment. After rinsing and drying, the tank was filled with 7 l of 70 psu trace-metal-clean seawater (Sunda et al., 2005) and 21 l of Milli-Q water for a total volume of 28 l and a final salinity of 22 psu. Blanks were taken from the aquarium at this point for background trace metal and DOM. 4 l of diatom culture was gently filtered from growth media into 4 l ASW. After filtering, the diatoms in ASW were added to the aquarium for a final volume of 32 l, then "Before Turbulence" samples were taken. The turbulence generation device was run for 45 s at different speeds to generate different turbulence energy levels. The actual turbulence levels, measured by the ADV, were designated as High, Medium, and Low. Immediately after turbulence exposure, "After Turbulence" samples were taken. We conducted four replicate experiments per turbulence level for *T. weissflogii* and three replicate experiments per turbulence level for *S. costatum*. One or more samples for each of the analyses described below were taken before and after each turbulence trial, except for trace metal samples, which were only collected for two of the *T. weissflogii* experiments due to logistical constraints. The total volume of samples taken before the turbulence experiment began was 460 ml, and each sample volume and number is given for each individual analysis. Blanks and experimental samples were used for each of the chemical analyses described below.

Mortality and cell size

The EB stain protocol was applied to three 10-ml samples taken before and after each turbulence trial for live/dead determinations. All stained samples were counted on a Sedgewick-Rafter counting slide (a minimum of 180 cells were counted). Additionally, for some randomly selected replicates, the Accuri C6 flow cytometer was used to obtain live/dead counts as well as cell size and abundance.

Trace metal content of diatoms

High resolution inductively coupled plasma mass spectroscopy (ICPMS; ThermoFisher Element2) was used to measure cellular trace metal and phosphorus content before and after turbulence. Our analysis focused on changes in Mn, Fe, Co, Cu, and P content of *T. weissflogii*. Due to logistical constraints, the analyses were only performed for two replicate experiments at each turbulence level. Two 125-ml samples from the experimental aquarium were taken in LDPE bottles and vacuum filtered in a trace-metal-clean room through acid washed, pre-weighed 8 μm polycarbonate filters. Algal cells were washed of surface-complexed metals and metal hydroxide precipitates using established methods (citrate/oxalate/EDTA wash) followed by an ultrapure, pH-buffered salt rinse (Tovar-Sanchez et al., 2003; Tang & Morel, 2006). Filters were dried at 60°C for at least 24 h and weighed, and kept refrigerated until acid digestion. To digest the algal biomass for ICPMS analysis, filters were placed in pre-weighed LDPE lock-top centrifuge tubes to which 0.67 ml HCl and 0.33 ml HNO_3 were added. After 12 h, samples were shaken and capped, weighed, then incubated in acid for 7 days. Digests were diluted 1:11 (200 ml sample: 2,000 ml acid + Indium standard) in trace-metal-clean sample cups, and then injected directly into the ICPMS using indium as an internal standard. Metal concentrations were externally calibrated using NIST-traceable multi-element standard solutions (Inorganic Ventures). Accuracy was confirmed using standard reference material NIST 1643e, which gave recoveries within 10% of the certified value.

DOM content of water

A Shimadzu RF-1501 Spectrophotofluorometer was used to qualitatively characterize optically reactive DOM in the ambient water (Stedmon et al., 2003). Three 5-ml samples were taken before and after each turbulence trial. It was not intended that this method would yield the exact quantity or identity of DOM in the samples; rather, a qualitative comparison of the fluorescence spectrum before versus after turbulence exposure would indicate if turbulence caused release of DOM. A control experiment without phytoplankton was conducted to account for any non-phytoplankton source of DOM.

Photosynthetic efficiency

A Pulse Amplitude Modulating (PAM) fluorometer (Walz Company) was used to assess photosynthetic efficiency of the diatoms. Three 25-ml samples were taken before and after each turbulence trial. Samples were diluted with ASW (10 ml sample: 40 ml seawater) and injected into the PAM. Three separate F_o/F_m pulse readings and one saturation pulse reading per sample were obtained before draining the sample. The instrument was rinsed with distilled water between samples. The following equation was used to calculate the photosynthetic efficiency of the cells:

$$F_v/F_m = (F_m - F_o)/F_m,$$

where F_m is the maximum fluorescence, F_o the minimum fluorescence, and F_v the variable fluorescence (Juneau & Harrison, 2005). F_v/F_m is used here as a proxy for the efficiency of Photosystem II (PS II), and is proportional to the quantum yield (i.e., molecules of CO_2 fixed per photon absorbed).

Calculating turbulent energy dissipation rate

Turbulent kinetic energy (k) is the mean kinetic energy per unit mass produced by turbulent flow, and is given as:

$$k = \frac{1}{2}\left(\left(\overline{u_1'}\right)^2 + \left(\overline{u_2'}\right)^2 + \left(\overline{u_3'}\right)^2\right)$$

where u_1', u_2', and u_3' are deviations from mean velocity measurements in the x, y, and z directions (Libby, 1996). Once k is calculated, the value is used to calculate turbulent energy dissipation rate (ε) in the aquarium:

$$\varepsilon = c_\mu^{3/4} k^{3/2} l^{-1},$$

where c_μ is assumed to be 0.09 (Libby, 1996) and l is the maximum size of an eddy possible given the physical constraints of the system:

$$l = Kz,$$

where z is the length of the water mass monitored by the ADV and K represents von Karman's constant (0.41), a dimensionless value that describes the fluid velocity gradient from a no-slip boundary layer. In this study, $z = 15$ cm. All calculations were performed in MATLAB.

Results

Evans Blue stain protocol

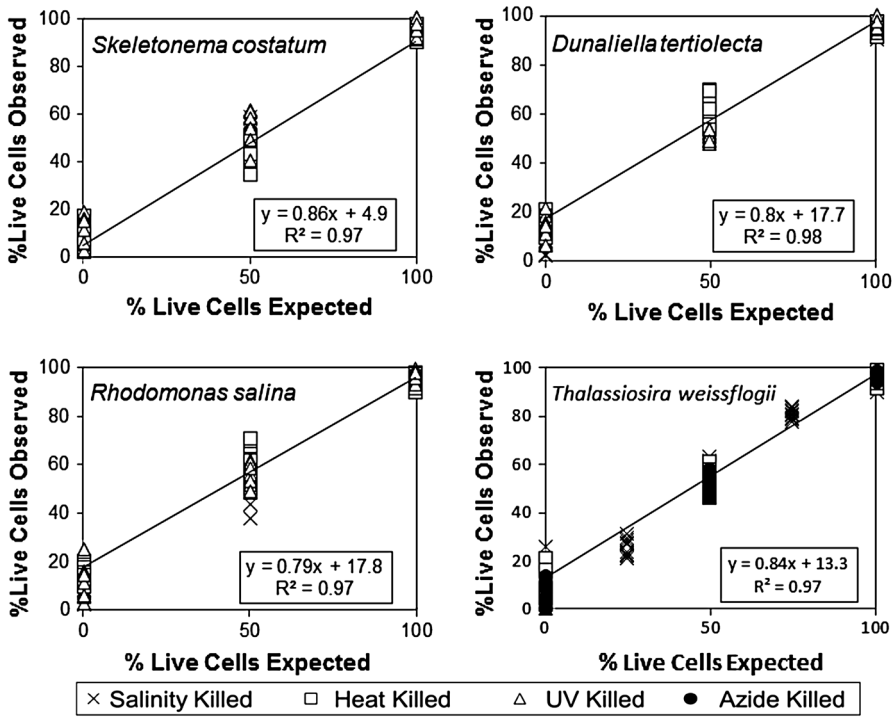
A significant linear relationship between expected percentages of live cells and observed percentages of live cells was obtained for all four phytoplankton species and different killing methods (Fig. 1). Without staining, the flow cytometer was unable to distinguish a sample of live cells (Fig. 2a) from either a sample of dead *T. weissflogii* cells (Fig. 2b) or a 1:1 live/dead mixed sample of *T. weissflogii* cells (Fig. 2c). When EB was applied, stained cells fluoresced at a different (higher) frequency than unstained cells, creating a second peak in the emission spectrum and enabling live/dead differentiation of *T. weissflogii* with the flow cytometer (Fig. 2d). A sample of only dead stained cells produced a peak comparable to the second peak in the 1:1 live/dead mixture (Fig. 2e).

EB-stained samples stored at room temperature or refrigerated showed a significant decrease in the percentage of stained cells after 24 h ($P = 0.01$) and 48 h ($P = 0.03$) (Fig. 3a, b). EB-stained samples preserved with formalin showed no significant change in the percentage of live cells counted 24 h, 48 h, or 7 days after staining ($P = 0.7$; Fig. 3c), indicating good stain retention by dead cells.

Turbulence experiments

Energy dissipation rate (ε), Kolmogorov length scale (L_K), and Batchelor microscale (L_B) were calculated for each turbulence level from the ADV data (Table 1). High, medium and low turbulence had energy dissipation rates of 4.0, 2.5, and 1.1 $cm^2\ s^{-3}$, respectively, which were analogous to turbulence levels recorded in intermittently stormy conditions (Gargett, 1989) and well below those produced by waves breaking on shore (Agrawal et al., 1992). The smallest L_K, 224 μm, and the smallest L_B, 55 μm, were produced by the high turbulence treatment (Table 1).

In the low turbulence treatment, the percentages of live *T. weissflogii* and *S. costatum* cells did not change significantly after turbulence exposure (paired t test; $P = 0.703$ and 0.092, respectively) (Fig. 4). Medium turbulence caused a 20% decrease in live cells in *T. weissflogii* ($P = 0.002$), and a significant decrease in percent live *S. costatum* (10%; $P = 0.009$). High



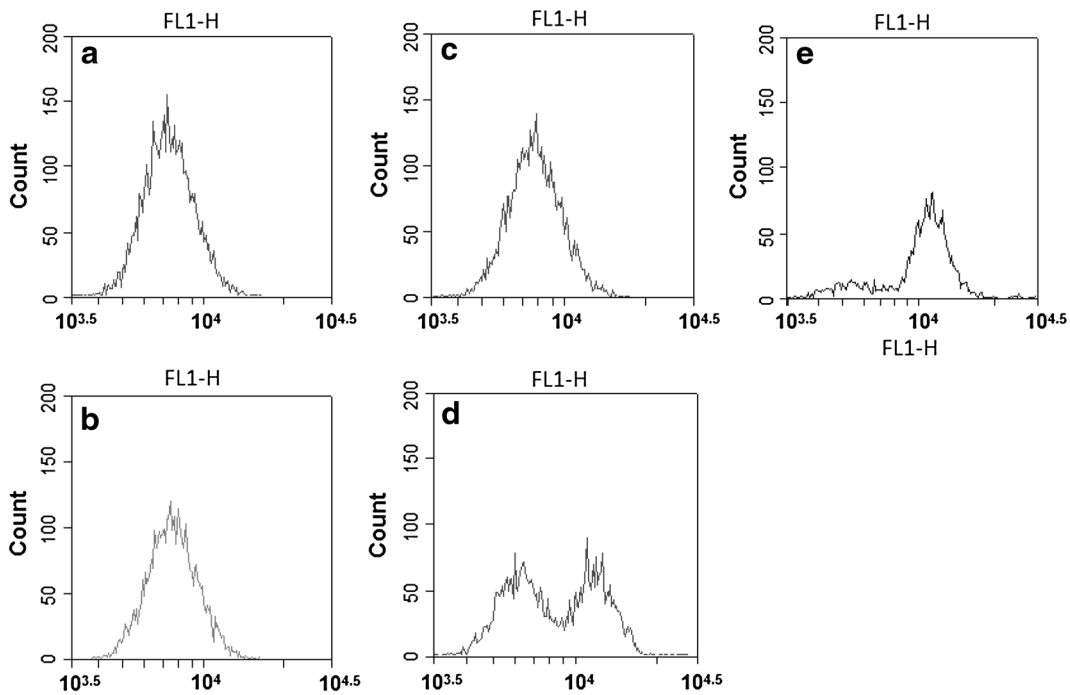


Fig. 1 Relationships between expected percentages of live cells and observed percentages of live (unstained by EB) cells. EB staining results were consistent across several killing methods and phytoplankton species ($n = 10$ per killing method). Linear regressions significantly describe the relationships in all cases ($P < 0.01$)

Fig. 2 Flow cytometer plots of fluorescence wavelengths detected by the 530 nm band filter (FL1-H) and cell count for **a** live *T. weissflogii* cells, **b** dead (azide-killed) *T. weissflogii* cells, **c** unstained 1:1 live/dead mixture, **d** stained 1:1 live/dead mixture, and **e** stained dead cells

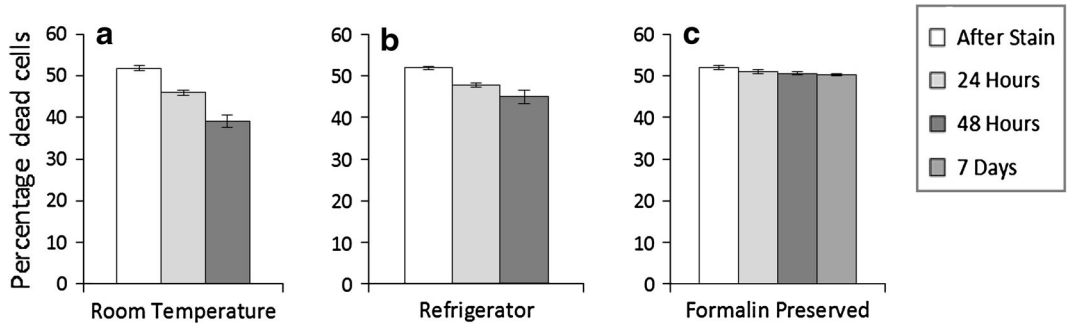


Fig. 3 Observed percentages (mean ± SD) of dead cells (stained by EB) in samples that were **a** kept at room temperature, **b** refrigerated without preservative, or **c** preserved in formalin. ANOVA indicated that there was a significant decrease in the observed percent dead cells in the room temperature samples ($n = 3$, $P = 0.01$) and refrigerated samples ($n = 3$, $P = 0.03$) over time, but the observed percent dead cells in formalin-preserved samples did not change significantly over 7 days ($n = 3$, $P = 0.7$)

Table 1 Propeller speeds used in the experiments and the corresponding energy dissipation rates (ε), Kolmogorov length scales (L_K) and Batchelor microscales (L_B). L_B were calculated for dispersion of metals in seawater under standard conditions

Propeller speed	ε (cm^2 s^{-3})	L_K (μm)	L_B (μm)
High	4	224	55
Medium	2.5	251	61
Low	1.1	309	76

turbulence caused a significant decrease in percent live *S. costatum* (15%; $P = 0.005$), but not in *T. weissflogii* ($P = 0.391$).

The total cell abundance decreased after exposure to high and medium turbulence (Fig. 5). After high turbulence, cell concentration decreased 17% in *T. weissflogii* (paired *t* test; $P = 0.002$) and 30% in *S. costatum* ($P = 0.028$). Medium turbulence also caused a significant decrease in the total abundance of *T. weissflogii* (15%; $P = 0.023$) and *S. costatum* (13%; $P = 0.021$). There was no significant change in the cell counts for either *T. weissflogii* ($P = 0.418$) or *S. costatum* ($P = 0.918$) at low turbulence.

Active fluorescence showed no significant change in quantum yields after exposure to low and medium turbulence (Fig. 6a, b, d, e). High turbulence was the only treatment that caused a significant reduction in photosynthetic efficiency: F_v/F_m decreased by 25% from 0.50 to 0.37 in *T. weissflogii* ($P = 0.02$), and by 12% from 0.56 to 0.50 in *S. costatum* ($P = 0.01$; Fig. 6c, f).

Spectrofluorometry showed changes in the fluorescence signatures of reactive DOM after turbulence (Fig. 7). Reactive DOM prior to turbulence exposure

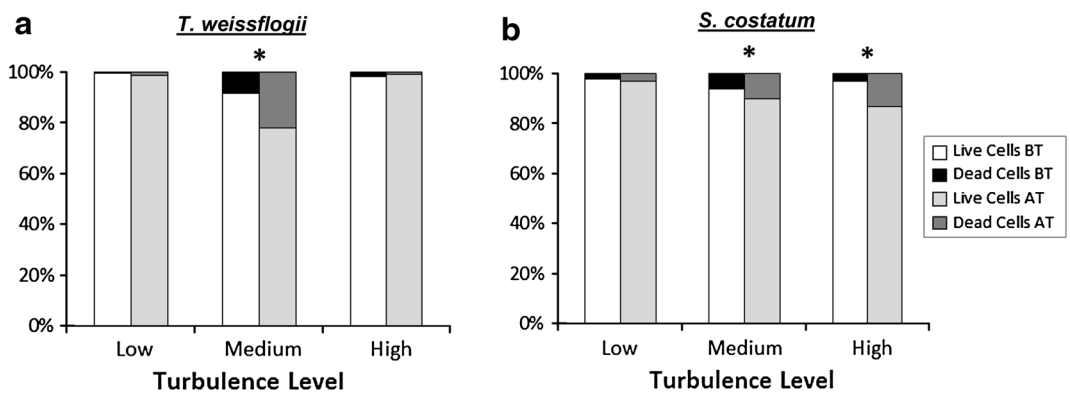


Fig. 4 Percentages of live and intact dead cells (stained by EB) before and after exposure to low, medium and high turbulence for **a** *T. weissflogii* ($n = 4$) and **b** *S. costatum* ($n = 3$). *Significant difference between before (BT) and after (AT) turbulence treatments (paired *t* test; $P < 0.05$)

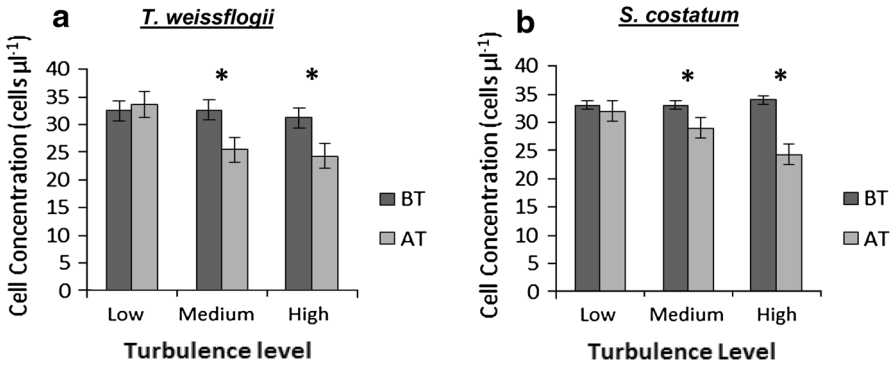


Fig. 5 Total cell concentrations (mean ± SD) before (BT) and after (AT) exposure to low, medium, and high turbulence for **a** *T. weissflogii* ($n = 4$), and **b** *S. costatum* ($n = 3$). *Significant difference between before and after turbulence treatments (paired t test; $P < 0.05$)

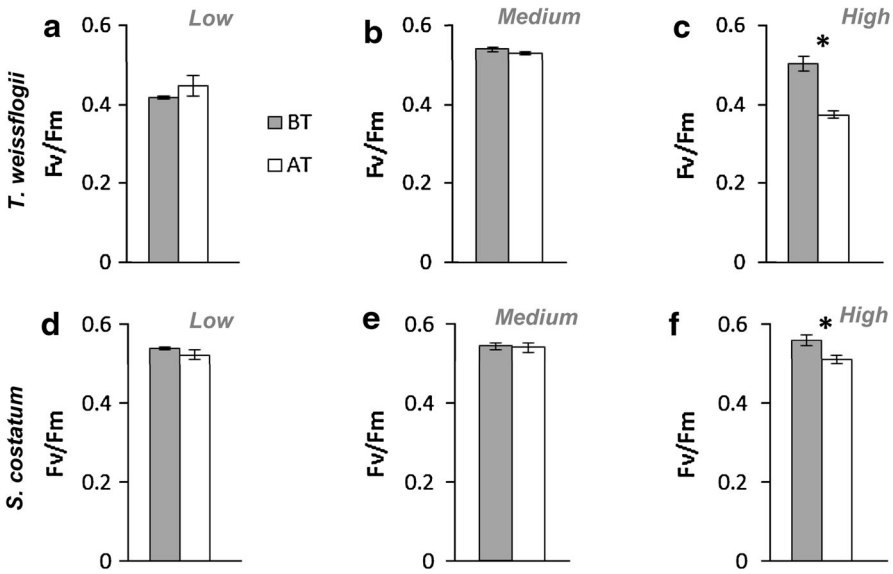


Fig. 6 Photosynthetic efficiency (mean ± SD) of **a**–**c** *T. weissflogii* ($n = 4$) and **d**–**f** *S. costatum* ($n = 3$) before (BT) and after (AT) exposure to **a**, **d** low, **b**, **e** medium, and **c**, **f** high turbulence. *Significant difference between before and after turbulence treatments (paired t test; $P < 0.05$)

was low in fluorescence intensity and number of peaks detected. In the low turbulence treatment, the DOM spectra for *T. weissflogii* and *S. costatum* showed a conspicuous peak at 325 and 315 nm, respectively, after turbulence exposure. Exposure to medium turbulence generated two conspicuous peaks in both species: 319 nm and 684 nm in *T. weissflogii*; 319 and 686 nm in *S. costatum*. At medium turbulence, the *T. weissflogii* samples also showed elevated levels of "background" DOM, which may have contributed to the peaks present (Fig. 7). Exposure to high turbulence also resulted in two conspicuous peaks similar to the medium turbulence treatment. Peak heights were higher in the high turbulence treatment than in the medium turbulence treatment for *S. costatum*, but not for *T. weissflogii*. The fluorescence spectrum showed negligible change in the control experiment, indicating that there was no significant source of DOM other than the diatoms.

ICPMS results showed different levels of metal reduction between trials and metal species (Fig. 8). In the high and medium turbulence treatments, Mn, Fe, and Co per gram dry weight of *T. weissflogii* decreased, while Cu per gram dry weight decreased at medium turbulence and increased at high turbulence. Low turbulence produced a positive effect on cellular Mn, Fe, Co, and Cu contents. Cu and Fe levels were below detection limits in one medium turbulence replicate and one low turbulence replicate, so statistical comparison was not performed for those trials. After low turbulence, P content of the diatoms increased, while after medium turbulence exposure, P contents decreased. After high turbulence exposure, P content did not change significantly.

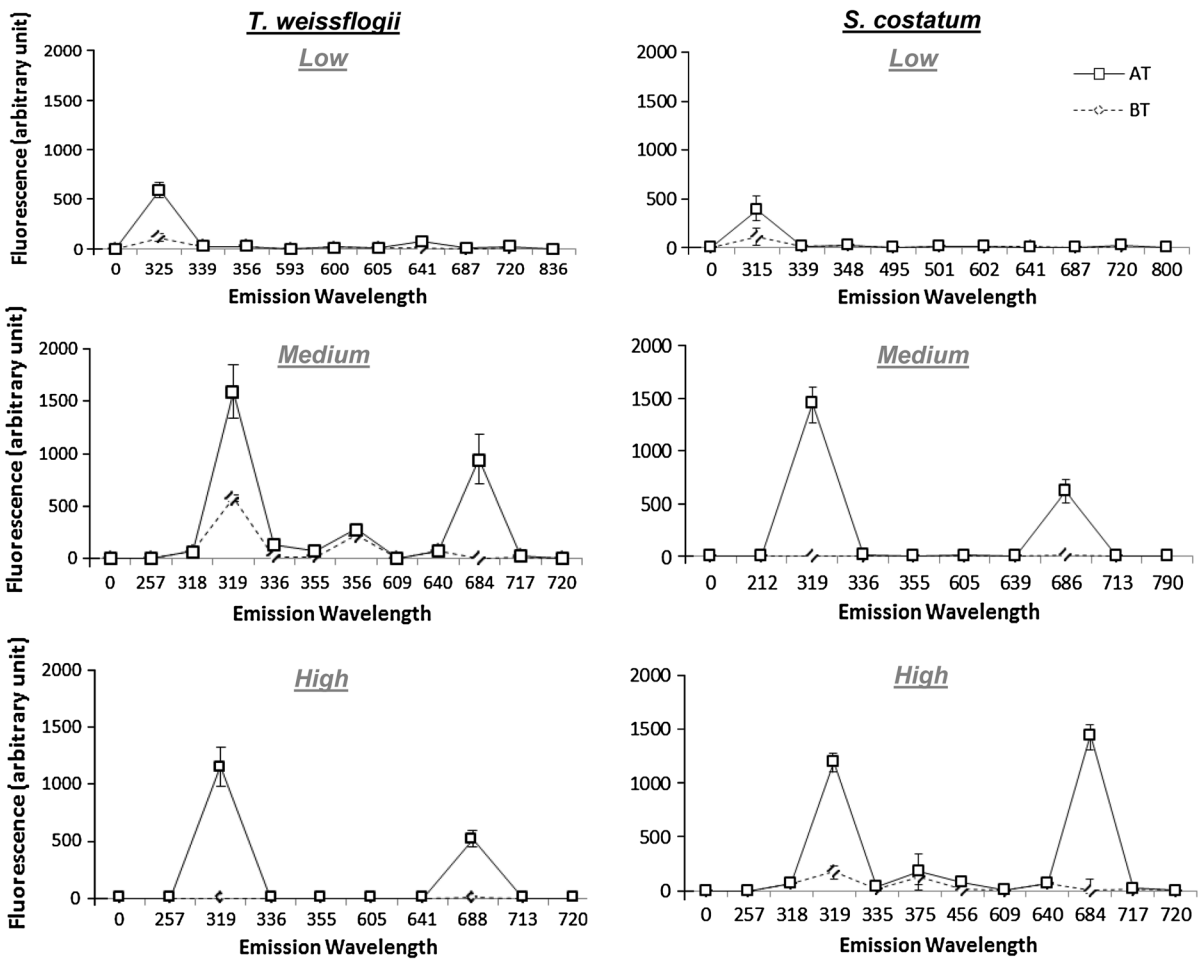


Fig. 7 Spectral emissions of ambient water in *T. weissflogii* experiments ($n = 4$) and *S. costatum* experiments ($n = 3$) before (BT) and after (AT) exposure to low, medium, and high turbulence. *Error bars* indicate 1 SD. Wavelengths obtained by auto-scanning emissions from 220 to 900 nm (ex = 280 nm)

Discussion

Turbulence experiments

Mortality

The percentages of live *T. weissflogii* and *S. costatum* cells, and total cell abundances were not significantly changed after exposure to low turbulence, indicating that low turbulence did not cause mortality. At medium turbulence, the percentage of intact dead *S. costatum* (stained by EB) increased significantly by 10%. Despite the fact that *T. weissflogii* cells were smaller than *S. costatum* cell chains, medium turbulence produced a 20% increase in intact dead *T. weissflogii* cells. High turbulence did not result in an increase in the percentage of intact dead *T. weissflogii* cells, but there was a significant decrease in total cell abundance, suggesting mortality via cell destruction. High turbulence caused a significant increase in intact dead cells and a decrease in total cell abundance in *S. costatum*.

Medium and high turbulence produced a 15 and 17% decrease in *T. weissflogii* cell abundance, and a 5 and 30% decrease in *S. costatum* cell abundance, respectively, indicating some differences in turbulence effects between the two species. *S. costatum* is a chain-forming diatom (9–12 μm solitary cells) and was often present in chains of three to four cells in our culture, giving an effective chain size of 36–48 μm,

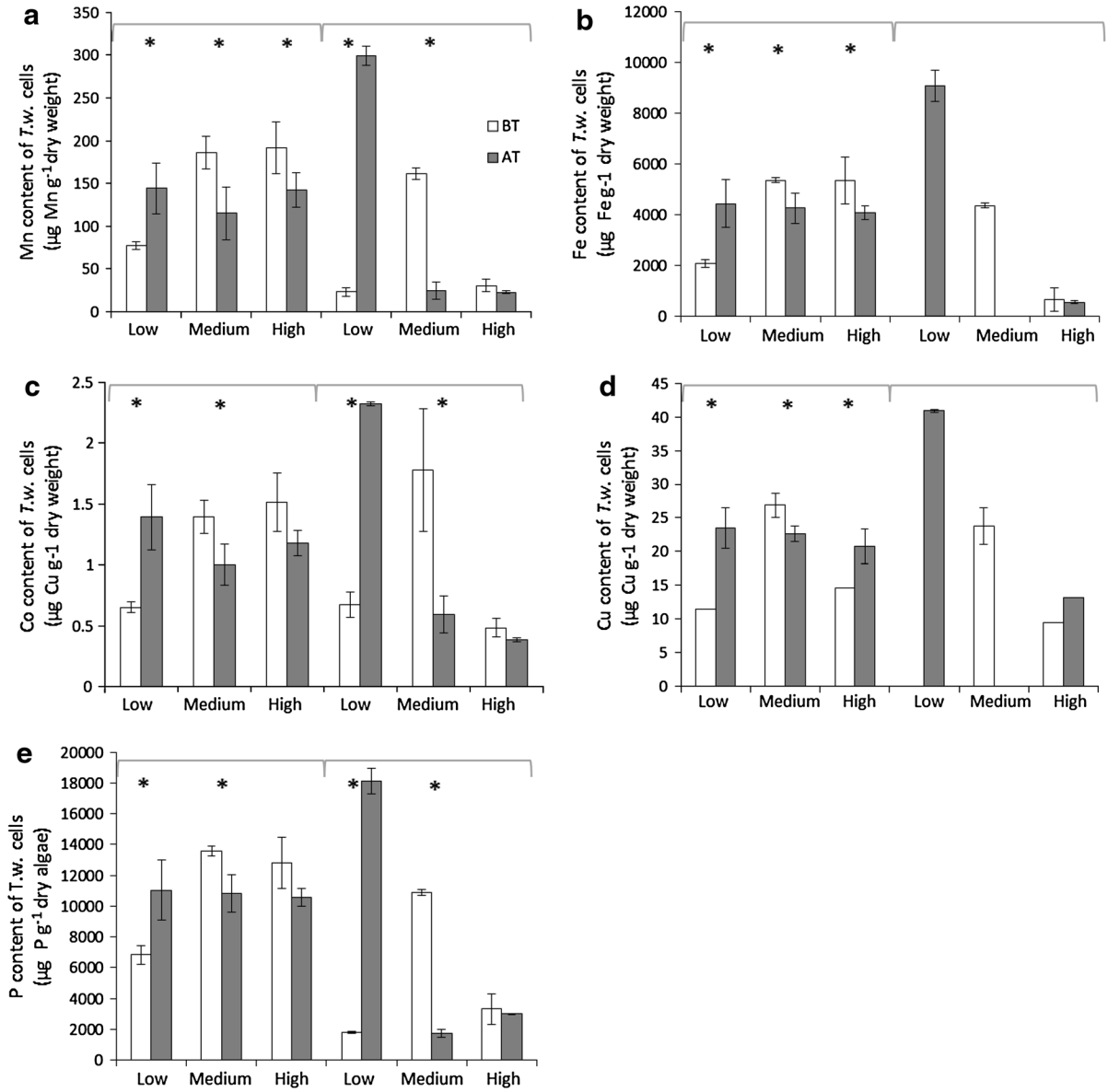


Fig. 8 ICPMS Analysis results for **a** manganese, **b** iron, **c** cobalt, **d** copper, and **e** phosphorus content of *T. weissflogii* cells before (BT) and after (AT) turbulence treatments. Each turbulence level was tested twice (indicated by *brackets*), and *error bars* indicate one standard deviation of duplicate samples. *Significant difference between before and after turbulence treatments (paired *t* test; $P < 0.05$)

whereas *T. weissflogii* single cells averaged 21 µm. After high and medium turbulence exposure, chain size of *S. costatum* was typically reduced to two cells. Low turbulence did not produce a significant decrease in cell abundance in either species. Since the effective size of *S. costatum* chains was larger than individual *T. weissflogii*, they may be more vulnerable than *T. weissflogii* to shear from turbulent eddies or velocity fields, causing a higher destruction of cells at high turbulence. However, *S. costatum* experienced a lower rate of cell destruction at medium turbulence, which may be attributed to the breakage of cell chains rather than destruction of individual cells.

Episodic turbulence appeared to affect the larger diatoms to a greater extent, causing a preferential reduction of the abundance of larger cells. Shear

forces at medium and high turbulence were strong enough to cause reduction in cell abundance, suggesting that episodic turbulence events in the marine environment may cause mechanical cell destruction, but only above a certain shear threshold and effective cell size. Phytoplankton size is important in determining the structure of marine food webs. A system dominated by small cells will recycle more biomass in the upper ocean as part of the microbial loop (Azam et al., 1983; Legendre & Le Fevre, 1995), whereas a system dominated by larger cells allows a greater transfer of energy to higher trophic levels (Cushing, 1989) and export to depth (Michaels & Silver, 1988). As such, episodic turbulence may intermittently alter the species dynamics within the phytoplankton community, and the associated trophic and flux processes.

Photosynthetic efficiency

In both *T. weissflogii* and *S. costatum*, active fluorescence showed that F_v/F_m was significantly reduced at high turbulence but not at low or medium turbulence. Similar reduction in photosynthetic efficiency has been reported for dinoflagellates, which in general are more sensitive to turbulence than diatoms, at an even lower turbulence level. For example, in a study of *Gonyaulax polyedra* (a dinoflagellate sensitive to turbulence effects), chlorophyll-specific photosynthetic rate (μmol C μmol chl^{-1} h^{-1}) decreased at a turbulence level of 0.18 cm^2 s^{-3} (the highest tested by Thomas et al., 1995, but still much lower than in our experiments). Rapidly changing physical regimes reduce photosynthetic efficiency transiently (Lewis et al., 1984), but when the regime change or perturbation persists over a period of hours, phytoplankton are able to acclimate their photochemical apparatus to optimize photosynthetic yield (Cullen & Lewis, 1988). The duration of episodic turbulence is short—on the order of seconds to minutes in the case of waves and boat propeller disturbances. Phytoplankton may be unable to adapt their photosynthetic machinery on this short timescale, thus leading to a reduction in photosynthetic capacity, as observed at high turbulence intensity in our experiments.

DOM release

DOM excretion is common even in healthy phytoplankton, and often consists of low molecular weight molecules such as amino acids, some polysaccharides, and nitrogenous compounds (Watt, 1969; Mague et al., 1980; Myklestad et al., 1989). Spectrofluorometer results showed an increase in optically reactive DOM at wavelengths 319–325 nm, especially in the high and medium turbulence experiments, a signature consistent with tyrosine-like amino acids and small proteins (Fellman et al., 2010; Ferrari and Mingazzini 1995). A second peak at ~688 nm appeared after both medium and high turbulence; this might represent humic-like compounds with low reactivity derived from larger cell fragments (Coble, 1996; Matthews et al., 1996).

One possible mechanism for this DOM release is the elimination of boundary layers by turbulent shear; i.e., in the absence of a stable boundary layer, diffusion gradients between the cell and ambient water increase and cause elevated DOM extrusion (Malits et al., 2004). We did not monitor the fate of materials released via cell destruction and DOM extrusion, but it is likely that the labile materials (319–325 nm) would be taken up by bacterial cells (Arin et al., 2002; Malits et al., 2004). Episodic turbulence may therefore cause increased flux of phytoplankton-derived organic materials to the microbial loop.

Trace metal release

The expected maximum amounts of metals (μg g^{-1} dry weight) in *T. weissflogii* cells are 9,151 for Fe, 28,650 for Mn, 600 for Co, and 915 for Cu (Ho et al., 2003; Finkel et al., 2006). Fe, Mn, Co and Cu levels detected in our experiments were well below their expected maximum values.

While the absolute amounts of cellular metals should be viewed with caution, comparison of before- and after-turbulence treatments did reveal some effects of turbulence on cellular trace metal content. *T. weissflogii* cells displayed significantly lower Mn, Co, and Fe concentrations after high and medium turbulence treatments, suggesting that the cells expelled the trace metals during turbulence exposure. In diatom cells, Mn and Fe are primarily located in chloroplasts as part of the photosynthetic machinery (Nuester et al., 2012), while Co, a component of carbonic anhydrase, is located in the cytoplasm (Twining & Baines, 2013). If episodic turbulence destroyed a diatom cell or if cellular matter was extruded as a stress response, one or all three of these

metals would be released, resulting in a decrease in metal amount per gram dry weight. P levels decreased at medium turbulence, indicating biomass loss, and tended to decrease but did not change significantly after high turbulence. Low turbulence may have stimulated increased uptake of P, while medium and high turbulence damaged cells, causing P to decrease through biomass loss. P is located in many components of the cell including the cytoplasm and cell membrane, so it is difficult to parse out the direct cause of these measurements.

Cu per gram dry weight, on the other hand, decreased after medium turbulence, but *increased* after high turbulence. Unlike Mn, Fe, and Co, Cu is primarily located in the cell membrane as part of a high-affinity iron uptake molecule (Twining & Baines, 2013). If turbulence caused a loss of cytoplasmic materials but Cu-rich frustules and membrane fragments remained in the particulate residues, that could lead to an increase in Cu per gram dry weight. Exposure to high turbulence caused 17% destruction of *T. weissflogii* cells, and we did find many cell fragments in the samples. This observation may explain the increase in the measured Cu per gram dry weight.

Bioavailable forms of Mn, Fe, Co, and Cu are rare in the euphotic zone due to relatively low supply and high uptake by phytoplankton (Danielsson et al., 1985; Coale & Bruland, 1988; Saito & Moffett, 2002). Bioavailable metals released from cells due to episodic turbulence may readily be removed by phytoplankton and microbes, which also benefit from release of labile DOM during turbulence (Malits et al., 2004). Metals that remain within intact dead cells may be transported to depth as part of the sinking flux (Fisher & Wente, 1992). Other phytoplankton genera of similar size or larger than the species we tested are expected to have a similar response to the mechanical shearing action produced by episodic turbulence.

Evaluation of the use of Evans Blue

A diverse suite of stains are available for analysis of marine plankton, such as SYBR-green for assessing cell cycle stage and other parameters, SYTOX-green for viability of phytoplankton, and Nile red for lipid evaluation (Yentsch & Campbell, 1991; Zetsche & Meysman, 2012). These stains are often only applicable to larger size classes of phytoplankton and mesozooplankton, and may exhibit high interspecific variability (Buma et al., 1995). EB was anecdotally tested earlier for differentiating live and dead phytoplankton, but the efficacy of the stain was not fully evaluated (Crippen & Perrier, 1974). To confirm usability across phytoplankton taxa, we tested the stain on two diatoms and two non-siliceous species. We also tested several killing methods (biocide, osmotic stress, UV, and heat) to ensure that the cause of death would not alter EB stain efficacy. Overall, the staining protocol yielded consistent and reliable results across all trials, making it particularly useful for environmental monitoring where mixed phytoplankton taxa and multiple mortality factors are expected.

Our results showed there was a significant linear relationship between expected and observed percent live phytoplankton cells; the regression also showed a positive *y*-intercept and a slope of 0.82 on average, suggesting that the stain underestimated the number of dead cells. Therefore, the results of EB staining should be treated as conservative estimates of phytoplankton mortality.

Cells as small as 6 μm produced visible stain color, but smaller cells were not tested. It is conceivable that application of EB stain on smaller cells would be difficult if their cytoplasms are not readily visible via light microscopy. When photographing stained samples, it is recommended that the samples be rinsed with filtered seawater after staining. Residual EB stain in the water decreases the amount of light that reaches the camera, producing a darkened image. EB staining in combination with flow cytometry provides fast quantification of live/dead phytoplankton. A portable flow cytometer may be used to directly assess the stained samples in the field, but EB-stained samples preserve well with formalin, allowing the samples to be examined at a later date.

The stain protocol is a simple and inexpensive way to quantify live/dead phytoplankton. It works on both diatoms and non-diatoms regardless of the mortality source, so long as the cells remain intact. The staining method, when combined with preservation and flow cytometry, should greatly expand its applications for laboratory and field studies as automated methods of enumerating and evaluating phytoplankton become increasingly available (Veldhuis & Kraay, 2000). Ecological and water quality monitoring, such as that in ballast water treatments, are potential applications of the stain. EB staining can provide insights into the

live/dead status of natural plankton assemblages that would be otherwise difficult to quantify.

Future perspectives

Conventionally, dinoflagellates are considered to be the primary phytoplankton group negatively influenced by turbulence (Thomas & Gibson, 1990; Estrada & Berdalet, 1997). However, the turbulence levels tested in this study—1.1–4.0 cm^2 s^{-3}—produced intact dead cells and lowered total cell abundance in both *T. weissflogii* and *S. costatum*, indicating that strong turbulence represents a source of mortality to diatoms. Furthermore, photosynthetic efficiency dropped 20–30% after high turbulence, indicating that photosynthesis may be inhibited by intense turbulence. DOM and trace metal release stimulated by turbulence may represent a flux of nutrients to heterotrophic bacteria, shunting organic material into the microbial loop. In localities that frequently experience intense turbulence, diatom mortality and nutrient release should be taken into consideration when evaluating the ecology and primary production of the system.

As storms and other extreme weather events are predicted to increase due to global climate change (Meehl et al., 2000), the results of this study will improve our understanding of how these events may affect phytoplankton. The mechanisms described may already play a role in diatom populations and nutrient cycling in regions that regularly experience elevated turbulence levels, such as coastal regions and areas that experience strong winds. Turbulence is a rule rather than an exception in the world's oceans and its role in altering the physical environment of individual phytoplankton is largely unknown. Rising sea surface temperature, decreasing pH (due to higher CO_2), and changing nutrient cycles related to climate change will all impact phytoplankton (Karl & Trenberth, 2003; Doney et al., 2009). Episodic turbulence may have an even stronger effect on phytoplankton when acting synergistically with those stressors. We suggest that more studies of sublethal and lethal effects of episodic turbulence in concert with other climate-related stressors will be a valuable addition to our understanding of the impacts of climate change.

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