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Lethal Effects of Pulsed High-Voltage Discharge on Marine Plankton and *Escherichia coli*

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Abstract Ballast seawater is considered globally as a major vector for invasions of non-indigenous organisms. Several technologies have been tested for their ability to remove organisms from ballast water. In the present study, we constructed a novel pulsed high-voltage discharge (PHVD) system that could operate in either high current mode with several hundred amperes or shockwave generating mode with relatively lower current in seawater. In laboratory-scale experiments, the PHVD system with shockwave-generating mode was found to be more effective in killing zooplankton (1.9- to 4.0-fold) and phytoplankton (3.3-fold) than high current mode at discharge with 300–500 pulses at 7.1 kV. Further experiments were carried out at different voltages and pulse-numbers to examine effects of the shockwave-generating PHVD system on viabilities of one zooplankton larva, two phytoplankton species, and an indicator bacterium suspended in seawater in a static chamber. For zooplankton, live cells were not detected at discharge with 400 pulses at 13 kV. For phytoplankton, the initial live cells of a dinoflagellate was decreased by 77±0.5%, and the initial chl *a* concentration of a diatom was decreased by 76±6% at discharge with 700 pulses at 13 kV. For an indicator bacterium *Escherichia coli*, live cells were not detected at discharges with 200 or 700 pulses at 13 kV. Measurements of ATP content of organisms showed congruent results with those obtained by the above methods, suggesting it may be a rapid method for evaluating treatment efficiency. Though further scale-up studies are necessary, these results suggest that the PHVD system have a high potential for applying to ballast seawater treatment.

Keywords Pulsed high-voltage discharge · Zooplankton · Phytoplankton · *Escherichia coli* · Ballast seawater treatment

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1 Introduction

Ships' ballast water is considered as the principal vector responsible for transferring and introducing non-indigenous organisms to new environments throughout the world (Cohen and Carlton 1998). It has been reported that invasions of non-indigenous organisms via ship's ballast water could cause destruction of indigenous aquatic habitat, losses of biological diversity and commercial fisheries, and increase of health risks (Ruiz et al. 2000). In response to those problems, the International Maritime Organization (IMO) made an International Convention for the Control and Management of Ships' Ballast Water and

Sediments, which was adopted in 2004 (IMO 2005). Once the convention is ratified by the member states, the convention will impose the performance standard for discharge of ballast water with a phased implementation schedule through 2016 (IMO 2005).

Many technologies to remove organisms in seawater have been developed using filtration, heating treatment, ultraviolet radiation, deoxygenation, ozone, and various biocides (Waite et al. 2003; Quilez-Badia et al. 2008; Wright et al. 2009). However, no single technology is exclusively superior to others, indicating that there remains scope for an effective treatment that is highly effective, easily applied and environmentally benign at discharge (Gregg and Hallegraeff 2007). In this study, we investigated the use of pulsed high-voltage discharge (PHVD) for potential ballast water treatment.

PHVD systems have been applied to freshwater treatment and liquid foods sterilization (Anpilov et al. 2002; Chang 2003; Sato 2008). These methods were found to effectively kill viruses, bacteria, yeast cells, and macroorganisms (e.g., brine shrimp) (Mizuno and Hori 1988; Schoenbach et al. 1997; Anpilov et al. 2002; Vilkov et al. 2004). However, those studies were conducted using deionized waters or low level saline liquids (i.e., a salinity of ≤1.5%; NaCl as a sole salt) as the suspension medium. In seawater (e.g., a salinity of ~3.4%), the discharge generation becomes technically difficult due to the high leakage current via conductive seawater, requiring input powers in excess of a few megawatts. In the present study, we constructed a PHVD system that could operate in two different modes, one with high current and the other with low current followed by the generation of intense pressure wave. First, removal efficiencies by the PHVD system in the two modes were compared using zooplankton and phytoplankton. As judged by these experiments, the more effective PHVD system was further evaluated using four different organisms, one zooplankton larva, two phytoplankton species, and *Escherichia coli* suspended in seawater in a static chamber.

2 Materials and Methods

2.1 Electrode System and Pulsed High-Voltage Generator

An experimental setup to generate intensive shockwave by high-voltage pulse is shown in Fig. 1a. The discharge system consisted of high-voltage DC power supply (Glassman PK50R80, 50 kV, 80 mA), a charging capacitor (Maxwell 37330, 0.1 μF), mechanical switches, and discharge reactor containing seawater (85 ml) and the electrodes. It is noted that the entire reactor wall (made of stainless steel) was used as a ground electrode for electrical safety. The electrical properties of the discharge reactor were measured using a high-voltage probe (Tektronix P6015A) for the voltage across the electrodes and a current sensor (Ion Physics, CM-10-H) for a discharge current. Typically, the capacitor was charged with the positive high voltage, on the order of 5–20 kV. Once the charging was completed, the mechanical switch was closed, and the high-voltage power was applied to the discharge reactor.

In this study, two different power electrodes were used, and each generated distinctively different electrical properties (Fig. 1b–d). The PHVD system operated with very high current and generated practically no pressure wave when the unshielded stainless steel was used as a power electrode. In comparison, the high-voltage powers created intense discharges while operating at much lower current, indicated by the emission of bright lights and the generation of intense pressure waves when the power electrode was partially shielded with the insulator (polyaryl ether ether ketone, PEEK) that was punched a hole (3 mm in diameter) at the tip of the electrode.

The experiments were conducted using the high-voltage pulse generator with the following parameters: voltage applied to the load was 7.1 kV, 10 kV, and 13 kV, each of which delivered to a load 2.5 J, 5.0 J, and 8.5 J per shot. Due to low repetition rate limited by the mechanical switch, high-voltage pulse applied was fixed at 0.5 Hz. The total numbers of pulses varied from 100 to 700.

2.2 Sampling

The static chamber of the system was washed once by 10% HCl solution and rinsed three times by deionized distilled water (DDW), prior to filling the chamber with seawater for each new experiment. Effects of the system on plankton and an indicator bacterium in seawater were assessed as the following: in sterilized 2 l polycarbonate transparent bottles (Nalgene), individual organisms were diluted to appropriate densities (see the results of each organism) with 1 l of 0.2-μm filtered and autoclaved seawater

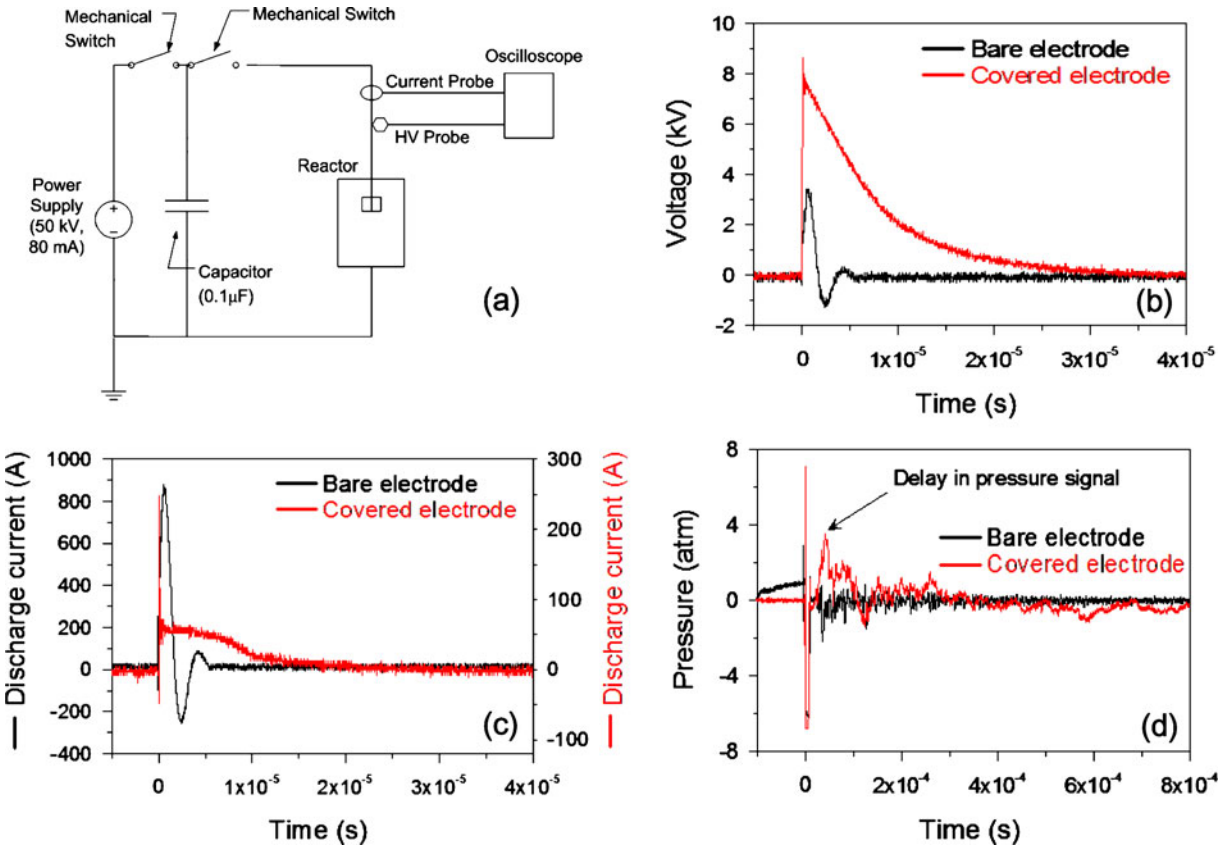

Fig. 1 Description of pulsed high-voltage system. **a** Schematic diagram of the system and a treatment reactor. **b** Voltage and **c** current waveform of two different discharges at 7.1 kV. Note that the voltage of the bare electrode goes up to only 3.5 kV due to the high leakage current. **d** Pressure measurements using a PCB piezotronics sensor (S113B)

(30–33 psu). After gentle mixing, 85 ml of seawater containing individual organisms was added to the static chamber. After treatment by the system, the seawater in the static chamber was gently mixed. The whole seawater sample was dispensed into three sterilized 50 ml conical tubes (SPL Life Science). The tubes were subjected to biological analyses (see below). For the control sample, 85 ml of seawater containing individual organisms was added to the static chamber as above and remained untreated for 23.5 min, which is almost equivalent to a time of 700 pulses.

2.3 Zooplankton Analysis

To determine the effect of pulsed high-voltage discharge on zooplankton, we used brine shrimp (*Artemia* nauplii) as a model zooplankton because of convenience: eggs of *Artemia* nauplii are commercially available (Ocean Star International Inc. PRO 80™). *Artemia* nauplii were hatched from ca 0.02 g eggs in 1 l of autoclaved seawater and incubated at 20 °C for 1–2 days. Three- to 4-day-old larvae approximately 1 mm in length were used for experiments.

Abundance of live and dead zooplankton was measured as described by Crippen and Perrier (1974). An aliquot (10 ml) of sample was stained with neutral red (final conc. of 0.001%). After 50-min incubation at room temperature, the sample was fixed by unneutralized formalin (final conc. of 3.5%). The organisms were filtered from the solution on 20 μm mesh screen and were placed in autoclaved seawater (10 ml); 400 μl of an acetic acid-sodium acetate stock solution made up of equal parts of 1 N acetic acid and 1 N sodium acetate were added. The formalin addition (final conc. of 3.5%) was repeated, and the samples were stored overnight at 4 °C prior to live–dead determinations. Live (deep red in color) and dead (white to pink in color) organisms were enumerated by a stereomicroscope (SZX9, Olympus).

2.4 Phytoplankton Analysis

We used two phytoplankton species, *Prorocentrum micans* (dinoflagellate) and *Skeletonema costatum* (diatom), which were purchased from the Korean Marine Microalgae Culture Center (strain KMMCC D-008) and the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (strain CCMP782), respectively. The organisms were incubated in f/2 medium (Guillard and Ryther 1962) based on 0.2-μm filtered and autoclaved seawater. The cultures were incubated at 20°C under continuous light condition (~120 μE $m^{-2}s^{-1}$). The organisms in exponential growth phase were used in experiments.

Abundance of live and dead phytoplankton for *P. micans* was measured using the green nucleic acid dye SYTOX® Green (Molecular Probes) as described by Veldhuis et al. (1997), with modifications for use with epifluorescent microscopy. An aliquot (1 ml) of sample was stained with SYTOX® Green (final conc. of 0.5 μM) and stored at the dark for 10 min. Then, the sample was filtered on 0.8 μm pore-size polycarbonate black filter (Whatman). Live (red) and dead (green) cells were counted by using a blue filter set on an epifluorescence microscope (BX60, Olympus) at ×400. However, the above method could not be employed to *S. costatum* owing to its poor differentiation between live and dead cells. To see if chlorophyll *a* (chl *a*) determination is a good index for viability assessment for our experiment, concentrations of chl *a* for *S. costatum* were measured spectrophotometrically according to Parsons et al. (1984); an aliquot (10 ml) of sample was filtered on GF/F filter (Whatman). Chl *a* was extracted from the filter with acetone (90%) in the dark at 4°C overnight and analyzed using a UV–Visible spectrometer (Ultrospec 2000, Pharmacia Biotech).

2.5 Indicator Bacteria Analysis

E. coli KACC 13821^T was obtained from the Korean Agricultural Culture Collection (KACC). The cells of *E. coli* were cultured at 37°C in LB medium (Bertani 1951). A preparation of cells to use in experiments was made as follows: cells grown overnight were centrifuged at 3,000×*g* for 3 min. To remove growth medium, a supernatant was discarded, and a pellet was resuspended with 0.2-μm filtered and autoclaved seawater. The centrifugation and washing procedure were repeated three times.

Viability of bacteria was assessed by three different methods, including two culture-based methods (membrane filter method and enzyme substrate method, which are approved by the Standard Methods for the Examination of Water and Wastewater; American Public Health Association 1999) and one culture-independent method (live and dead cells counts using epifluorescence microscopy) as follows. For the membrane filter method, samples were diluted with 0.2-μm filtered and autoclaved seawater (final conc. of 10–100 cells ml^{-1}). On a sterilized 0.2 μm membrane filter, 5 to 10 ml of the diluted sample was filtered (Toyo Roshi Kaisha, Ltd.). The filter was placed on Endo medium (Difco) and incubated at 37°C for 24 h. Colonies that are pink to dark red and have a greenish-gold metallic sheen were counted as live *E. coli* (mEndo method; American Public Health Association 1999). For the enzyme substrate method, a sample was diluted with 100 ml of 0.2-μm filtered and autoclaved seawater (final conc. of 1–10 cells ml^{-1}). Live cells of *E. coli* were enumerated using the Colilert kit (IDEXX Laboratories), according to the manufacturer's instruction. For the live and dead cell counts using epifluorescence microscopy (Choi et al. 1996), an aliquot of a sample (1 ml) was fixed with 0.2-μm filtered glutaraldehyde (final conc. of 4%). The sample was stained with both SYTO 9 and propidium iodide dyes (final conc. of 0.3% of each) supplied in live/dead BacLight kit (Molecular Probes) and incubated at room temperature in the dark for 20 min. The sample was filtered through 0.2 μm polycarbonate black filter (Whatman), then the filter was washed twice with isopropanol (1 ml). Live (green) and dead (red) cells were counted by using a blue filter set on an epifluorescence microscope (BX60, Olympus) at ×1000.

2.6 Biochemical Analysis for Assessing Viability of Organisms

In parallel with the above methods, adenosine triphosphate (ATP) content was used to assess the effects of the pulsed high-voltage system on viability of organisms. Determination of ATP is basically based on luciferin–luciferase assay (Hamilton and Holm-Hansen 1967). An aliquot (10–25 ml) of the samples was filtered on an appropriate pore-size

cellulose acetate membrane filter (Advantec): 5 μm, 1 μm, and 0.2 μm for zooplankton, phytoplankton, and bacteria, respectively. Autoclaved seawater passed through a 0.2-μm filter served as a blank. The filter was quickly placed in a 15-ml conical tube, and 5 ml of boiling TE buffer (100 mM Tris–HCl, 4 mM EDTA, pH 7.75) was added to the tube. After heating at 100°C for 5 min, to allow for complete extraction of ATP, the tube was chilled to room temperature. After centrifugation at 3,000 rpm for 15 min, a triplicate of 50 μl of a supernatant was dispensed to a 96-well plate. ATP standard (10^{-5}–10^{-10}M; ATP bioluminescence assay kit CLS II, Roche) was also dispensed to the 96-well plate. ATP was analyzed with the luciferin–luciferase assay using the ATP bioluminescence assay kit and a luminometer (Panomics Inc.). ATP contents of samples were calculated based on the relationship between ATP standard and light intensity.

2.7 Other Analyses

Seawater temperature before and after treatment was measured using a digital thermometer (APPA 51, APPA Technology). Salinity was measured using a Temperature/Conductivity/Salinity Instrument (YSI 30, YSI Inc.). Data for statistical analyses were inspected by examining residuals and corrected for normality using the natural log transformation. Regression analyses and *t* tests were carried out using the SPSS software for Windows (Version. 16.0, 2007).

3 Results

3.1 Zooplankton

Initially, we compared the effects of the PHVD for two different modes at 7.1 kV, one with high current (~800 A) and the other with the intense discharge and the shock wave generation at low discharge current (~65 A). The results indicated that the intense discharge mode was superior (1.9–to 4.0-fold) to the high current mode (Table 1). Similar results have been observed for phytoplankton when two different types of discharge modes were compared. Based on these results, we decided to focus treatment experiments only on the intense discharge mode, while employing higher voltages of 10 kV and 13 kV to enhance the efficiency of PHVD treatment.

Table 1 Lethal effects of pulsed high-voltage discharge system with two electrodes on zooplankton (*Artemia* nauplii) and phytoplankton (*Prorocentrum micans*). Voltage (7.1 kV) applied to a capacitor (0.1 μF) and pulse-frequency (1 Hz) were identical for the two electrodes. Numbers of pulse were 500 and 300 for zooplankton and phytoplankton, respectively. Killing effects were calculated as percentages of the initial cell counts or ATP contents; mean±SD (n=3)

	High current-generating electrode (bare electrode)	Pressure waves-generating electrode (partially covered electrode)
Zooplankton		
Viable count (%)	45±6	85±3
ATP method (%)	20±2	80±2
Phytoplankton		
Viable count (%)	15±4	50±3
ATP method (%)	15±3	50±3

Abundance of live zooplankton was 11–15 cells ml^{-1} (dead cells were <1% of total cells) in untreated (control) samples. The effects of the pulsed high-voltage system on live cell abundances were similar at the applied voltages of both 10 kV and 13 kV (Fig. 2a). The initial abundance of live zooplankton decreased by 57±7% at discharge with 100 pulses at 13 kV, and live cells were not detected at discharge with 400 pulses at 13 kV (Fig. 2a). The results obtained by measurements of live cell abundance were similar to those obtained by ATP analysis (Fig. 2b).

3.2 Phytoplankton

For the dinoflagellate, *P. micans*, abundance of live cells was 4.5–5.1×10^3 cells ml^{-1} (dead cells were 7–9% of total cells) in untreated (control) samples. Based on measurements of live cell abundance, the pulsed high-voltage system at 13 kV seems to be more effective than at 10 kV in removing live cells (Fig. 3a); the initial abundance of live cells decreased by 58±1% with 400 pulses at 13 kV, and by 77±0.5% with 700 pulses at 13 kV (Fig. 3a). Based on measurements of ATP content, the initial ATP content decreased by 57±5% with 400 pulses at 13 kV, and by 87±2% with 700 pulses at 13 kV (Fig. 3b).

For the diatom *S. costatum*, the initial chl *a* concentration decreased by 76±6% with 700 pulses

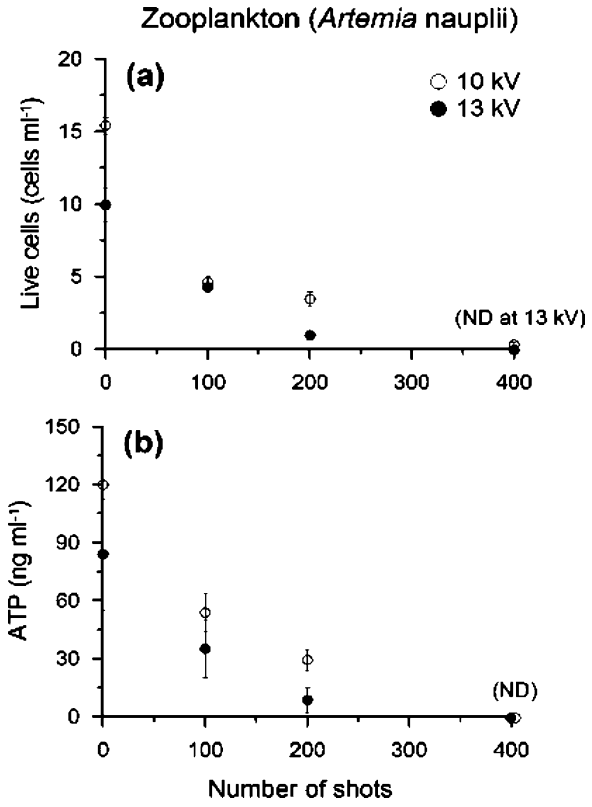

Fig. 2 Effects of applied voltages and pulse numbers on viability of zooplankton (*Artemia* nauplii) determined by **a** live and dead cell counts and **b** ATP content measurements. When bars are not shown, 1 SD is less than size of the symbol (n=3). ND; not detected

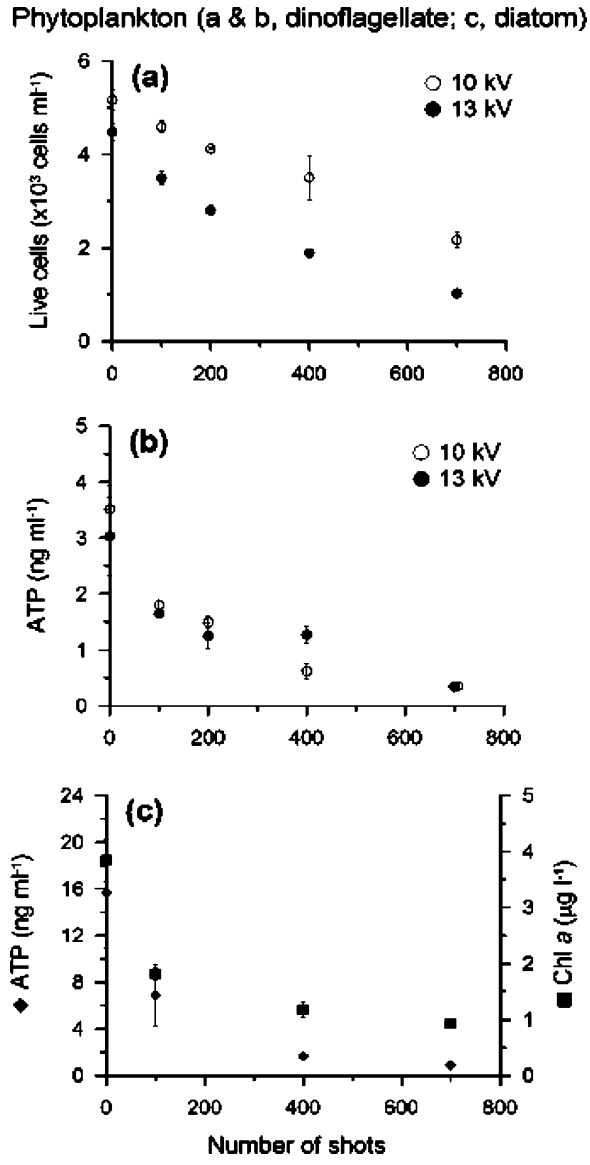

Fig. 3 Effects of applied voltages and pulse numbers on viability of phytoplankton determined by **a** live and dead cell counts and **b** ATP content measurements for the dinoflagellate of *Prorocentrum micans*; **c** ATP content and chl *a* measurements for the diatom of *Skeletonema costatum* at discharge of 13 kV. When bars are not shown, 1 SD is less than size of the symbol (n=3)

at 13 kV (Fig. 3c). A similar decrease (97±2%) was found in measurements of ATP content with 700 pulses at 13 kV (Fig. 3c).

3.3 Indicator Bacteria

For *E. coli*, abundance of live cells measured by epifluorescence microscopy using the live/dead BacLight kit was 1.3–1.8×10^5 cells ml^{-1} (dead cells were 10–15% of total cells) in untreated (control) samples. The initial abundance of live cells determined by epifluorescence microscopy decreased by 94±1% with 700 pulses at 13 kV (Fig. 4a). Live cells determined by the mEndo method were not detected with 200 pulses at 13 kV and with 400 pulses at 10 kV (Fig. 4b). Live cells determined using the Colilert kit were not detected with 700 pulses at 13 kV (Fig. 4c). The initial ATP content decreased by 99±2% with 700 pulses at 13 kV (Fig. 4d).

4 Discussion

The present PHVD system has shown to be effective in treating tested organisms in seawater. The effectiveness of the system was measured by separate independent methods, two for phytoplankton and zooplankton, and four for indicator bacteria. The killing effects of the

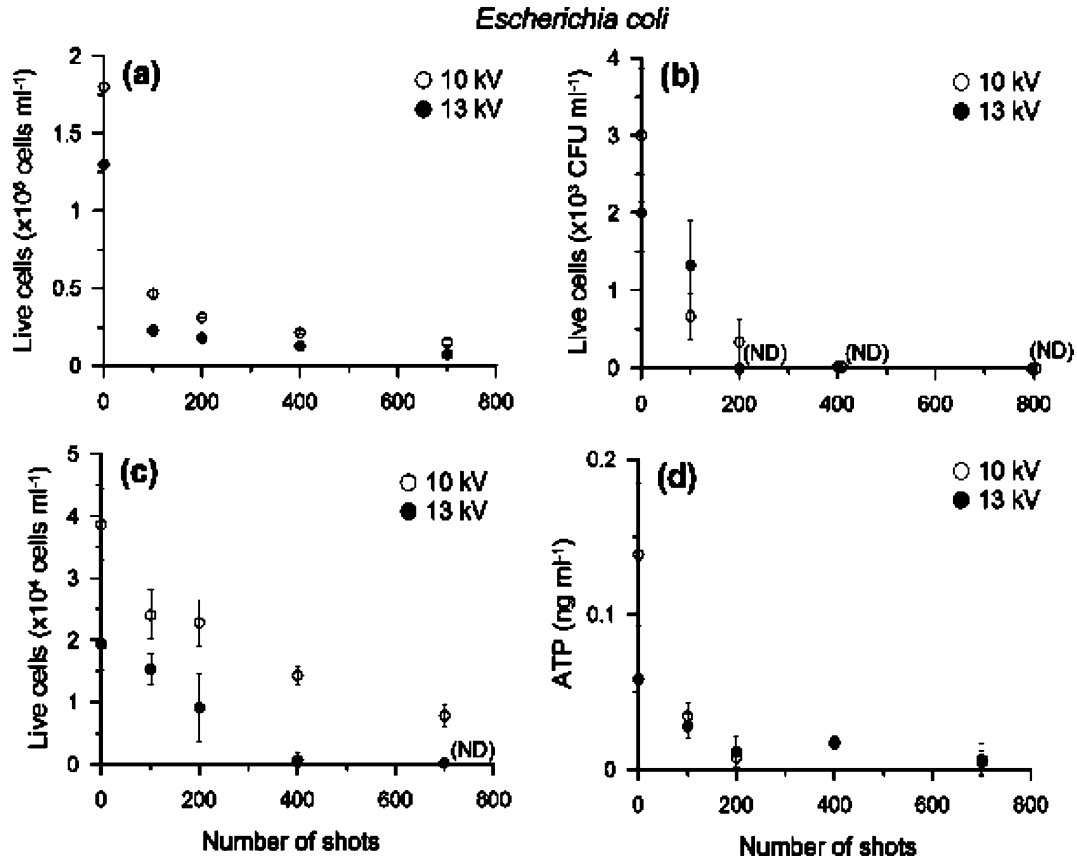

Fig. 4 Effects of applied voltages and pulse numbers on viability of *Escherichia coli* determined by **a** live and dead cells counts, **b** membrane filter method (mEndo), **c** enzyme substrates method (Colilert kit) and **d** ATP content measurements. When bars are not shown, 1 SD is less than size of the symbol (n=3). CFU; colony forming unit, ND; not detected

system were pronounced for zooplankton and indicator bacteria (i.e., live organisms were not detected in certain treatment conditions; Figs. 2 and 4), indicating that the system was effective in killing the corresponding organisms in seawater. The effects of the system on phytoplankton were less pronounced than those observed in other organisms in the PHVD system.

It is, however, noted that biological effectiveness of ballast water treatments might vary depending on assessed methods since no standard methods for measuring abundance of live organisms in the Ballast Water Performance Standard (IMO 2005). The present study employed the conventional methods for evaluating effects of ballast water treatment on zooplankton (neutral red staining, Waite et al. 2003; Quilez-Badia et al. 2008), phytoplankton (SYTOX® Green staining, Veldhuis et al. 2006; Gregg and Hallegraeff 2007), and *E. coli* (using mEndo or Colilert kit, Waite et al. 2003). Interestingly, measurements of ATP content of organisms showed congruent results with the conventional methods, suggesting it may be a rapid method for evaluating treatment efficiency for ballast water.

In addition, the efficiency of ballast water treatment on organisms could be affected by abundance of the organisms to be treated: for seawater samples supplemented with higher numbers of *E. coli* (i.e., the density was ~370-fold higher than that was used in Fig. 4), PHVD treatment efficiencies in killing *E. coli* were slightly (i.e., 14.7%) lower (data not shown).

In comparisons of the methodologies employed in the present study, two results were notable; first, effects of the novel system on a diatom using measurement of chl *a* were somewhat but significantly lower ($p<0.05$, t test, slope comparison; Fig. 3c) than those using measurement of ATP content. A possible explanation for the difference is that chl *a* may exist for

some time (e.g., hours to days) in dead phytoplankton, while ATP content quickly disappears upon the death of a cell (Waite et al. 2003). Thus, measurements of chl *a* might slightly underestimate killing effects for phytoplankton. Second notable result was that live indicator bacteria were still observed by culture-independent methods (i.e., using live/dead BacLight kit and ATP content; Fig. 4a, d) at conditions that live cells were not detected in culture-dependent methods (i.e., using membrane filtrations and substrate utilization; Fig. 4b, c). The inconsistency suggests that monitoring of indicator bacteria in the treated samples should be based on detecting viable but non-culturable bacteria (VNCB). The presence of live bacteria that are uncultured by conventional methods after treatment would pose a problem.

In pulsed high-voltage systems, one of the factors affecting viability of organisms may be pressure (Anpilov et al. 2002) rather than current. As shown in Table 1, the partially covered electrode-generating pressure wave (7.1 kV, ~65 A) was found to be more effective in killing zooplankton (1.9- to 4.0-fold) and phytoplankton (3.3-fold) than a bare electrode generating high current (7.1 kV, ~800 A) in the system we tested. Elevated seawater temperature by pulsed high-voltage discharge may not be a crucial factor affecting viability of organisms in the system. Although initial seawater temperature (ca 20 °C) was increased up to 25.1 °C by discharge with 700 pulses at 13 kV, the increased temperature was in the range of growth temperature of the assessed organisms (Browne and Wanigasekera 2000; Okaichi 2004; Scheutz and Strockbine 2005; Takabayashi et al. 2006). In addition, it was reported that such short-time heat treatments (e.g., ~30 °C for <0.5 h) did not exert lethal effects on plankton and bacteria (Choi et al. 2002; Quilez-Badia et al. 2008). Changes of pH value in seawater samples after pulsed high-voltage discharge were negligible (data not shown). It is noted that there are other factors that may affect the viability of organisms in the pulsed high-voltage discharge (e.g., chemically active substances and UV radiation; Anpilov et al. 2002). We plan to include these factors in the future study as well as investigating the scalability and 'real world' applicability of the PHVD technology.

One of the major issues related to scalability is the cost and technical complexity of high-voltage pulsed power system. The typical cost is about US $5–10 per watt of average power for a custom pulsed power system, and we believe that a minimum of 10 kW of power will be needed to investigate the scalability properly. A number of other factors such as flow rate, pressure wave propagation, and damping should be considered to ensure the efficiency of the PHVD system. Operating the PHVD technology in a flowing system would be more desirable while the ship is ballasted (or de-ballasted) rather than treating ballast water in the ballast tanks during a voyage. Finally, the minimum treatment rated capacity (200 m^3h^{-1}) and the safety of the ship and personnel for the operation of the system (e.g., electric leakage circuit breaker and installation of suitable by-passes or overrides in the event of an emergency) should be fulfilled to meet IMO standards and conditions (IMO 2008). To sum up, the PHVD system with a shockwave-generating mode has a high potential for applying to ballast seawater treatment.

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