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Nucleic acids-based tools for ballast water surveillance, monitoring, and research

John A. Darling [a,*], Raymond M. Frederick [b]

[a] National Exposure Research Laboratory, U.S. Environmental Protection Agency, Research Triangle Park, NC, USA
[b] National Risk Management Research Laboratory, U.S. Environmental Protection Agency, Edison, NJ, USA

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ABSTRACT

Understanding the risks of biological invasion posed by ballast water—whether in the context of compliance testing, routine monitoring, or basic research—is fundamentally an exercise in biodiversity assessment, and as such should take advantage of the best tools available for tackling that problem. The past several decades have seen growing application of genetic methods for the study of biodiversity, driven in large part by dramatic technological advances in nucleic acids analysis. Monitoring approaches based on such methods have the potential to increase dramatically sampling throughput for biodiversity assessments, and to improve on the sensitivity, specificity, and taxonomic accuracy of traditional approaches. The application of targeted detection tools (largely focused on PCR but increasingly incorporating novel probe-based methodologies) has led to a paradigm shift in rare species monitoring, and such tools have already been applied for early detection in the context of ballast water surveillance. Rapid improvements in community profiling approaches based on high throughput sequencing (HTS) could similarly impact broader efforts to catalogue biodiversity present in ballast tanks, and could provide novel opportunities to better understand the risks of biotic exchange posed by ballast water transport—and the effectiveness of attempts to mitigate those risks. These various approaches still face considerable challenges to effective implementation, depending on particular management or research needs. Compliance testing, for instance, remains dependent on accurate quantification of viable target organisms; while tools based on RNA detection show promise in this context, the demands of such testing require considerable additional investment in methods development. In general surveillance and research contexts, both targeted and community-based approaches are still limited by various factors: quantification remains a challenge (especially for taxa in larger size classes), gaps in nucleic acids reference databases are still considerable, uncertainties in taxonomic assignment methods persist, and many applications have not yet matured sufficiently to offer standardized methods capable of meeting rigorous quality assurance standards. Nevertheless, the potential value of these tools, their growing utilization in biodiversity monitoring, and the rapid methodological advances over the past decade all suggest that they should be seriously considered for inclusion in the ballast water surveillance toolkit.

Contents

* Corresponding author.
E-mail address: darling.john@epa.gov (J.A. Darling).

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1. The importance of ballast water monitoring in research and regulatory contexts

Maritime trade has long been recognized as a major driver of invasive species spread globally, and ballast water continues to serve as a primary vector for non-indigenous marine species introductions (Drake and Lodge, 2004; Keller et al., 2011; Seebens et al., 2013). To address the environmental, economic and public health impacts of ballast water-borne invasive species, a number of government entities have enacted comprehensive regulations involving the use of best management practices and the application of treatment technologies for use on-board commercial vessels (Costello et al., 2007). Ballast water discharges are currently regulated at multiple levels of governance by multiple agencies, including internationally by the International Maritime Organization (IMO) and within the U.S. by the Coast Guard (USCG) and the Environmental Protection Agency (EPA). These policies are the outcome of decades of research and concerted effort by the global scientific and shipping communities to understand the risks posed by ballast water-borne invasions and to determine the management strategies best positioned to mitigate those risks.

In February 2004, the IMO, through its Marine Environmental Protection Committee and participating member nations, enacted the International Convention for the Control and Management of Ship's Ballast Water and Sediments. More widely known as the BWM Convention (Lloyd's Register, 2016), it is intended to help reduce the risk of new invasions of non-native species through the implementation of an interim ballast water exchange Standard (D-1) and a more restrictive numerical organism discharge Standard (D-2) for shipboard treatment systems. The technology performance standard targets two size classes of plankton in addition to limits for toxigenic *Vibrio cholerae*, *Escherichia coli* and intestinal enterococci. Ship owners required to meet the D-2 standard must have on-board treatment systems retrofitted to existing vessels or designed into the ballasting systems of new build vessels according to a prescribed timeline. On September 8, 2016 the total world shipping tonnage of ratifying states reached the 35% minimum required to trigger the one-year period for the BWM Convention to enter into force on September 8, 2017.

Ballast water discharges in the U.S. are regulated under the Nonindigenous Aquatic Nuisance Prevention and Control Act of 1990 (NANPCA), as amended by the National Invasive Species Act of 1996 (NISA). The USCG is the responsible agency for developing and enforcing regulations under this act. The USCG published a "Notice of Proposed Rule Making" in 2009, and after reviews and public comments, the "Standards for living organisms in ships' ballast water discharged in U.S. waters; final rule" was published in the Federal Register on March 23, 2012 (USCG, 2012). Both the IMO and USCG discharge standards for treatment systems are identical, as follows:

1. For organisms greater than or equal to 50 μm in minimum dimension: discharge must contain fewer than 10 live organisms per cubic meter (m^3) of ballast water.
2. For organisms <50 μm and greater than or equal to 10 μm in minimum dimension: discharge must contain <10 living organisms per milliliter (ml) of ballast water.
3. Indicator organisms must not exceed:
   a. For toxigenic *V. cholerae* (serotypes 01 and 0139): a concentration of <1 colony forming unit (cfu) per 100 ml.
   b. For *Escherichia coli*: a concentration of fewer than 250 cfu per 100 ml.
   c. For intestinal enterococci: a concentration of fewer than 100 cfu per 100 ml.

That document also contains a specific timetable for installation of treatment technology based on the vessel's construction date and ballast capacity. In December of 2016 USCG issued its first type approval for a ballast water management system (BWMS) to Optimarin AS of Norway. USCG continues to evaluate additional BWMS at several USCG accepted testing facilities.

Since 2008, EPA has also regulated ballast water discharges and other discharges incidental to the normal operation of vessels under the Clean Water Act (CWA) through the Vessel General Permit (VGP) (USEPA, 2013). Sixteen states also have specific ballast water management requirements imposed either through separate regulations or CWA Section 401 Certifications for the VGP Program. EPA had previously exempted these discharges from its regulations, citing the fact that USCG had promulgated and administered ballast water regulations pursuant to its Congressional mandate in NISA. However, a 2005 court decision vacated that exemption, leading to implementation of the first VGP in 2008 (*Northwest Environmental Advocates v. EPA*, Northern District Court of California, 2005). In March 2013, EPA issued a revised VGP replacing the previous 2008 version, and requiring existing vessels to meet the IMO D-2 and USCG ballast water discharge performance standard. The VGP includes monitoring requirements for installed BWMS that address functionality depending on the technology used by the treatment system, and periodic biological organism monitoring using total heterotrophic bacteria, *E. coli*, and enterococci as indicators of treatment performance.

Regulatory agencies and ship owners need assurance that treatment technology will perform successfully in a wide range of water quality and under harsh environmental conditions on board ships. Developing appropriate testing procedures to verify biological treatment efficacy at land-based facilities has proven to be a challenging endeavor. Since 2001, USCG and EPA have participated in a joint activity under EPA's Environmental Technology Verification Program (ETV) to develop performance verification protocols for BWMS. Assisted by stakeholders and technical panel input, a working draft was produced in 2004. Additional research and testing by the USCG at the Naval Research Laboratory resulted in significant improvements to the draft which was eventually released by EPA as the document entitled "Generic protocol for the verification of ballast water treatment technology" (USEPA, 2010). The ETV protocol contains a biological assay for enumerating live organisms based on a combination of two vital fluorescence stains (fluorescein diacetate [FDA] and 5-chloromethylfluorescein diacetate [CMFDA]) with direct microscope observation and probing for motility verification. Although vital fluorescent stains have been demonstrated useful in evaluation the efficacy of BWMS, the ETV protocol recommends testing facilities validate the effectiveness of stains with ambient populations before use.

The USCG is required to periodically monitor the performance of BWMS installed on commercial vessels. It is impractical for inspectors to perform shipboard biological testing to the degree conducted at land-based facilities. Therefore, portable and rapid test kits are being developed by at least seven manufacturers to enable inspectors to determine if a sample of ballast water appears to have been successfully treated, or if a condition of "gross exceedance" exists. The inspector can then arrange for a more detailed biological test if time permits, or notify the ship operator to have the treatment system fully evaluated at the next port of call. The USCG is currently evaluating several candidate test kits based on different assay technologies.

The ETV protocol recognizes that improvements in biological assays and instrumentation for BWMS testing are expected to occur in the coming years. USCG and EPA remain committed to updating and improving the ETV protocol periodically to include such advances in procedures and technology which will enhance the quality and cost effectiveness of the BWMS testing program. In addition, USCG and EPA continue to encourage research aimed at better understanding the relationship between the number of viable propagules discharged with ballast water, and the risk of non-native species establishment—the so-called "risk release" relationship (Carlton et al., 2011). These efforts are rooted in concerns expressed by some that existing discharge limits are not sufficiently protective of water quality, concerns highlighted by recent court ruling challenging currently adopted U.S. standards

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