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Letter to *Management of Biological Invasions*

Response to Letter to the Editor from Hull and Linden (2020)

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Abstract

We appreciate Natalie M. Hull and Karl G. Linden's interest in our recent article in which we examined a novel application of a CyQuant®-based approach for examining DNA concentrations of UV- and chlorination-treated organisms. Hull and Linden questioned our work, which was conducted to inform considerations of regulating ballast water discharges on the basis of the number of viable organisms, and several concerns were identified. Here we provide our response to the specific concerns raised about experimental design, procedure, and interpretation.

Key words: DNA assay, cell reproduction, treated ballast

Introduction

Hull and Linden (2020) identified several points of concern regarding our recent study (Molina et al. 2019). We consider each of the issues below, but first we offer some context to this study beyond what was written in the article. As we noted, the approval of a ballast water management system (BWMS) for operation in U.S. waters depends on its demonstrated performance in a series of land-based and shipboard trials (USCG 2012). These trials are intended to verify that the concentration of organisms in treated water meets the U.S. discharge standard under a set of challenging, yet not extreme, test conditions. Proposed viability methods rely on changes in chlorophyll concentrations to indicate population growth in ballast water samples (MacIntyre et al. 2019). Consequently, such methods (1) only measure viability of the autotrophic organisms in the sample, and (2) generally require long incubation periods to accumulate sufficient chlorophyll to be detected by natural fluorescence intensity. It is in this light that we examined the cell proliferation assay; to our knowledge, it is a novel application of the CyQuant®-based approach for examining DNA concentrations of UV- and chlorination-treated organisms. The work was conducted to inform considerations of regulating ballast water discharges

on the basis of the number of viable organisms. We agree that this work—by itself—is not sufficient to support its adoption as a required method. Below, we provide our responses to specific concerns raised about the experimental design and procedure.

Experimental Design

Consistency

Hull and Linden (2020) stated that the experiment design lacks consistency in application of experimental conditions. We note that the experimental conditions were changed in an iterative process during the study. These adjustments were part of an adaptive management strategy to extend and implement the knowledge gained in each previous run of an individual experiment. Changes in the experimental design reflected the results of each previous experiment. For example, when eight dilution factors were determined to be more than necessary after the first experiment (Figure 1A), six dilution factors were used in subsequent experiments (Figure 1B and 1C). Similarly, when 300 mWs cm^{-2} UV treatment did not have the expected effect on *Tetraselmis marina* (Figure 1B), the treatment dose was increased to 800 mWs cm^{-2} (Figure 1C).

Bacterial Contamination and Controls

Hull and Linden (2020) suggested that the experimental design and the subsequent results were confounded by bacterial contamination resulting from inadequate control between experiments for both UV and chlorination treatments. As stated in the article, filtration was conducted merely to *minimize* bacterial interference within the cell proliferation assay, not to completely remove it (Feng et. al. 2018; Sobsey 2002). Cultures were non-axenic, so bacteria associated with the cultured organisms would inoculate sterile media and water used for dilutions. Because of this procedure, bacteria associated with individual algal cells were still expected to be present in samples. Filtration of axenic cultures in the laboratory was performed to simulate filtration of samples in the field.

The filtration step was added to the procedures to investigate fluorescence response and was intended to reduce free-floating bacterial concentrations in the culture. Similar to field samples, natural bacteria attached to cells in culture were likely still present throughout the experiments (Fang 2018). There was only an increase in fluorescence in treated samples compared to controls with *T. marina*. There was not an increase with *Prorocentrum micans*. This increase in fluorescence could have been attributed to the tendency of *Tetraselmis* sp. to be more robust against UV treatment as opposed to *Prorocentrum* sp. (First and Drake 2014; First et al. 2016; Fon-Sing and Borowitzka 2016; Liu et al. 2016; Montero et al. 2011). Post-treatment, bacterial survival did not occur. This

was evident by a decrease in fluorescence in the same total residual oxidant (TRO) treatment for both *P. micans* (with a decrease in concentrations to near and below zero), and *T. marina* (resulting in concentrations close to starting concentrations; see Figure 2B and 2C of Molina et al. 2019). Any surviving and reproducing bacteria would have shown an increase in fluorescence in the tests, which was not the case. These results showed that bacteria were not necessarily affected by the treatment itself, but by filtration.

Hull and Linden (2020) stated that n = 3 trials were technical replicates rather than independent experimental replicates. In our experiments, n = 3 trials were three independent trials in that each sample used for the experiment was independently grown as its own culture. Each independent trial used a fresh culture grown overnight in its own vessel; therefore, each was an individual sample. All sample cultures were initially seeded from a heterogeneous stock culture, from which the same volume aliquot was added to fresh growth media in separate vessels. Three replicate populations used per experiment were sampled so as to be comprised of cultured cells representing the same age and growth phase. Therefore, differences in growth rates amongst the sample vessels were the result of the growth within each sample vessel itself and not a consequence of the experimental design.

Experimental Procedure

Simulated Treatments

Hull and Linden (2020) questioned the measurement accuracy of the UV radiation and chlorination treatments used in our experiments. Our experiments employed standardized treatment and doses for consistency among independent trials. For UV radiation, we developed a treatment approach and estimated fluence (UV dose) using a radiometer calibrated to standards set by the National Institute of Standards and Technology (NIST) to ensure irradiance was accurately measured. After chlorination, residual oxidants may decrease with holding times and with varying temperature (Alfa Laval n.d.; Wang et al. 2008), therefore, neutralization may not occur if concentrations of biocidal oxidants are below detection limits. This scenario was considered in our experiment, as samples were held for 14 days. The concentrations of organisms increased over this time, so remaining oxidants did not suppress cell growth. We recognize that this is but one of many scenarios of treatment type, hold time, and neutralization.

Light and Dark Incubations

Our study shows that *T. marina* exhibited cell proliferation in all UV treatments, although no cell proliferation was detected for *T. marina* or *P. micans* in any chlorination treatment. In general, all UV treatments showed increases in DNA concentrations over a 14-day incubation, but in

only one case (800 mW cm^{-2}, 0% dilution) were these differences significant. In contrast, chlorine-treated samples exhibited lower DNA concentrations relative to initial values following treatment and 14-day incubation.

Hull and Linden (2020) commented that light incubations would typically be used for most probable number (MPN) enumeration of photosynthesizers and therefore questioned our experimental 14-day incubation in darkened conditions. Because our study examined the plausibility of a cell proliferation assay as a new viability detection method, both light and dark incubations were performed. An incubation period of 14 days was used to simulate a holding time of treated water in a ballast tank after treatment. These dark incubation experiments were an alternative to the dilution, grow out method, incubated in light conditions.

CyQuant Assay

The primary objective of our paper was to adapt and evaluate a cell-based assay for use with cultured phytoplankton cells. This approach, if effective, could provide more rapid results than approaches that monitor biomolecules of a subset of organisms (e.g., chlorophyll fluorescence). As an initial investigation into the method, our scope was limited. The CyQuant cell proliferation assay—initially developed for mammalian cells—measures changes in DNA concentrations. Given that DNA is extractable and that other cell characteristics do not interfere with reactions, other cell types (in this case, phytoplankton) could be a target of the assay. We performed dilutions of the samples to measure the fluorescence signal strength at several concentrations. If some of the challenges and uncertainties we (as well as Hull and Linden 2020) identified are addressed, the assay could be used to measure growth via an MPN approach.

Hull and Linden (2020) objected to the exclusion of the RNA component of fluorescence detected within the cell proliferation assay, suggesting that RNA interference may explain the slight increase in fluorescence in UV treated samples at Day 0. The data do not support this hypothesis. All measurements at Day 0 remained very near or below the measurements for the other remaining days. The increase in fluorescence due to upregulation of RNA is not seen as a response in our data.

Hull and Linden (2020) commented that, at a minimum, a standard curve is required to warrant the use of the assay. A standard curve was not performed to compare cell numbers and fluorescence. The initial intent was to test the capability of the assay to detect growth of cells over time, and in this case, any significant increase in the fluorescence signal would indicate that the cells were viable, and thus, the treatment may not have been effective. An absence of growth could indicate either absence of organisms or responsiveness to treatment. Consequently, for these

experiments, a standard curve was not needed to show a relationship between cell numbers and fluorescence.

Hull and Linden (2020) commented on limit of detection issues, including their statement that concentrations of cells used for experiments had a detection limit well above the International Maritime Organization (IMO) limit of < 10 viable organisms per mL. In preliminary studies, *T. marina* was not detectable at concentrations < 5% of the stock concentration. Based on results of those studies, the detection limit was set to this dilution for subsequent experiments to determine the overall capability of the assay to detect *growth* in treated samples. The purpose of the assay used in these experiments tests the efficacy of the treatment to render organisms non-viable; therefore, the concentration of organisms within each dilution does not necessarily mean a standard limit failure. The density of organisms remaining viable at the end of the incubation period would be the concentration put in question by the international standard. In contrast, if the dilutions within the assay *were* questioned, although, *T. marina* did not meet the limit of < 10 mL^{-1} U.S. and IMO discharge standard for organisms in ballast water, *P. micans* did meet the limit for dilutions of 5% and 10%. The goal of the assay was to detect the growth of heterotrophs and autotrophs, hopefully quicker than other approaches, including those using fluorescence.

Author Interpretation

Emphasis on UV treatment and Photorepair

Hull and Linden (2020) suggested that photorepair of cell DNA could have affected the results, potentially increasing the number of organisms that would then be viable following treatment. Specific testing for the detection of UV-induced damage and repair mechanisms was not included in the scope of this study. As stated in our paper, unexpected increases in DNA concentrations with increased UV dose may also depend on individual species' tolerances and environmental conditions. In this case, replication was more likely attributed to species adaptation and environment, and not necessarily photorepair. Although this is true, we agree with the statement that the assay cannot differentiate whether increases of DNA are driven by a few surviving individuals capable of fast reproduction after treatment or photorepair. Either (or both) of these processes may have driven the observed increase in DNA concentrations.

Comparison to Growth Assays

Hull and Linden (2020) stated that the method proposed in our study does not allow for an MPN calculation. Our study was not intended to develop a method that would require an MPN calculation. The study was conducted to determine the applicability of a cell viability assay to detect increased

concentrations of cells over time after treatment. Therefore, the detection of growth above that of initial Day 0 would indicate the treatment was unsuccessful in reducing the density of viable cells. The reduction of cell numbers to one cell by dilution was not the anticipated purpose of the assay, nor was the intention to adapt the assay for use with non-destructive chlorophyll autofluorescence. Hull and Linden (2002) stated that the conventional MPN monitoring of phytoplankton using autofluorescence of phototrophs is a more selective measurement for organisms in this size fraction than a DNA-based measurement, which can be contaminated by other organisms including bacteria. Although our studies only attempted to eliminate free-living, unattached floating bacteria, bacteria attached to phytoplankton could have contributed to the total DNA concentration of all organisms within the 10–50 µM size class.

Conclusion

We appreciate interest in our study from the scientific community, and we welcome input from fellow scientists. Our study was part of initial research and development efforts undertaken to identify potential alternate methods of assessing organism viability in treated ballast water. The work in this study—or any single study on a new method or a novel application of the method—cannot sufficiently support its adoption as a standard method. However, preliminary tests may identify potential candidates for future, more comprehensive investigations. Our use of the CyQuant DNA assay to detect phytoplankton growth identified some opportunities and numerous challenges, many of which were well described by Hull and Linden (2020). Future modifications of the method, if performed, may improve its performance. Redesigning the assay to allow for MPN-based analysis or including additional measurements (variable fluorometry, immunofluorometry) may improve the method's utility. We expect that any actual or potential weaknesses or issues in the testing approach could be evaluated and addressed in subsequent studies, and we look forward to engaging with others involved in similar work.

Disclaimer

The contents of our original article—as well as the responses provided herein—do not represent the official policy of the U. S. Coast Guard or the U. S. Navy.

References

Alfa Laval (n.d.) Making sense of ballast water management. A guide to international ballast water regulations and compliance alternatives. https://www.alfalaval.com/globalassets/documents/microsites/pureballast/pdf/ballast_water_management.pdf (26 October 2020)

Fang T, Cui Q, Huang Y, Dong P, Wang H, Liu W, Ye Q (2018) Distribution comparison and risk assessment of free-floating and particle-attached bacterial pathogens in urban recreational water: Implications for water quality management. *Science of the Total Environment* 613–614: 428–438, https://doi.org/10.1016/j.scitotenv.2017.09.008

First MR, Drake LA (2014) Life after treatment: detecting living microorganisms following exposure to UV light and chlorine dioxide. *Journal of Applied Phycology* 26: 227–235, https://doi.org/10.1007/s10811-013-0049-9

First MR, Robbins-Wamsley SH, Riley SC, Drake LA (2016) Towards minimizing transport of aquatic nuisance species in ballast water: Do organisms in different size classes respond uniformly to biocidal treatment? *Biological Invasions* 18: 647–660, https://doi.org/10.1007/s10530-015-1036-7

Fon-Sing S, Borowitzka MA (2016) Isolation and screening of euryhaline *Tetraselmis* spp. suitable for large-scale outdoor culture in hypersaline media for biofuels. *Journal of Applied Phycology* 28: 1–14, https://doi.org/10.1007/s10811-015-0560-2

Liu L, Chu XN, Chen PY, Xiao Y, Hu JY (2016) Effects of water quality on inactivation and repair of *Microcystis viridis* and T*etraselmis suecica* following medium-pressure UV irradiation. *Chemosphere* 163: 209–216, https://doi.org/10.1016/j.chemosphere.2016.08.027

MacIntyre HL, Cullen JJ, Rastin S, Waclawik M, Franklin KJ, Poulton N, Lubelczyk L, McPhee K, Richardson TL, Van Meerssche E, Petri B (2019) Inter-laboratory validation of the serial dilution culture-most probable number method for enumerating viable phytoplankton. *Journal of Applied Phycology* 31: 491–503, https://doi.org/10.1007/s10811-018-1541-z

Molina V, Riley SC, Robbins-Wamsley SH, First MR, Drake LA (2019) Applicability of a cell proliferation assay to examine DNA concentrations of UV-and chlorine-treated organisms. *Management of Biological Invasions* 10: 255–266, https://doi.org/10.3391/mbi.2019.10.2.03

Montero MF, Aristizabal M, Reina GG (2011) Isolation of high-lipid content strains of the marine microalga *Tetraselmis suecica* for biodiesel production by flow cytometry and single-cell sorting. *Journal of Applied Phycology* 23: 1053–1057, https://doi.org/10.1007/s10811-010-9623-6

Sobsey MD (2002) Water, Sanitation and Health Department of Protection of the Human Environment World Health Organization Geneva. Managing Water in the Home: Accelerated Health Gains from Improved Water Supply. Report No. WHO/SDE/WSH/02.07, https://apps.who.int/iris/handle/10665/67319

USCG (2012) Standards for living organisms in ships' ballast water discharged in U.S. waters, final rule. *Federal Register* 77: 17254–17320

Wang, J-T, Chen M-H, Lee H-J, Chang W-B, Chen C-C, Pai S-C, Meng, P-J (2008) A Model to Predict Total Chlorine Residue in the Cooling Seawater of a Power Plant Using Iodine Colorimetric Method. *International Journal of Molecular Sciences* 9: 542–553, https://doi.org/10.3390/ijms9040542