Detection of UV-treatment effects on plankton by rapid analytic tools for ballast water compliance monitoring immediately following treatment

Johanna Bradie[a,*], Claudio Gianoli[b], Jianjun He[c], Alberto Lo Curto[d], Peter Stehouwer[e], Marcel Veldhuis[f], Nick Welschmeyer[g], Lawrence Younan[h], André Zaake[i], Sarah Bailey[a]

[a] Great Lakes Laboratory for Fisheries and Aquatic Sciences, Fisheries and Oceans Canada, 867 Lakeshore Rd., Burlington, Ontario L7S 1A1, Canada
[b] SGS Italia Spa, Via Campodoro 25, 35010 Villafranca Padovana (PD), Italy
[c] Technical Division, Satake Corporation, 2-30 Saijo Nishihonmachi, Higashi-Hiroshima-shi, Hiroshima-ken 739-8602, Japan
[d] Aqua-tools, 26 Rue Charles Edouard Jeanneret, 78300 Poissy, France
[e] SGS Institut Fresenius GmbH, Rödingsmarkt 16, 22087 Hamburg, Germany
[f] Marine Eco Analytics, Havenkade 1A, 1779 GS Den Oever, The Netherlands
[g] Moss Landing Marine Laboratories, 8272 Moss Landing Rd., Moss Landing, CA 95039, USA
[h] Turner Designs, Inc., 1995 N. 1st Street, San Jose, CA 95112, USA
[i] bbe Moldaenke GmbH, Preetzer Chaussee 177, 24222 Schwentinental, Germany

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ABSTRACT

Non-indigenous species seriously threaten native biodiversity. To reduce establishments, the International Maritime Organization established the Convention for the Control and Management of Ships' Ballast Water and Sediments which limits organism concentrations at discharge under regulation D-2. Most ships will comply by using on-board treatment systems to disinfect their ballast water. Port state control officers will need simple, rapid methods to detect compliance. Appropriate monitoring methods may be dependent on treatment type, since different treatments will affect organisms by a variety of mechanisms. Many indicative tools have been developed, but must be examined to ensure the measured variable is an appropriate signal for the response of the organisms to the applied treatment. We assessed the abilities of multiple analytic tools to rapidly detect the effects of a ballast water treatment system based on UV disinfection. All devices detected a large decrease in the concentrations of vital organisms $\geq 50\,\mu m$ and organisms $< 10\,\mu m$ (mean 82.7–99.7% decrease across devices), but results were more variable for the ≥ 10 to $< 50\,\mu m$ size class (mean 9.0–99.9% decrease across devices). Results confirm the necessity to choose tools capable of detecting the damage inflicted on living organisms, as examined herein for UV-C treatment systems.

1. Introduction

Non-indigenous species (NIS) are one of the greatest causes of species endangerment, (Lawler et al., 2006) and have caused significant direct and indirect economic consequences (Pimentel et al., 2005; Colautti et al., 2006). Ballast water, which is used to control ships' trim and stability, provides a vector for the movement of NIS around the globe as a by-product of global shipping operations. Ballast water has historically been one of the most important introduction vectors for aquatic NIS establishments (Carlton, 1985; MacIsaac et al., 2002), with over 3500 million tonnes of ballast water and entrained biota moved around the globe annually (Endresen et al., 2004).

The Convention for the Control and Management of Ships' Ballast Water and Sediments, (2004; BWMC) was established by the International Maritime Organization (IMO) to reduce the threat of NIS establishments from shipping. The BWMC limits allowable concentrations of organisms in ballast water discharge under Regulation D-2[7] to (i) < 10 viable organisms $/m^3 \geq 50\,\mu m$ in minimum dimension, (ii) < 10 viable organisms $/ml < 50\,\mu m$ in minimum dimension and $\geq 10\,\mu m$ in minimum dimension (hereafter, 10–50 μm), and also limits discharge concentrations for certain indicator microbes as a human health standard (i.e. toxigenic *Vibrio cholerae*, *Escherichia coli*, Intestinal Enterococci) (International Maritime Organization, 2004). The BWMC has been fully ratified and will enter into force on September 8, 2017. The United States is not a signatory to the convention, but instead has its own discharge standard. The limit values of the USCG ballast water discharge standard mirror those of the D-2 Ballast Water Performance Standard, except that the D-2 standard states 'viable' organisms while

the USCG standard states 'living' organisms. The timelines by which ships must meet the standards also varies slightly, according to the age and certification or dry-docking requirements of each ship, such that most ships will have to meet the standards by 2021 (US) or 2024 (IMO).

The BWMC (and parallel US regulations) has led to efforts worldwide to develop ballast water management systems (BWMS) to treat ballast water to eliminate or render harmless any organisms contained therein. Most BWMS consist of two stages: a mechanical solid-liquid separation (e.g. filtration, hydrocyclones) and a subsequent disinfection procedure, either by chemical (e.g. chlorination, ozonation) or physical treatment (e.g. ultraviolet (UV) irradiation, deoxygenation) (Tsolaki & Diamadopoulos, 2010). Indeed, numerous treatment technologies have been explored including filtration, biocides, heat exposure, electric pulse treatment, UV irradiation, ultrasound, magnetic fields, deoxygenation, and antifouling coatings (Tsolaki & Diamadopoulos, 2010; Mamlook et al., 2007). Nearly 70 BWMS have received IMO Type Approval Certification by their respective Administrations (resolutions MEPC.175(58) and MEPC.228(65)) (International Maritime Organization, 2016) following 'Guidelines for approval of ballast water management systems' established by the International Maritime Organization (International Maritime Organization, 2008). Type Approval signifies that the BWMS has been demonstrated to meet Regulation D-2 discharge standards when subjected to rigorous testing, including both shore-based and shipboard phases (Lloyd's Register, 2015). Further, Type Approval indicates that the BWMS has necessary documentation and complies with prescribed requirements for installation onboard of ships. The US requires its own Type Approvals separate from the IMO process; since 2016 the USCG has issued 5 BWMS with US Type Approvals (as of August 2017). Type Approved systems are expected to effectively manage ballast water, facilitating compliance with discharge standards when used operationally on ships. Nonetheless, it is important that Port State Control Officers have the ability to assess the ballast water of vessels for compliance to ensure that BWMS are being operated and maintained correctly and are functioning consistently over time.

Given the recent ratification of the BWMC, there is a heightened interest in determining how compliance will be monitored by Port State Control Officers (Akram et al., 2015; Drake et al., 2014). A number of 'indicative' analytic tools have been developed that can quickly and easily be used to measure water samples and provide either numeric or, more typically, biomass-based estimates of the concentration of living organisms (First & Drake, 2013; Bradie et al., 2017). In contrast, detailed methods can provide a direct measure of numeric concentrations, but require extensive expertise, a longer timeframe, and costly equipment. Ideally, indicative tools will be used to rapidly indicate whether ballast water is non-compliant with Regulation D-2 and therefore unacceptable for discharge. Recent studies have compared detailed methods and indicative tools to determine how well the results of the various methods agree e.g. (Bradie, 2016). Generally, 'indicative' methods are based on assessing vital signs, which are indicative of the viability of the organisms. These methods belong to one of three main types: tools that (i) measure chlorophyll fluorescence activity (CFA), (ii) measure adenosine triphosphate (ATP), and (iii) use fluorescein diacetate (FDA). Each type measures a different aspect of life, specifically (i) the ability of photosynthetic autotrophs to execute photosynthesis (Veldhuis et al., 2006; Wright, 2012), (ii) the ability of cells to produce cellular energy, ATP (Wright, 2012), and (iii) the integrity of metabolic processes, specifically, enzyme activity of non-specific intracellular esterases (Welschmeyer & Maurer, 2011), respectively. Thus, these tools measure specific indicators of vitality that are positively correlated with the abundance or concentration of intact cells (Hammes et al., 2010; Falkowski & Kiefer, 1985; Maurer, 2013), and hence are used as 'biomass indicator estimates'. The use of vitality indicators can pose the following issues for compliance monitoring after treatment: 1) if treatment does not lead to immediate cell death, indicative tools may not detect the damage inflicted by treatment if the targeted biomass indicator is not immediately affected by treatment, 2) if the treatment does cause immediate cell death, it is important that the targeted

biomass indicators are rapidly degraded with cell death, so there is no residual signal due to indicators that remain post-mortem (i.e. no time delayed effects). Further, it is important that there is no interference of treatment chemicals with the indicators. Since the active mechanisms utilized by different treatment technologies may affect organism vitality or viability by disrupting different vital cellular processes that eventually, but not necessarily immediately, lead to cell death, it is possible that tools may perform differently on treated samples depending on the type of treatment applied and how the treatment disrupts cellular processes.

For these reasons, it has been recognized that the appropriate analysis method for a sample may be treatment-specific, such that the indicative analytic tool applied should measure a vital sign that is rapidly influenced by the applied treatment (First & Drake, 2014). Thus, it is important to distinguish between tools that measure different vital signs (e.g. homeostasis, metabolism, growth or reproduction), and identify those that are able to recognize treatment effects immediately after application so that compliance monitoring can be conducted in a timely manner. Herein, we assess the ability of rapid, analytical tools and detailed methods to detect the effects of a UV-C treatment in water samples collected immediately after treatment. Importantly, while IMO discharge standards relate to viability, defined as the ability to successfully generate new individuals in order to reproduce the species, none of the indicative analytic tools measure viability directly. Herein, our assessment focuses on tools that detect vitality.

2. Methods

2.1. Sample collection

Samples were collected and analyzed on board the RV Meteor while transiting across the North Atlantic Ocean (see Bradie et al., 2017) for details. The RV Meteor is equipped with an Optimarin OBS80 BWMS consisting of a filter (125FS Filtersafe filter; 40 μm), UV reactor (capacity 0–2500 W/m^2), and multiple sampling ports to facilitate sample collection for research and compliance monitoring. The Optimarin system has been type approved by both the IMO and USCG (although note that the USCG-approved Optimarin system uses a different filter type, and the Optimarin system with Filtersafe filter (used here) here is pending testing for USCG Type Approval). Depending on dosage, UV treatment predominantly damages deoxyribonucleic acid (DNA) rendering organisms unable to reproduce (Quek & Hu, 2008), or in combination with other vital cell processes can result in immediate mortality (Cullen & MacIntyre, 2016). When exposed to light, UV treated phytoplankton will disintegrate in a matter of hours. In the dark, the phytoplankton can survive for up to two weeks after treatment but will again disintegrate after exposure to light showing that repair mechanisms are unable to cope with the damage caused by UV (Liebich, 2013). Our first trial was run with 'power control' UV dosage wherein the optimal UV dosage is determined based on UV transmission in the water, whereas the latter two trials were run with 'unregulated' UV dosage to more closely mimic the operational setup used to meet requirements for USCG Type Approval. Since our objective was to determine whether compliance monitoring tools can detect UV treatment effects immediately after treatment is applied, rather than to test the BWMS efficacy, this change in treatment application should not affect our results. Indeed, given that type approvals have already been granted, our trials assume that the Optimarin system does effectively reduce the concentration of organisms in ballast water, and our trials solely focused on testing whether the indicative analytic tools can detect the decrease. We did not observe any differences in tool performance between 'power control' and 'unregulated' trials.

For each trial, standard ballasting procedures were used to pump sea water through the ship's ballast water system. Three separate trials were run with uptake events occurring ~300 km off the coasts of Portugal and Spain. Note that these regions are generally characterized
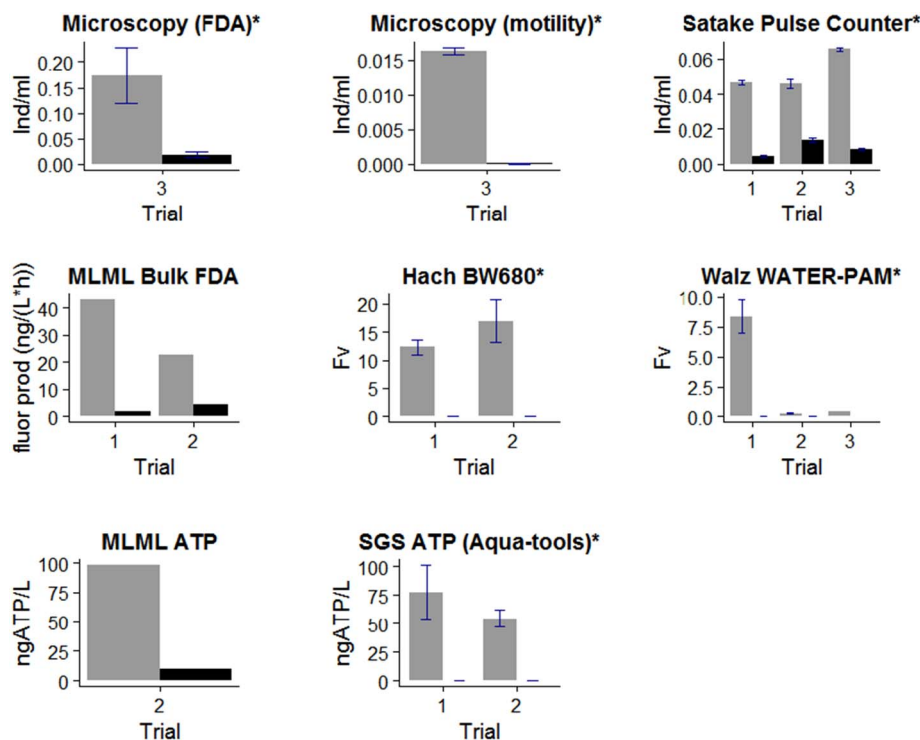
Fig. 1. Bar graphs of estimated mean concentrations (± SD) before and after treatment with the Optimarin BWMS for ≥ 50 μm organisms. Error bars are not indicated for MLML ATP and MLML Bulk FDA when replicate samples were not measured. Grey bars show estimated concentrations before treatment, and black bars show concentrations after treatment. (*) indicates that a significant decrease in live organism concentrations was detected after treatment (One-way paired *t*-tests on log + 1 transformed data with Welch's correction for unequal variance; all p < 0.05).

as 'blue water' with low organism densities, few particles, and low organic matter. The sampling ports on the ballast water system allowed for the simultaneous collection of paired samples of water before and immediately after treatment with the BWMS.

In each trial, samples were obtained by filtering water through a plankton net (mesh size: 50 μm in diagonal) within a wetted sample tub. Each net filtered ~1000 L of water for analysis of ≥ 50 μm size class that was concentrated to 1 L, with the actual volume of water filtered quantified using a Seametrics WMP104-100 magnetic flow meter so that appropriate concentrations could be back-calculated for sample comparisons. Additionally, between 13 and 16 L of water was collected directly from the containment drum after passing through the plankton net for analysis of 10–50 μm organisms and microbes. The 10–50 μm sample was filtered on a 10 μm Sterlitech polyester track etch (PETE) membrane filter with retained particles resuspended in filter-sterile seawater to eliminate particles < 10 μm and to concentrate the sample (up to 16 × times) for analysis. Individual sample bottles were prepared for each analytic tool by mixing each sample 5 times by gentle inversion, half-filling each sample bottle and repeating this procedure to fill the bottles.

For each trial, a common batch of particle free seawater was prepared for use as rinse water during sample collection and sample analysis. Rinse water was prepared by filtering local sea water obtained using the vessel's seawater taps through successive 1000 μm, 500 μm, 35 μm and 8 μm plankton mesh filters, and finally, through a 0.2 μm passive (gravity-fed) filter cartridge (Whatman Polycap TC). After each trial, all sampling gear and sample bottles were cleaned in a dilute (100–200 ppm) or concentrated (2500 ppm) bleach bath (depending on equipment hardiness) using Natriumhypochlorid 12% Chlor aktiv bleach to prevent cross-contamination of living organisms between tests. After bleaching, all equipment was rinsed with MilliQ water three times and nets were soaked in rinse water and hung to dry. Prior to reuse, all sample bottles were rinsed with filtered sea water three times to

remove any residues.

2.2. Sample analysis

Samples were analyzed in parallel using a number of detailed and indicative analytic methods for ≥ 50 μm, 10–50 μm, and < 10 μm size classes. While the < 10 μm size class is not explicitly referenced in the D-2 or US regulations, we include it here since the regulated bacteria belong to this size class. Detailed analytic methods included visual microscopy using a dissecting microscope (≥ 50 μm) and epifluorescence microscopy of samples stained using FDA (≥ 50 μm and 10–50 μm). The indicative analytic tools tested represented three main types: (i) tools that measure chlorophyll fluorescence activity (CFA), including Walz WATER-PAM, Turner Designs' BallastCheck-2™, bbe 10 cells, and Hach BW680, (ii) tools that measure adenosine triphosphate (ATP), including Moss Landing Marine Labs (MLML) ATP, and SGS ATP (aqua-tools), and (iii) tools that use fluorescein diacetate (FDA), including MLML bulk FDA and the Satake Pulse Counter. A full description and Standard Operating Procedures for each device is available in (Bradie et al., 2017). Additional methods for sample analysis are available, such as flow cytometry and culture based methods, but these were not used here as we restricted our analyses to tools that can be used rapidly to detect non-compliance. Microscopy was included as the historical standard (baseline) for detailed analysis of ballast water samples.

Three replicate subsamples were measured for each sample, with all analysis steps performed independently on each replicate. Due to time constraints and operational considerations (e.g. limited materials), not all analytic methods could be performed for all trials.

2.3. Statistical analysis

All results were standardized to equivalent concentrations in ballast

Table 1
Decrease in live biomass (%) ± SE for ≥ 50 μm organisms, 10–50 μm organisms, and < 10 μm organisms detected by various analytic tools after treatment with a filtration + ultraviolet (UV) BWMS and number of trials and the total number of repeats for each analytic method.

Analytic method	Size class	Live biomass reduction ± SE (%)	Trials (n)	Total repeats (n)
Hach BW680	≥ 50 μm	99.7 ± 0.2	2	6
Microscopy (FDA)	≥ 50 μm	89.3	1	3
Microscopy (motility)	≥ 50 μm	99.9	1	3
MLML ATP	≥ 50 μm	89.6	1	1
MLML bulk FDA	≥ 50 μm	88.5 ± 7.1	2	2
Satake pulse counter	≥ 50 μm	82.7 ± 10.8	3	9
SGS ATP (aqua-tools)	≥ 50 μm	99.5 ± 0.1	2	6
Walz WATER-PAM	≥ 50 μm	99.4 ± 1.1	3	7
bbe 10cells	10–50 μm	98.7 ± 1.1	3	9
Hach BW680	10–50 μm	97.9 ± 2.5	3	10
Microscopy (motility)	10–50 μm	82.7	1	3
MLML ATP	10–50 μm	55.2 ± 41.5	3	9
MLML bulk FDA	10–50 μm	9.0 ± 24.5	3	9
Satake pulse counter	10–50 μm	89. ± 15.2	3	9
TD BallastCheck2	10–50 μm	97.1 ± 0.7	3	9
Walz WATER-PAM	10–50 μm	99.9 ± 0.4	3	9
Hach BW680	< 10 μm	92.9 ± 0.8	2	7
MLML ATP	< 10 μm	92.5 ± 1.4	2	6
MLML bulk FDA	< 10 μm	85.8 ± .50	2	6
TD BallastCheck2	< 10 μm	89.7 ± 7.9	3	9
Walz WATER-PAM	< 10 μm	99.7 ± 0.4	3	9
SGS ATP (aqua-tools)	0.7–2.7 μm	96.3 ± 2.1	3	9

water prior to analysis (i.e. concentration steps during sample processing or analysis were taken under consideration to determine organism concentrations; final concentration = measured concentration / sample concentration). One-way paired t-tests on log + 1 transformed data with Welch's correction for unequal variance were used to determine if a significant decrease in organism concentration was detected by each analytic method following treatment. Shapiro-Wilk normality tests were used to verify that the transformed data did not violate assumptions of normality. P-values < 0.05 were deemed to be significant.

To compare the magnitude of reduction in concentration detected by each analytic method, we calculated the percent reduction in concentration as: reduction (%) = 1 – Measurement after treatment / Measurement before treatment. While each technique measures a different variable (e.g. numeric concentration, various biomass indicators), this metric allows us to compare the magnitude of reduction in concentration after treatment across analytic methods. Hereafter, we will use 'numeric concentration' for measures estimating a numeric concentration of organisms, 'biomass concentration' for measures estimating a concentration of a biomass based on an indicator where measurements are provided in units other than numeric concentrations, and simply 'organism concentration' to reference all measurements (i.e. inclusive of 'numeric' and 'biomass' concentrations). To distinguish between measurements where the full complement of vital (hereafter, live) and non-vital (hereafter, dead) organisms in the sample was measured versus those that measure only live organisms, we will use 'total' and 'live', respectively. This allows us to report results of analytic methods where separate measures of total and vital biomass concentration after treatment were available (Walz WATER-PAM and TD Ballast Check2™; F_0 and Fv respectively). Standard deviation (SD) is used to report variability between replicates and standard error (se) is used to report variation between trials. All analyses were performed in the R statistical programming environment (R Core Team, 2015).

3. Results

Almost all analytic methods showed a consistent decrease in concentration of live organisms after treatment across trials. For the ≥ 50 μm size class, samples were analyzed using microscopy (both with

an epifluorescent microscope for FDA stained samples and using a dissecting microscope to detect motility), the Satake Pulse Counter, MLML bulk FDA, Hach BW680, Walz WATER-PAM, MLML ATP and SGS ATP (Aqua-tools). A significant decrease in live organism concentrations after treatment was detected by the majority of analytic methods (Fig. 1; one-way paired t-tests on log + 1 transformed data with Welch's correction for unequal variance; all p < 0.05). The MLML bulk FDA and MLML ATP methods did not detect a significant decrease in live biomass concentrations; since these techniques were not used in all trials, statistical power may have been insufficient to detect a difference at the desired significance level (Fig. 1). The mean decrease in live organism concentrations across trials varied between 82.9 and 99.8% across analytic methods (Table 1).

Samples for the 10–50 μm size class were analyzed using epifluorescent microscopy, Satake Pulse Counter, MLML ATP, MLML bulk FDA, TD BallastCheck2™, bbe 10 cells, Hach BW680, and Walz WATER-PAM. For this size class, there was greater variability between analytic tools in the average detected decrease in live biomass concentrations (Table 1). A significant decrease in live organism concentrations after treatment was detected by each analytic method (Fig. 2; one-way paired t-tests on log + 1 transformed data with Welch's correction for unequal variance; all p < 0.05), except for the MLML bulk FDA method (p > 0.05). All CFA tools showed greater than a 97% decrease in the live (phytoplankton) biomass concentration (i.e. bbe 10cells, Hach BW680, TD BallastCheck2, Walz WATER-PAM). The Satake Pulse Counter and microscopy, both of which are based on the FDA staining method, performed similarly with an 89.1% (se = 8.7%) and 82.7% (1 trial only) reduction in numeric concentration, respectively. In contrast, MLML ATP showed a modest 55.2% decrease in live biomass concentration, with a considerable variability between trials (se = 24%), and MLML bulk FDA showed very little decrease (9%).

Although not part of the IMO nor US Regulations, organisms in the size class < 10 μm were included in the analysis. All analytic methods detected a significant decrease in live organism concentrations in this size class of organisms (Fig. 3; one-way paired t-tests on log + 1 transformed data with Welch's correction for unequal variance; all p < 0.05), which were measured using MLML ATP, SGS ATP (Aqua-tools), MLML bulk FDA, TD BallastCheck2, Hach BW680, and Walz WATER-PAM. Each method detected > 85% decrease in live biomass
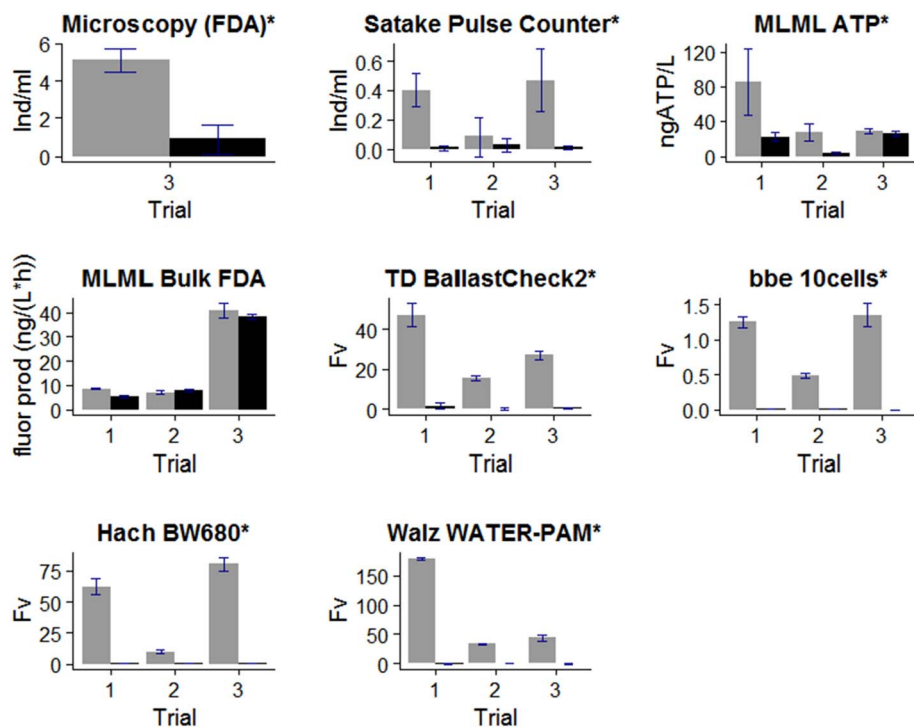
Fig. 2. Bar graphs of estimated mean concentrations (± SD) before and after treatment with the Optimarin BWMS for 10–50 μm organisms. Grey bars show estimated concentrations before treatment, and black bars show concentrations after treatment. (*) indicates that a significant decrease in live organism concentrations was detected after treatment (One-way paired *t*-tests on log + 1 transformed data with Welch's correction for unequal variance; all $p < 0.05$).

concentrations after treatment (Table 1). Note that SGS ATP (Aquatools) measures a subset of < 10 μm organisms, focusing specifically on particles between 0.7 and 2.7 μm (i.e. bacteria).

Two CFA tools, the Walz WATER-PAM (all size classes) and the TD BallastCheck2™ (< 50 μm only), provided estimates of both the total reduction in phytoplankton chlorophyll biomass, measured by change

in F_0 (i.e. chlorophyll autofluorescence), and the reduction in live biomass, measured by change in F_v (i.e. active chlorophyll fluorescence). For the ≥ 50 μm size class, there was a decline of 44.9% (se = 0.3%) of the total chlorophyll biomass compared to 99.4% (se = 0.01%) of the live biomass after treatment (Walz WATER-PAM). For the 10–50 μm size class, much of the total biomass concentration in
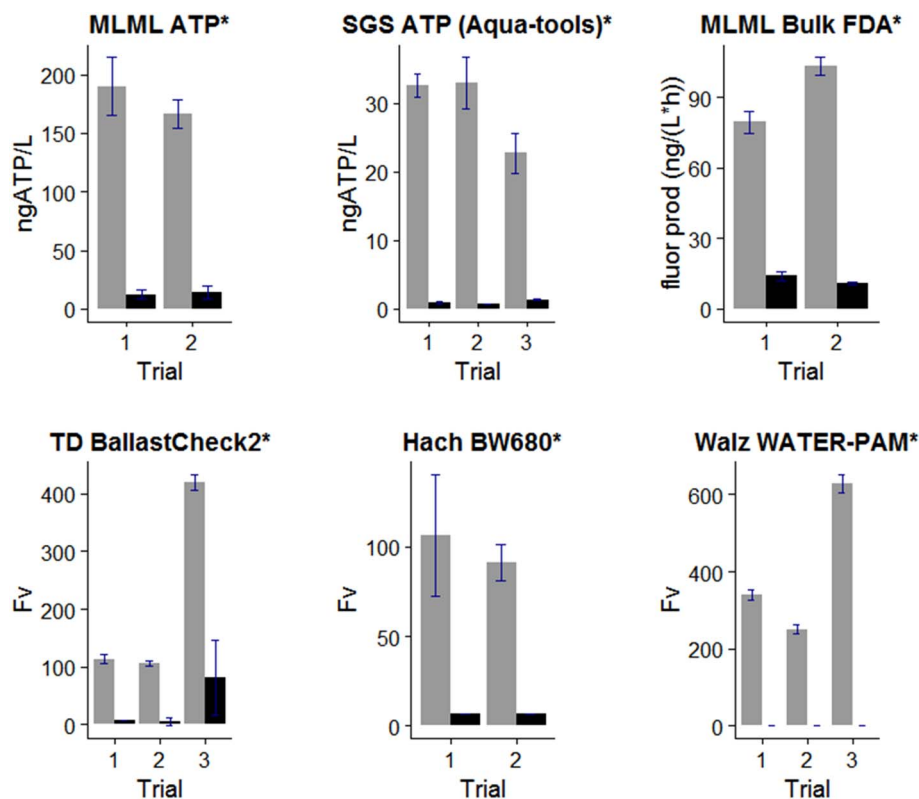

Fig. 3. Bar graphs of estimated mean concentrations (± SD) before and after treatment with the Optimarin BWMS for < 10 μm organisms. Grey bars show estimated concentrations before treatment, and black bars show concentrations after treatment. (*) indicates that a significant decrease in live organism concentrations was detected after treatment (One-way paired *t*-tests on log + 1 transformed data with Welch's correction for unequal variance; all $p < 0.05$).

Table 2

Total biomass $\pm$ SE (%) and live biomass $\pm$ SE (%) remaining after treatment for ≥ 50 µm organisms, 10–50 µm organisms, and < 10 µm organisms. Total biomass was measured as change in baseline fluorescence under dark adaptation (F_0), and live biomass was measured as total active chlorophyll fluorescence (F_v) by Walz WATER-PAM and TD BallastCheck2 as indicated.

Analytic method	Size class	F0: Total biomass remaining after treatment $\pm$ SE (%)	Fv: Live biomass remaining after treatment $\pm$ SE (%)
Walz WATER-PAM	≥ 50 µm	55.1 $\pm$ 40.0	0.6 $\pm$ 1.2
TD BallastCheck2	10–50 µm	76.6 $\pm$ 12.2	2.9 $\pm$ 0.7
Walz WATER-PAM	10–50 µm	106.7 $\pm$ 19.1	0.1 $\pm$ 0.4
TD BallastCheck2	< 10 µm	21.7 $\pm$ 5.1	10.3 $\pm$ 7.9
Walz WATER-PAM	< 10 µm	33.8 $\pm$ 5.5	0.3 $\pm$ 0.4

the sample remained present after UV-treatment (mean remaining biomass was 76.7% and 106.7%, for TD BallastCheck2 and Walz WATER-PAM, respectively), but there was a large decrease in live biomass concentrations ($< 3\%$ remaining for both tools) (Table 2; TD BallastCheck2 and Walz WATER-PAM results). Finally, for the < 10 µm size class, there was a large decrease in both total phytoplankton biomass remaining after treatment (mean 21.7% and 33.8% total chlorophyll fluorescence remaining for TD BallastCheck2 and Walz WATER-PAM, respectively) and the amount of live phytoplankton biomass remaining after the treatment (mean 10.3% and 0.3% live biomass remaining) (Table 2).

4. Discussion

Our results indicated that nearly all tools applied detected a large decrease in organism concentrations immediately following UV treatment (exception: MLML bulk FDA for 10–50 µm organisms). Importantly, there was a contrast in results between the size classes. For ≥ 50 µm and < 10 µm organisms, all tools were able to immediately detect a large decline in organism concentrations (≥ 50 µm: mean across methods 93.4%, range 82.7–99.9%; < 10 µm: mean 93.3%, range 85.8–99.7%), whereas there was more variability among tools for 10–50 µm organisms (mean across methods: 73.4%, range 9–99.9%). These results are not surprising given the two-phase treatment process: larger organisms (≥ 50 µm) are largely removed from ballast water by means of the combined filtration at intake and UV-treatment, so the bulk of these organisms would be entirely absent from samples collected after treatment and the biomass indicator targeted by the analytic device would be irrelevant. In contrast, the effects of the BWMS on smaller organisms (< 50 µm) would largely depend on the UV treatment (Tsolaki & Diamadopoulos, 2010). In general, UV treatment damages cells and depending on the resistance of the organisms, the damage can lead to immediate mortality, delayed mortality, or potentially be reversible. The ability for organisms to tolerate the effects of UV is influenced by a number of factors, but notably, there is a size-dependency whereby smaller organisms are more susceptible to UV than larger organisms (Newman & Clements, 2008). For organisms < 50 µm, our results showed that a large decrease in live organism concentrations was detected by all methods for the smaller fraction (< 10 µm), whereas only some tools detected a large decrease for the 10–50 µm organisms. Specifically, MLML ATP detected only a 55.2% reduction in live biomass concentration for 10–50 µm organisms and a 92.5% reduction in live biomass concentration for the < 10 µm organisms. Similarly, MLML Bulk FDA detected a 9% reduction in live biomass concentration for 10–50 µm organisms and an 85.8% reduction in live biomass concentration for < 10 µm organisms. This may indicate that < 10 µm organisms are being completely disintegrated by UV treatment whereas the 10–50 µm organisms are still present, albeit dead or damaged and non-viable. This hypothesis is supported by our results that showed that much of the total biomass for the 10–50 µm size class remained after treatment (76.7–106.7%) compared to relatively little for the < 10 µm size class (21.7–33.8%), and also by past research that showed that UV is a commonly applied technique to disinfect bacteria present in water (Laroussi et al., 2002; Bolton & Linden, 2003). Our

results do not allow us to determine whether the analytic tools are measuring a delayed signal from a biomass indicator that has not degraded despite organism death, whether the analytic tools are measuring cellular function from living, but non-viable, organisms, or both.

Practical differences in the methods may be responsible for differences in the tools' respective abilities to immediately detect the effects of UV radiation. This may have connotations for which analytic tools can be used in combination with certain BWMS technologies. In addition to microscopy, three main approaches were employed in the tools used herein: ATP measurement, CFA measurements, and FDA methods. ATP is a key element in energy metabolism for all living organisms and can be quantified to determine the biomass of living micro-organisms present in a sample (Wright, 2012). ATP detection involves an enzymatic reaction where luciferase produces light in the presence of the chemical luciferin and ATP, and the amount of light energy released is directly proportional to the quantity of ATP present. While the first ATP-metry tools appeared in the 1970s (Hodson et al., 1976), recent advances, including microfiltration, have greatly increased the sensitivity and accuracy of measurements (First & Drake, 2013). While UV may not directly affect ATP, cell damage inhibits the organism's ability to generate new ATP. Normally, the pre-existing ATP inside the cell lasts for only few hours before it is exhausted leading to organism death. Both the SGS ATP (Aqua-tools) method and the MLML ATP method measure intracellular ATP, so any cells that have released their ATP due to cellular damage will not be detected. Thus, the tests are capable of detecting the effects of UV in less than one hour to determine whether a UV treatment system is functioning.

CFA methods measure the natural photosynthetic activity of chlorophyll-containing cells (Veldhuis et al., 2006; Wright, 2012), and thus measure only photosynthetic autotrophs, which we conveniently refer to as 'phytoplankton' (i.e. CFA methods cannot detect heterotrophs). The CFA devices, Walz WATER-PAM, bbe 10cells, TD Ballast-Check2, and Hach BW680, have been shown in previous work to perform well as compliance monitoring tools (Bradie et al., 2017; Gollasch et al., 2015). In this study, all were able to detect a significant difference in live phytoplankton biomass concentrations immediately after treatment and showed the greatest decrease across all tool types and for all size classes including 10–50 µm organisms ($> 97\%$). These tools measure baseline fluorescence under dark adaptation (F_0), and maximal fluorescence (F_m) under saturating light to estimate total active chlorophyll fluorescence (F_v) in the subsample ($Fv = Fm - Fo$) (Wright et al., 2015). Changes in F_0 can be used to estimate change in total biomass, whereas changes in F_v are related to changes in live biomass. Thus we are able to obtain measurements of total biomass and an indication of health of the cells using CFA devices. Notably, since these tools only measure photosynthetic autotrophs, they do not provide any indication of whether treatment has eliminated heterotrophs from the ballast water, however, these tools show much promise for verification that a treatment system is operational. However, it should be noted that within the size class of 10–50 µm phytoplankton is by far the dominant group ($> 95\%$ of total abundance, Welschmeyer & Veldhuis, personal observations).

FDA methods measure enzymatic activity of non-specific intracellular esterases that hydrolyse FDA resulting in the formation of a fluorescent molecule (fluorescein) (Rotman & Papermaster, 1966).

Fluorescein fluoresces green when exposed to blue light at an intensity proportional to the total living biomass in the samples (Welschmeyer & Maurer, 2011) and requires an intact cell membrane for stain accumulation (Rotman & Papermaster, 1966). This principle is applied in different ways depending on the specific analytic method. For instance, epifluorescence microscopy with FDA and the Satake Pulse Counter use both enzyme activity and membrane integrity to indicate viability. Specifically, they employ a counting procedure to enumerate the number of living cells which are identified by positive fluorescence (i.e. enzyme activity necessary for cells to fluoresce, membrane integrity is necessary for fluorescein to remain in the cell) (First & Drake, 2013). Both of these methods were able to detect a significant decrease in the number of live cells after UV treatment. In contrast, MLML bulk FDA relies solely on enzyme activity, cellular and extra-cellular. This offers benefits in some scenarios since it eliminates the necessity for a counting procedure, but it reduces its utility for analyzing UV-treated samples, since it measures both internal and external fluorescein accumulation. Indeed, false positives are possible for two main reasons: (i) remnant enzymes inside dead, dying or DNA-damaged (UV) organisms could cause FDA to hydrolyze to fluorescein, and (ii) FDA can hydrolyze to fluorescein abiotically in alkaline solutions (Welschmeyer & Maurer, 2011), with greater hydrolysis occurring with increasing pH (Alarcón-Gutiérrez et al., 2008). This may occur if the sample itself had alkaline pH or if there was residual cellular alkalinity due to cellular ATP production which can create a proton gradient on opposite sides of certain biological membranes. Notably, FDA can also stain organic detritus which could cause erroneous results for ballast water compliance monitoring, but is unlikely to influence our results here since low levels of detrital material were observed in our samples.

We note four caveats of our results. First, instruments can differ in their detection limits. It is important to keep this in mind when comparing results, since instruments that are unable to detect low organism concentrations may appear to show a greater effect of treatment simply because they are registering zero values when only a few organisms remain. This is unlikely to be a large issue for compliance monitoring since most devices function well at low concentrations. More specifically, we don't expect this to be an issue herein, because although prior work found that the minimum detection limit of the Hach BW680 was lower than that for other CFA devices (Bradie et al., 2017), our results do not show an inflated reduction in live organism concentrations for this device compared to the other CFA devices. Second, while all samples were prepared at the same time, sample analysis may have been completed anywhere between minutes and hours after the sample was prepared owing to the different processing times associated with each device. It is possible that this may contribute to differences in results, since the effects of UV could be more apparent to tools that have a longer processing time if organisms were damaged, but not destroyed, by treatment. Regardless, we expect that it shows a realistic scenario of the types of results that could be expected during compliance monitoring since the 'sample to result' timeline would be similar in practice. Third, as detailed in our methodology, the methods analyzed herein provide data as either numeric concentrations or biomass indicator concentrations. Only some of the biomass indicators can be converted to numeric organism concentrations, and where possible, conversions are subject to wide confidence intervals dependent on the community composition (Bradie, 2016). This provides an added complexity to the use of tools that rely on the use of biomass indicators, which merit further investigation, but may not be important if the purpose is to verify that a system is working rather than to quantify exact numeric organisms concentrations in ballast water. Fourth, our results only focus on whether there is a reduction in plankton concentrations immediately after treatment. We do not assess viability, monitor samples for regrowth, or consider the potential and consequences of DNA repair. For the purposes of this paper, we assume that the treatment technology is able to reduce organism concentrations to acceptable discharge limits

based on type approval, and simply examine which tools can detect the decrease in organism concentrations immediately after treatment is applied. This is useful for compliance monitoring purposes as samples would need to be taken and analyzed quickly in order to ascertain whether a ship is compliant with regulations and can discharge water. In-depth assessments of whether re-growth occurs are beyond the scope of our study.

In conclusion, we found differences across analytic tools and across size classes in the ability to detect UV treatment, and we suggest that multiple tools may be needed for compliance monitoring depending on the type of treatment that has been applied and the size class of organisms to be measured. While our results are specific to UV systems, the general premise should apply to all types of BWMS. Since different treatment processes affect viability in different ways, it will be important future work to assess the reliability of compliance monitoring tools with different types of BWMS systems.

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