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Development of an automated ballast water treatment verification system utilizing fluorescein diacetate hydrolysis as a measure of treatment efficacy

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ABSTRACT

Methods for verifying ballast water treatments in foreign vessels are needed to protect the Great Lakes from the discharge of live non-native organisms or pathogens. A prototype automated viability test system using fluorescein diacetate (FDA), a membrane permeable fluorogen, to differentiate live from dead bacteria and algae is described. The automated fluorescence intensity detection device (AFIDD) captures cultured algae or organisms in Detroit River water (simulated ballast water) on 0.2 μm filters, backwashes them from the filter into a cuvette with buffer and FDA for subsequent fluorescence intensity measurements, and washes the filters with sterile water for serial automated reuse. Preliminary manual versions of these procedures were also tested. Tests of various buffers determined N,N-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid, N,N-Bis(2-hydroxyethyl)taurine (BES) and 3-(N-morpholino)propanesulfonic acid (MOPS) at pH 7.0 to be the best buffers, causing the least spontaneous FDA breakdown without inhibiting enzymatic activity. Fluorescence in the presence of live organisms increased linearly over time, and the rate of increase was dependent on the sample concentration. Following simulated ballast water treatments with heat or chlorine, the fluorescence produced by Detroit River samples decreased to near control (sterile water) levels. Automated measurements of FDA hydrolysis with a reusable filter backwash system should be applicable to near real-time remote-controlled monitoring of live organisms in ballast water.

1. Introduction

The transportation of organisms between ports through ship ballast water is a major factor in the introduction of invasive species. Economic damages within the United States associated with the effects of non-native invasive species and their control amount to approximately $120 billion/year (Pimentel et al., 2005), with more than $14 billion in costs being attributable to non-indigenous wetland and aquatic species

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(Pimentel, 2005). The cost of aquatic invasive species in the Great Lakes region has been estimated at $5.7 billion per year (Pimentel, 2005). Major impacts of such invasions include changes in fisheries, increased costs to commercial users of raw water, decreased tourism, and human health impacts. Invasive species may also cause ecological disruptions such as the drastic declines in native mussel populations in response to invasions by zebra mussels (Strayer and Malcom, 2007). Ballast water is a potential vector of pathogens between ports. For example, epidemic *Vibrio cholera* reported in Peru in 1991 was transported to the United States in cargo ship ballast water (McCarthy and Khambaty, 1994). The introduction of invasive species into their non-native range has been recognized as an urgent concern.

In response to such problems, the International Maritime Organization (IMO), the United States Coast Guard (USCG), and the United States Environmental Protection Agency have developed regulations that, in effect, will require most commercial ships to perform ballast water treatment before discharge (Champ, 2002; US EPA, 2013). The IMO Convention (International Conference on Ballast Water Management for Ships, 2004; International Maritime Organization, 2004) sets discharge limits on densities of live organisms by size class of organism while the USCG and US EPA are proposing the same limits be applied through stricter United States regulations (US EPA, 2013; USCG, 2012). Great Lakes states also enforce their individual ballast water regulations as well. Currently, ballast water exchange is required for reducing the number of invasive species transported by ballast water from distant ports. However ballast water exchange is viewed as only an interim solution and implementation of ballast water treatment methods are a high priority (Tsolaki and Diamadopoulos, 2010). The greater emphasis on ballast water treatment systems as the most effective method of reducing the introduction of invasive species has fostered a need for robust ballast treatment and treatment verification systems.

Most treatment methods for ballast water can be categorized into several groups: chemical, mechanical and physical (California State Lands Commission, 2013). Examples of chemical treatments include hydrogen peroxide and chlorine treatments; mechanical treatments include filtration and hydrocyclonic separation; and physical methods employ lethal thermal, ultraviolet, and sonic treatments. Although dozens of commercial ballast water treatment systems exist, robust and rapid methods for verifying their effectiveness have not been described. To address this need, we developed an automated ballast water treatment verification system that utilizes fluorescein diacetate (FDA), a membrane permeable fluorogen, to differentiate live from dead organisms.

Hydrolytic cleavage of colorless FDA by non-specific esterases inside living organisms produces fluorescein, a green fluorescent compound which can be detected and correlated to the presence of viable organisms. FDA hydrolysis is widely accepted as an accurate and simple method for measuring total microbial activity in a range of environmental samples (Adam and Duncan, 2001). A current method of viable organism enumeration using FDA is to use the fluorogen as a staining agent and subsequently counting organisms microscopically (Steinberg et al., 2010). Diaper et al. (1992) claimed FDA was not an ideal fluorescent substrate for microscopy as bacteria retained fluorescein poorly, resulting in weaker staining of target cells and increased background fluorescence. Instead of using FDA as a staining agent, fluorescence resulting from fluorescein leaking into the incubation medium can be measured and correlated to the quantity of organisms. Welschmeyer and Maurer (2012, 2013) demonstrated quantifying viable marine microorganisms using FDA detection in a similar manner.

The objectives of this study are twofold. The first is to describe methods for using FDA as a broad spectrum fluorogen to detect viable organisms in freshwater environments. FDA hydrolysis was tested on a variety of organisms, including organisms from environmental freshwater as well as cultured microalgae. Buffers and incubation conditions were optimized to obtain linear FDA hydrolysis. The second objective of the paper is to perform the test method in an automated system, the Automated Fluorescence Intensity Detection Device (AFIDD). The rationale for building the AFIDD was to provide untrained cargo ship owners with an inexpensive, efficient, and simple to use platform that could potentially be used to verify the reduction of live organisms after operation of ballast treatment systems. Both laboratory and field experiments were conducted using the AFIDD system to verify the efficacy of simulated ballast water treatments.

2. Methods

2.1. Sources of samples

2.1.1. Environmental samples

Water samples comparable to untreated ballast water were collected from the Detroit River at Belle Isle Park midway between the Belle Isle Beach and the Detroit Yacht Club (42° 20′ 51.6012″ N, 82° 58′ 31.1772″ W). Water temperature, air temperature, and time of collection were recorded for each sample. All water samples were collected in 3 L aliquots and stored on ice until the samples arrived at the laboratory for testing. Water samples were usually tested immediately after bringing the sample to the laboratory from the collection site. For testing, the sample was kept at ambient temperature (20–22 °C) for the duration of the experiment. If collected water was not immediately used, it was stored in the dark in a 4 °C walk-in refrigerator for up to 24 h.

2.1.2. Algae samples

The algae culture utilized for experiments was *Ankistrodesmus falcatus* UTEX 748 (UTEX- University of Texas at Austin). Stock cultures were inoculated in Jaworski's medium (http://www.ccap.ac.uk/media/documents/JM.pdf, accessed 11/20/2013) and aerated with 0.2 μm filtered air. The algae culture was maintained at room temperature and cultured in a 12 hr:12 hr light:dark light cycle. Algae density counts were done using a hemocytometer. *A. falcatus* are rod-shaped cells about 1–2 μm in diameter and 15–40 μm long (microscopic observations for this study and http://nordicmicroalgae.org/taxon/Ankistrodesmus%20falcatus).