CORRECTED PROOF

Letter to *Management of Biological Invasions*

On applicability of a cell proliferation assay to examine DNA concentration of UV- and chlorine-treated organisms – a rebuttal of Molina et al. (2019)

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Abstract

In their 2019 study, Molina et al. evaluate a cell proliferation assay that fluorescently quantifies DNA for assessing ballast water treatment efficacy of organisms ≥ 10 to 50 µm. Because of concerns with the overall experimental design, procedures, and authors' interpretations, their conclusions do not appear to be justified and the assay used to arrive at those conclusions is not proven appropriate. Specific concerns we highlight include UV and chlorine dose calculations and exposure conditions, bacterial contamination issues, poor agreement between controls, high detection limit of the assay, and mismatch between conclusions in the abstract and statistical significance of the data. Their conclusions that "population maintenance or growth was evident … after UV treatment" and "photoreactivation could have been attributed to increased mean DNA concentrations" are therefore not scientifically defensible without further experimentation and verification. These concerns call into serious question the use of this study by the US Coast Guard or other governing bodies to (1) determine the applicability of this cell proliferation assay for assessing ballast water disinfection or (2) make conclusions regarding the suitability of UV treatment for inactivating organisms in ballast water.

Key words: ballast water management system, DNA quantification, MPN, photoreactivation, cell proliferation, DNA damage, UV disinfection

Introduction

In the experiments used by Molina et al. (2019) to assess a DNA quantification assay (CyQuant®) for quantifying ballast water treatment efficacy of ≥ 10 to 50 µm organisms there are several issues with experimental design, procedures, and interpretation. Given these issues, the conclusions presented are not supported and the ability to reproduce this work in another lab for verification is not possible.

Experimental Design Concerns

Consistency: The experimental design lacks consistency in parallel of application of experimental conditions, making it nearly impossible to compare between treatments, incubation conditions (light/dark), filtration

of treated samples, and taxa. The inconsistent design limits the utility of the study to determine the appropriateness of the assay for these ballast water treatment technologies and standard test organisms.

Bacterial Contamination and Controls: Filtration experiments were conducted to "reduce bacterial contamination". Bacterial contamination will be unavoidable in field samples just as bacteria were seemingly present in both the 5 µm filtered seawater used for treatments and the 0.22 µm filtered seawater used for dilutions. For example, for all the *T. marina* experiments (Figure 1 and Figure 2A), the Day 0 controls differ vastly and there was little signal for controls in Figure 1B. Additionally, in filtration experiments there was an unexplained increase in fluorescence in chlorine treated samples vs. controls. These differences could indicate the extent that bacterial contamination confounds results. These differences could also indicate that experimental conditions were not well controlled between experiments, or that n = 3 trials were technical replicates rather than independent experimental replicates. When comparing Figures 1B and C, greater fluorescence signal was reported in both controls and all samples treated with the higher UV dose in Panel C, negating the provided reasoning that adaptation, nutrients, or temperature impacted the results. Instead, results were confounded by bacterial contamination and/or inadequate control between experiments.

Experimental Procedure Concerns

UV Treatment: Authors make no mention of measuring sample UV absorbance, which is essential to accurately calculate UV dose. This is especially problematic with their setup, where 10 cm petri dishes and 30 mL sample volumes would result in a sample depth of 0.38 cm. In a similar study (Hull et al. 2017) absorbance of 60,000 *T. suecica* cells/mL was 0.1 cm^{-1}, which is a UV transmittance of 80%. It is unclear whether a separate radiometer with NIST-traceable calibration was used to measure irradiance, or if the built-in sensor (which can be less accurate and may not be regularly calibrated) of the UV apparatus was used. Additionally, the UV exposure apparatus only has one small lamp, which will result in uneven distribution of irradiance across the sample surface. However, authors don't indicate that spatially integrated irradiance measurements were used to correct for nonuniformity, nor do they report the "petri factor" (Bolton and Linden 2003). Authors also don't mention stirring samples, which is essential for uniform dose distribution. Nonuniform dose distribution coupled with no stirring may have resulted in zones of samples that were not evenly treated by UV, resulting in living organisms capable of growth in proliferation assays. Additionally, the limited height (8 ¾") of the UV apparatus (https://www.uvp.com/ultraviolet-crosslinkers) dictates that light divergence, and/or reflection or absorption off the collimating tube of unidentified material, may have impacted UV dose. As a result of these

factors, the UV doses applied are effectively unknown and were definitely not uniform throughout samples.

Chlorine Treatment: The chlorine treatments are expressed as TRO (total residual oxidant) concentration but not as CT (concentration × exposure time). CT is common practice in water treatment studies and is necessary to compare between studies because TRO includes both free and combined chlorine species which have vastly different inactivation efficacies. It is not possible to accurately describe or reproduce the chlorine dose without CT for individual chlorine species. Additionally, chlorine methods do not indicate any quenching of the remaining oxidant, indicating that much higher and undetermined chlorine doses were applied during incubations. In a control experiment with no cells, authors report a chlorine residual of 6 mg/L after the 30 min treatment, indicating that oxidant was present and may have provided additional disinfection during subsequent incubations. There was no reported temperature or pH, which are known to impact chlorine disinfection kinetics. Authors present no control studies to determine if chlorine or quenchants (if they were used) interfered with the fluorescence-based assay.

Light and Dark Incubations: Given that most experimental conditions were in the dark and the only comparison of dark versus light (Figure 1A and B) was inconclusive due to control concerns, the results obtained by the authors cannot be attributed to photorepair after UV exposure. In addition to large error bars found throughout the results, almost no significant differences were found in any subset of the data, so the statement that "*T. marina* exhibited cell proliferation in all UV treatments, although no cell proliferation was detected for *T. marina* or *P. micans* in any ClO^- treatment" is unfounded. Additionally, using a simple light *vs.* dark experimental design is insufficient to solely detect photorepair for the tested phototrophic *T. marina*, given the confounding additional metabolic processes that are impacted by photosynthetically active radiation and the potential dark repair processes that would happen during either incubation condition. Before incubations, the 30 min room temperature incubation in ambient light (assuming there was emission in the active region of the photoreactivating enzyme photolyase, which was not reported) would be sufficient to initiate rapid enzymatic light and dark repair processes (Hull et al. 2017) and impact the ability of the experimental design to solely assess dark repair mechanisms. Additionally, although dark 14-day incubations were selected to simulate ballast tank conditions and incubations for the most probable number (MPN) serial dilution assay, light incubations would typically be used for MPN enumeration of photosynthesizers.

CyQuant® Assay: The RNA component of fluorescence is known to impact CyQuant measurements (Invitrogen 2006) but was totally unaccounted in this study. This interference may explain the slight increase in fluorescence in UV treated samples at day 0 as cells may have responded by upregulating

transcription of stress response genes (Moeller et al. 2007). Similarly, authors present no control studies or statistics to assess whether UV treatment may have increased dye intercalation into DNA, which might explain increased day 0 fluorescence. Authors present no validation indicating appropriateness of this assay developed for mammalian cell lines for these marine flagellates. At a minimum, a standard curve is needed to show the relationship between cell numbers and fluorescence and to account for RNA contributions. Without this standard, there is no context as to how changes in fluorescence relate to changes in cell numbers. This standard is especially important for untreated controls and is standard laboratory practice; without it the authors cannot address the purpose of the study. Also, the sensitivity of the DNA assay is very low for these organisms, where detection limit issues were encountered for 5% dilution of samples in preliminary experiments, which would equate to 450 cells/mL for the reported mean *T. marina* concentration of 9000 cells/mL. Moreover, authors did not calculate these detection limits even though one of their stated objectives was to "determine optimal cell proliferation thresholds and detection limits". This detection limit is well above the International Maritime Organization (IMO) limit of < 10 viable organisms upon discharge in this size fraction. The detection limits here and the interference of bacterial contamination indicate that this type of assay may not be useful for BWTS testing for organisms in this size fraction.

Author Interpretation Concerns

Undue Emphasis on UV Treatment and Photorepair: Although authors state that "Surprisingly, population maintenance or growth was evident … after UV treatment", it is a well-defined characteristic that a single viable cell can reproduce to carrying capacity in growth media. In fact, growth kinetic studies have been used recently to assess UV-C treatment of ballast water (e.g., Romero-Martínez et al. 2016; MacIntyre et al. 2018). Instead of being an indication of any of the proposed repair phenomena in the discussion, the differences between their data could be explained by single viable cells colonizing microwells, lack of experimental controls and consistency, or by bacterial contamination. In general, the study incubations cannot distinguish between proliferation of viable cells capable of repair versus replication of undamaged survivors. Authors also state that "photoreactivation could have been attributed to increased mean DNA concentrations in tests incorporating light incubation (Figure 1A)" although this is not supported by their data in Figure 1 or by any statistical test.

Unsupported Comparison to Growth Assays: Without experimental comparison to a cell concentration standard curve, a traditional growth assay that more appropriately uses light incubation for photosynthesizers, or calculating a most probable number (MPN) from their results, authors claim that "This type of assay allows for increased sample replication,

smaller sample volumes, quicker preparation, and faster measurement than traditional growth assays." However, there are several issues with this statement. First, traditional growth assays with other endpoints besides DNA concentration (e.g., turbidity, autofluorescence, etc.) have been adapted into other formats with replication and volume advantages (MacIntyre et al. 2018). Although minimal dilutions were used in this study, this is because responses were only measurable at high detection limits (≈ 450 cfu/mL). On the other hand, diluting a sample to 1 viable organism per replicate is needed to resolve an MPN calculation, so the detection limit (which can be as low as 0.2 cfu/mL) and the range of the MPN assay are better. Related to detection limits is the assay incubation time, which depends both on growth rates and detector sensitivity for the assay endpoint. Both the assay presented here and traditional growth assays rely on amplifying viable cells to detection. Because the detection limit of the DNA-based measurement was so high, early measurements were not that useful. The conventional way of monitoring phytoplankton MPN assays uses chlorophyll autofluorescence, which can be non-destructively (allowing repeated kinetic monitoring for photoreactivation) and quickly read by common microplate readers. Additionally, 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. Increasing DNA concentration as bacteria proliferate during incubations will negatively impact implementation of this assay for assessing ballast water treatment systems for organisms in the ≥ 10 to 50 µm organisms size fraction, as in this study. Finally, culture-based assays with a non-specific endpoint such as total DNA concentration will be negatively impacted by varying treatment sensitivity, cultivability, and growth kinetics of diverse ballast water organisms.

Conclusions

Overall, the conclusions and interpretations stated by the authors do not align with their data, and the experiments are poorly controlled. The authors are urged to reconsider their findings, rescind conclusions that are not supported by their data, and repeat their experiments with proper controls and experimental design. In particular, authors' strong language that "unexpectedly, results nevertheless revealed an *increase* in DNA concentration after UV treatment" (which their data do not support) and discussion of UV as potentially "ineffective" (based on unsupported interpretations) largely promote misconceptions about UV treatment mechanisms and efficacy. For example, the IMO and the United States Coast Guard (USCG) differ in requirements for assessing ballast water management system efficacy – IMO guidelines are based on determining viability (including by culture-based assays measuring ability to reproduce such as MPN) whereas the USCG protocol requires a "living" determination

(by microscopy observations of movement and by fluorescent staining of enzymatic esterase activity). Given the differences between IMO and USCG, promoting these misconceptions serves to further confuse the issue and undermine evidence-based discourse to help resolve this difference on an important issue in international policy. The authors also ignore recent literature (the most recent citation for this 2019 article was from 2016) and a wealth of water treatment literature (including mechanistic studies of growth assays and studies of light and dark repair relevant to ballast water UV treatment). This is especially troubling because the USCG seeks "best available science" to approve protocols for testing ballast water treatment systems for type approval. As the current staining protocol is inappropriate for UV treatment, and the comment period on the Vessel Incident Discharge Act (VIDA) ended on September 30, 2019 (USCG 2019), the concerns with this article need to be addressed immediately for the benefit of the UV treatment industry, the ballast water community, and the USCG.

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