Chemosphere 258 (2020) 127412

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Improved toxicity analysis of heavy metal-contaminated water via a novel fermentative bacteria-based test kit

Heonseop Eom [a, 1], Woochang Kang [a, 1], Seunggyu Kim [a], Kangmin Chon [b], Yong-Gu Lee [b], Sang-Eun Oh [a, *]

[a] Department of Biological Environment, Kangwon National University, 192-1 Hyoja-dong, Gangwon-do, Chuncheon-si, 200-701, Republic of Korea
[b] Department of Environment Engineering, Kangwon National University, 192-1 Hyoja-dong, Gangwon-do, Chuncheon-si, 200-701, Republic of Korea

HIGHLIGHTS

- A novel fermentative bacteria-based microbial toxicity test kits is developed.
- Toxicity assessment is performed by inhibition of fermentative gas production.
- Hg^{2+}, Cu^{2+}, Cr^{6+}, and Ni^{2+} are highly toxic on fermentative gas production.
- Fermentative bacteria test kits warrant simple and reliable water toxicity tests.

ARTICLE INFO

Article history:
Received 27 April 2020
Received in revised form
10 June 2020
Accepted 11 June 2020
Available online 18 June 2020

Handling Editor: Willie Peijnenburg

Keywords:
Fermentative bacteria
Toxicity test kit
Heavy metals
Syringe method

ABSTRACT

The objective of this study was development of a simple and reliable microbial toxicity test based on fermentative bacteria to assess heavy metal (Hg^{2+}, Cu^{2+}, Cr^{6+}, Ni^{2+}, As^{5+}, or Pb^{2+})-contaminated water. The dominant species of test organisms used in this study was a spore-forming fermentative bacterium, *Clostridium guangxiense*. Toxicity of water was assessed based on inhibition of fermentative gas production of the test organisms, which was analyzed via a syringe method. Overall, the fermentative bacteria-based test kits satisfactorily identified increased toxicity of water as water was contaminated with high amounts of heavy metals; however, levels of inhibition were dissimilar depending on the species of metals. Inhibitory effects of Hg^{2+}, Cu^{2+}, Cr^{6+}, and Ni^{2+} were considerably greater than those of As^{5+} and Pb^{2+}. The 24 h half-maximum effective concentrations (EC_{50}) for Hg^{2+}, Cu^{2+}, Cr^{6+}, Ni^{2+}, As^{5+}, and Pb^{2+} were analyzed to be 0.10, 0.51, 1.09, 3.61, 101.33, and 243.45 mg/L, respectively, confirming that Hg^{2+}, Cu^{2+}, Cr^{6+}, and Ni^{2+} are more toxic to fermentative gas production than As^{5+} and Pb^{2+}. The fermentative bacteria-based toxicity test represents an improvement over other existing toxicity tests because of ease of end-point measurement, high reproducibility, and favorable on-site field applicability. These advantages make the fermentative bacteria-based test suitable for simple and reliable toxicity screening for heavy metal-contaminated water.

1. Introduction

Water toxicity is determined to assess the hazardous effects of contaminated water on the environment and ecosystems (Pritchard, 1993; Gunjan and Gargi, 2015). Conventionally, this determination has relied on physicochemical analyses of fundamental parameters of water quality, such as dissolved oxygen, turbidity, solids, and nutrients, and quantification of selected contaminants in water (Niemirycz et al., 2007; Tyagi et al., 2013; Bae and Park, 2014; Rahmanian et al., 2015). This approach is beneficial for obtaining characteristics of water and detailed information about particular toxicants (Brayner et al., 2011; Hassan et al., 2013; Gunjan and Gargi, 2015). However, physicochemical analyses are not able to identify all compounds causing toxicity due to the complex molecular structure of some contaminants (Lors et al., 2011; Hassan et al., 2013; National Research Council, 2014). Moreover, physicochemical analyses are limited in demonstrating biochemical effects of contaminants on living organisms, synergetic or antagonistic interactions between toxicants, and bioavailability

* Corresponding author.
E-mail address: ohsangeun@kangwon.ac.kr (S.-E. Oh).
[1] The authors equally contributed.

https://doi.org/10.1016/j.chemosphere.2020.127412
0045-6535/

of pollutants (Munawar et al., 1989; Hernando et al., 2005; Brayner et al., 2011; Lors et al., 2011). To overcome these drawbacks of physicochemical analyses and reveal actual threats of contaminated water to the environment and ecosystems, biological toxicity tests, which employ organisms to assess toxicity of contaminants, have been widely used as alternatives or as valuable supplements to physicochemical toxicity tests (Sponza, 2002; Farré and Barceló, 2003; Niemirycz et al., 2007).

Bioassays have served as one of the most efficient tools for biological toxicity tests (Brayner et al., 2011; Oh et al., 2011; Hassan et al., 2012). In bioassays, different trophic levels of organisms including invertebrates, fish, daphnia, algae, and bacteria are exposed to contaminated water and monitored for their physiological responses (Mishra et al., 2000; Cho et al., 2004; Moreira-Santos et al., 2004; Barata et al., 2008; Palma et al., 2008). Particularly, bacteria-based bioassays are desirable for simple and routine toxicity screening tests because they provide short test times, easy test protocols, high sensitivity, cost-effectiveness, and less ethical responsibility (Liu et al., 1989; Rönnpagel et al., 1995; Catterall et al., 2010; Oh et al., 2011). A variety of bacteria such as bioluminescent bacteria, nitrifying bacteria, iron-oxidizing bacteria, sulfur-oxidizing bacteria, oligotrophic bacteria, and *Escherichia coli* has been used as test organisms in microbial toxicity tests and has performed favorably in evaluating the toxicity of diverse inorganic and organic toxicants in water (Tada et al., 2001; Kim et al., 2003; Eilersen et al., 2004; Tencaliec et al., 2006; Zlatev et al., 2006; Oh et al., 2011).

The current study tested employment of fermentative bacteria in bioassays to develop simple and reliable microbial toxicity tests to assess heavy metal-contaminated water. To the best of our knowledge, this is the first report employing fermentative bacteria in biological toxicity tests. Fermentation is the metabolic process by which organic molecules are converted into gases, acids, and alcohols in the absence of oxygen (Medina, 2019). Regardless of the type of fermentation, common fermentation by-products include gases such as H_2 and CO_2 (Medina, 2019). In the presence of toxic substances, metabolic activity of fermentative bacteria is inhibited, resulting in less production of gas. The present study evaluated the toxicity of heavy metal-contaminated water based on inhibition of the gas production of fermentative bacteria.

For the current tests, we developed 25 mL vial-based kit-type bioassays. Test organisms obtained from glucose fermentation with heat-shocked soil inoculum were mostly spore-forming fermentative bacteria, *Clostridium guangxiense*. They were directly exposed to water spiked with mercury (Hg^{2+}), copper (Cu^{2+}), hexavalent chromium (Cr^{6+}), nickel (Ni^{2+}), arsenic (As^{5+}), or lead (Pb^{2+}) for 24 h in kit bioassays. The amounts of gas produced from test kits were determined via a syringe method and served as end-points for toxicity assessment. Expected benefits of this test protocol stem from the ease and reliability of end-point measurements. This is because the syringe method provides an easy and simple way to determine amounts of gas production using a glass syringe. The present test requires neither skilled personnel nor advanced equipment to determine end-points. In addition, unlike other microbial toxicity tests that adopt luminescence, fluorescence, and optical density as end-points and thus are potentially affected by conditions of test samples including turbidity, color, and nanoparticles (Rotini et al., 2017; Qiu et al., 2017), the current end-point measurement is independent of the characteristics of test samples, providing high reproducibility and reliability of test results. Based on the inhibitory effects of heavy metal-contaminated water on fermentative gas production, dose-response relations were developed. Half maximum effective concentrations (EC_{50}) for heavy metals were analyzed and compared to those from published microbial toxicity tests. Suitability and advantages for the employment of fermentative bacteria in biological toxicity tests are discussed.

2. Materials and methods

2.1. Fermentative bacteria culture reactor

Fermentative bacteria, used as test organisms in toxicity tests, were cultured in 125 mL serum bottles (Fig. 1). As inoculum, 0.5 g of heat-shocked soil was employed. The soil was collected from a local tomato farming area around Kangwon National University, Chuncheon, South Korea, and the heat treatment (heat shock) of the collected soil sample was conducted as described by Logan et al. (2002). Glucose (4 g/L) was used as an organic substrate, and 125 mL of nutrient solution composed as shown in Table 1 was employed as media. The nutrient solution was buffered with sodium phosphate (4.3 g/L of $NaH_2PO_4 \cdot 2H_2O$ and 4.0 g/L of Na_2HPO_4); working pH was approximately 6. Prior to incubating the culture

Table 1
Nutrients composition of media used for the culture reactor and toxicity tests.

Component	Amount
$NaH_2PO_4 \cdot 2H_2O$	4.33 g/L
Na_2HPO_4	2 g/L
NH_4Cl	0.5 g/L
$MgSO_4$	0.4 g/L
$CaCl_2 \cdot 2H_2O$	3.67 mg/L
$FeSO_4 \cdot 7H_2O$	2.5 mg/L
$MnCl_2 \cdot 4H_2O$	1.25 mg/L
$ZnSO_4 \cdot 7H_2O$	1.1 mg/L
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.25 mg/L
$CoCl_2 \cdot 6H_2O$	0.05 mg/L
$CuSO_4 \cdot 5H_2O$	0.05 mg/L

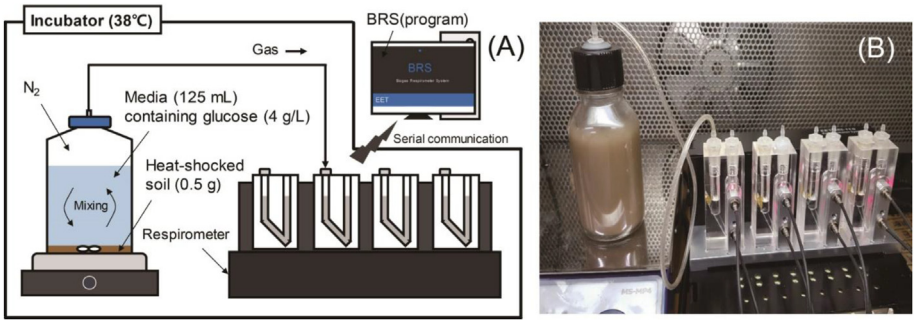

Fig. 1. Schematic diagram (A) and picture (B) of fermentative bacteria culture reactor system.

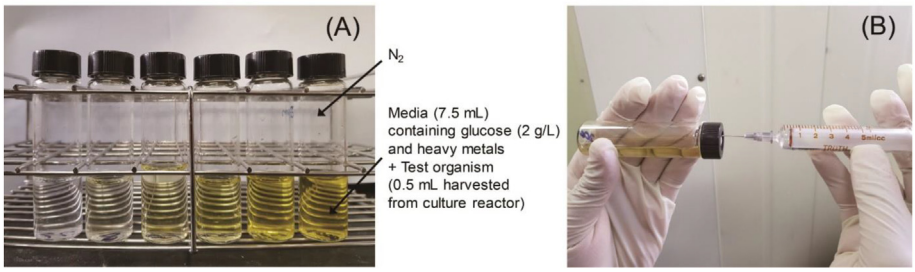

Fig. 2. (A) Fermentative bacteria-based toxicity test kit, (B) Syringe method.

reactor, N_2 gas was sufficiently sparged to the media and head-space of the culture reactor (for approximately 20 min) to remove O_2 and render conditions anaerobic. The culture reactor was maintained inside an incubator at 35 °C. Amounts of gas produced from the culture reactor were automatically monitored every 30 min by a respirometer (BRS-110, EET, Chuncheon, South Korea) during the entire operation of the culture reactor (All data were recorded by a computer connected to a respirometer). Percentages of H_2 and CH_4 in the produced gas were analyzed every 5 h. Concentrations of glucose in the samples collected at 0, 10, 15, 20, 25, and 30 h were determined. Amounts of volatile fatty acids (VFAs) and alcoholic compounds were measured at 25 h. Once the culture reactor reached maximum gas production, cells were harvested and provided to subsequent heavy metal toxicity tests.

2.2. Toxicity kit test

A fermentative bacteria test kit was developed based on a 25 mL glass vial equipped with a plastic cap and a rubber stopper (Fig. 2A). Details of preparing the toxicity kit test are as follows. First, 7.5 mL of media used in the culture reactor and containing glucose (2 g/L) was added to the test kit. Initial temperature of the media should be 35 °C. Mercury (Hg^{2+}), copper (Cu^{2+}), hexavalent chromium (Cr^{6+}), nickel (Ni^{2+}), arsenic (As^{5+}), or lead (Pb^{2+}) was individually spiked to the media in various concentration ranges (Table 2) but always less than 0.1 mL. After closing the cap, the media and head-space of the test kit were sparged with N_2 gas to eliminate O_2 from them. Specifically, two syringe needles were used to pierce the rubber stopper; N_2 gas was sparged through one syringe needle and the other needle was for releasing pressure. After sufficient N_2 gas sparging (for approximately 10 min), the two needles were removed. Then, the test organisms (0.5 mL) harvested from the culture reactor were added to the media within the test kit using a syringe. Lastly, before incubation of the test kit, the test kit's rubber stopper was pierced by a syringe with needle to completely release residual positive pressure. These preparation procedures were performed under anaerobic conditions, if possible.

The test kits were incubated for 24 h in a shaking water batch with a 100 rpm of mixing intensity at 35 °C. In mercury toxicity tests, amounts of gas production, percentages of H_2 and CH_4 in the produced gas, and concentrations of glucose, VFAs, and alcoholic compounds were analyzed at 0, 6, 12, 24, 28 h after beginning the incubations. In the other heavy metal tests, amounts of gas production were determined only at 24 h. All toxicity kit tests were conducted in triplicate, and their average values and standard deviations are reported in the results.

Table 2
Ranges of doses of introduced heavy metals for toxicity tests.

Heavy metals	Range (mg/L)
Hg^{2+}	0.05, 0.1, 0.2,0.5
Cu^{2+}	0.5, 1, 1.5, 2
Cr^{6+}	0.5, 1, 2, 5
Ni^{2+}	1, 5, 10, 15
As^{5+}	50, 100, 200, 500
Pb^{2+}	50, 100, 200, 500

2.3. Metagenomic 16S rRNA gene sequencing

A biomass sample was collected from the culture reactor at 30 h and its 16S rRNA sequencing library was developed to identify members of the community of microorganisms. Genomic DNA was extracted from the collected sample using a DNeasy PowerSoil Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. The extracted DNA was quantified using a Quant-IT PicoGreen dsDNA assay kit (Invitrogen, Waltham, MA, US). An Illumina 16S Metagenomic Sequencing Library protocol (Illumina, San Diego, CA, US) was employed for the library preparation. The extracted DNA served as a template for the 1st PCR to amplify the V3-4 region of the 16S rRNA with universal primers 341F and 805R. The 1st PCR was conducted in 25 μL of a reaction volume containing 25 ng of the gDNA, 500 nM of each F/R primer, 2.5 U of Herculase DNA polymerase (Agilent Technologies, Santa Clara, CA, US), 1 nM of dNTP mix, and 5 μL of 5× Herculase reaction buffer (Agilent Technologies, Santa Clara, CA, US). Amplification processing for the 1st PCR had the following thermal conditions in a Bio-Rad T100 thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA, US): initial denaturation at 95 °C for 3 min; 25 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s; then final extension at 72 °C for 5 min. The 1st PCR product was purified with AMPure beads (Agencourt Bioscience, Beverly, MA, US). Following purification, 2 μL of the 1st PCR products was again PCR-amplified for the final library construction using Nextera XT DNA Library Prep Kit (Illumina, San Diego, CA, US). Thermal conditions for the 2nd PCR were identical to those of the 1st PCR except for 10 cycles. The 2nd PCR products were also purified with AMPure beads (Agencourt Bioscience, Beverly, MA, US). The final purified products were then quantified with qPCR according to the qPCR Quantification Protocol Guide (KAPA Library Quantification kits for Illumina Sequencing platforms) and qualified with TapeStation D1000 ScreenTape (Agilent Technologies, Waldbronn, Germany). The amplified 16S rRNA genes were sequenced with MiSeq Reagent Kit V3 (600 cycles; Illumina, San Diego, CA, US). Database homology searches for 16S rRNA were conducted using the BLAST program in the National Center for Biotechnology Information (NCBI). Dominance of specific microorganisms was determined based on the ratio of the number of specific sequence reads to the total number of overall sequence reads. A phylogenetic tree was developed with MEGA4 software using a neighbor-joining method.

2.4. Chemicals and analyses

In the present study, only analytic grade chemicals requiring no further purification were used. For the heavy metal toxicity tests, mercury chloride ($HgCl_2$), copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$), potassium dichromate ($K_2Cr_2O_7$), nickel chloride hexahydrate ($NiCl_2 \cdot 6H_2O$), sodium arsenate dibasic heptahydrate ($Na_2HAsO_4 \cdot 7H_2O$), and lead nitrate ($Pb(NO_3)_2$) were used (Sigma-Aldrich, St. Louis, MO, US). VSS concentrations were determined according to Standard Methods (2540D and E) (APHA, 2005). Amounts of gas production from the test kits were measured via a syringe method using a 10 mL glass syringe (Fig. 2B; Perfektum Syringes, Popper & Sons, INC., NY, US). This syringe method is based on the gas volume measuring method described in Owen et al. (1979). The syringe method determines changes in gas volume based on movement of a syringe plunger. Prior to usage, a syringe plunger was lubricated with 50 mL of deionized water containing 2–3 drops of detergent and its ease of movement confirmed. The needle of the syringe was injected into the test kit parallel to the ground through the rubber stopper without contacting the liquid. The syringe plunger was then allowed to move and equilibrate between the gaseous phase of the test kit and atmospheric pressure (Fig. 2B). The volume of movement of the syringe plunger represents the amount of gas produced from the test kit.

Analyses for H_2 and CH_4 gases were performed using a gas chromatograph (GC; SRI 8610C, SRI Instrument, Torrance, CA, US) equipped with a thermal conductivity detector. The analytical column used in GC analyses was a 6 ft × 2.1 mm stainless steel molecular sieve column (13 × 60/80, Supelco/Sigma-Aldrich, Bellefonte, PA, US). The operational temperature of the GC injector, oven, and detector were 100, 40, and 100 °C, respectively. Argon was employed as the carrier gas with a flowrate of 70 mL/min. Percentages (%) of H_2 and CH_4 in the produced gas from the culture reactor and test kits were determined by comparing peak areas under chromatograms of sample gases with those of 100% pure H_2 and CH_4 gases. Data acquisition, storage, and treatment were controlled by PeakSimple software (Version 3.29, SRI Instrument, Torrance, CA, US).

Analyses for glucose, VFAs (acetic, malic, lactic, propionic, succinic, and tartaric acids), and alcoholic compounds (ethanol, acetone, propanol, and butanol) were conducted using a Waters high performance liquid chromatography (HPLC) system (Waters Corporation, Milford, MA, US). The HPLC system consists of a pump (Waters 515), autosampler (Waters 717), column oven (Waters Alliance 2695), and UV detector (Waters 2487) connected in series with a refractive index detector (Waters 2414). Prior to analyses, samples were filtered through a 0.45 μm syringe PVDF filter (SPF4525-1, Woongki Science Co., LTC, Seoul, South Korea). The analytical column used in HPLC analyses was a 300 mm × 7.8 mm ion exclusion column (Aminex HPX-87H, Bio-Rad Laboratories, Inc., Hercules, CA, US), and sulfur acid (5 mM) was employed as the mobile phase. A sample of 20 μL was injected by autosampler into the column at a flowrate of 0.6 mL/min. Analyses of glucose and alcoholic compounds were performed with a refractive index detector; VFAs were analyzed by a dual wavelength absorbance UV detector at 210 nm. Concentrations (mg/L) of glucose, VFAs, and alcoholic compounds were determined by comparing peak areas under chromatograms of samples with those of standard solutions. The data acquisition, storage, and treatment were controlled by Empower pro software (Waters Corporation, Milford, MA, US).

Toxic response of the test organisms to heavy metals was determined by the inhibition rate (%) based on Eq. (1).

$$Inhibition(\%) = \left(1 - \frac{Amounts\ of\ gas\ for\ 24\ h\ in\ samples}{Amounts\ of\ gas\ for\ 24\ h\ in\ controls}\right) \times 100 \quad (1)$$

Dose-response relations and EC_{10} and EC_{50} values, representing 10 and 50% inhibition of the test organisms, respectively, were analyzed based on the Hillslope equation (Eq. (2)) using Prism 8 (GraphPad Software Inc., San Diego, CA, US)

$$Y = Bottom + \left(\frac{(Top - Bottom)}{\left(1 + 10\right)^{((logEC_{50} - X) \times Hillslope)}}\right) \quad (2)$$

where X is the dose of heavy metal, Y is the toxic response (inhibition rates), *Top* is the maximum toxic response, and *Bottom* is the minimum toxic response.

ANOVA analysis was conducted to assess statistical significance among the data. A p-value less than 0.05 indicated statistical significance.

3. Results and discussion

3.1. Cultivation of fermentative bacteria

Results from operation of the fermentative bacteria culture reactor are shown in Fig. 3. Consumption of initial glucose (4 g/L) was 30.4% at 15 h, 63.9% at 20 h, 83.6% at 25 h, and 100% at 30 h after the culture reactor was started. There was a rapid increase in gas production after a lag phase of 11 h until 25 h, after which no more increase in gas production was observed (Fig. 3A). The total amount of gas produced from the culture reactor was approximately 101 mL, and the maximum gas production rate was estimated to be 5.2 mL/h. Fig. 3B illustrates the percentages (%) of H_2 gas in the head-space of the culture reactor. The highest percentage of H_2 gas, 70.9%, was achieved at 20 h. The low percentages of H_2 gas in the early stage of the culture reactor are most likely due to dilution of H_2 gas by N_2 gas initially existing in the head-space. The maximum H_2 yield of the culture reactor accounted for 1.2 mol H_2/mol glucose. Assuming a theoretical maximum H_2 yield of 4 mol H_2/mol glucose (Gottschalk, 1986; Oh et al., 2003), the culture reactor's conversion efficiency of glucose to H_2 was 30%, which is in the range of findings (22–45%) from previous studies that investigated H_2 gas production from glucose fermentation with heat-shocked soil inoculum (Logan et al., 2002; Oh et al., 2003, 2004; Iyer et al., 2004; Park et al., 2005). CH_4 gas was not detected at all during the entire operation of the culture reactor; accordingly, the other produced gas is thought to be CO_2. A number of earlier studies have likewise reported that fermentation with heat-shocked soil inoculum results in no generation of CH_4 gas (Lay et al., 1999; Van Ginkel et al., 2001, 2005; Logan et al., 2002; Oh et al., 2003). They found that heat treatment (heat shock) of ordinary soils can effectively remove non-spore-forming bacteria such as hydrogen-consuming methanogens and enrich the soil with spore-forming bacteria, preventing interspecies hydrogen transfer leading to methanogenesis (Lay et al., 1999; Van Ginkel et al., 2001, 2005; Logan et al., 2002; Oh et al., 2003). Moreover, the slightly acidic pH (6) and short retention time (3 d) of the current culture reactor would also repress growth of methanogens. Fig. 3C illustrates amounts (based on COD equivalents) of other fermentation by-products including VFAs and alcohols in the culture reactor at 25 h. Among VFAs, acetic acid (626.1 mg COD/L) accounted for the most dominant component, followed by lactic (231.1 mg COD/L),

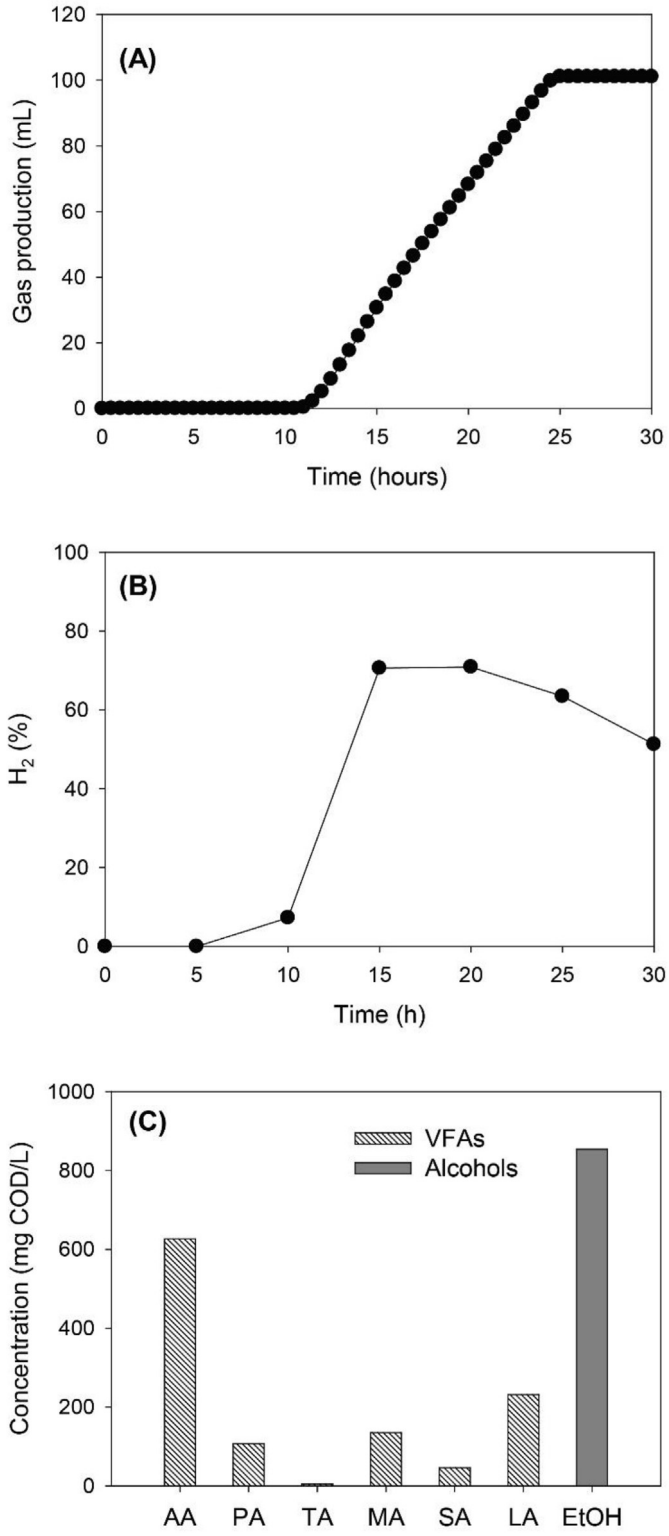

Fig. 3. Fermentative by-products from culture reactor. (A) Gas, (B) Percentages of H_2 gas in head-space, (C) VFAs and alcoholic compounds at 25 h (AA, PA, TA, MA, SA, LA, and EtOH stand for acetic acid, propionic acid, tartaric acid, malic acid, succinic acid, lactic acid, and ethanol, respectively).

malic (134.9 mg COD/L), and propionic acids (107.1 mg COD/L). Minor amounts of succinic (45.6 mg COD/L) and tartaric (4.5 mg COD/L) acids were produced. Ethanol (853.9 mg COD/L) was the only alcoholic compound detected.

To identify species of microorganisms grown in the culture reactor, a metagenomic 16S rRNA gene sequencing library for the sample collected at 30 h was developed. Upon analysis, 94.9 and 4.9% of microorganisms existing in the culture reactor were found to be *Clostridium guangxiense* and *Leclercia adecarboxylata*, respectively. Methanogens were not detected at all, which supports the above finding of no production of CH_4 gas from the culture reactor. The dominance of specific fermentative bacteria and absence of methanogens in the community of the test organisms accord with our intention. We expected that if diverse fermentative bacteria and methanogens exist in the test organisms, microbial reactions in toxicity tests would become complex and gases (H_2 and CO_2) produced from fermentation can be highly consumed via methanogenesis, leading to unreliable toxicity tests. As discussed above, heat shock treatment of soils is an efficient way to make specific fermentative bacteria, particularly spore-forming fermentative bacteria, dominant and eliminate non-spore forming bacteria including methanogen from bacterial community. Moreover, Logan et al. (2002) and Oh et al. (2003) reported that fermentation with heat shock-treated soils can result in fairly similar gas production, regardless of the kinds and characteristics of soils (if identical carbon source is employed), potentially providing high reproducibility to toxicity tests. Accordingly, we performed heat-shock treatment of the inoculum soil used in the culture reactor in order to obtain test organisms suitable for the present test, resulting in dominance of spore-forming bacteria (*C. guangxiense*) and absence of methanogens in the community of the test organisms.

Fig. 4 demonstrates the phylogenetic location of *C. guangxiense* and *L. adecarboxylata*. *C. guangxiense* is known to be a rod-shaped gram-positive, strictly anaerobic, and spore-forming fermentative bacterium with terminal flagella (Zhao et al., 2017); its dominant by-products from glucose fermentation include H_2 gas, CO_2, acetic acid, and butyric acid (Zhao et al., 2017). *L. adecarboxylata* was reported to be a motile rod-shaped gram-negative and facultatively anaerobe of the Enterobacteriaceae family (De Mauri et al., 2013). In spite of information that *L. adecarboxylata* is not a spore-forming bacterium (Muratoğlu et al., 2009), this bacterium enables glucose fermentation when electron acceptors are not available and produces acids, H_2 gas, and CO_2 as fermentation by-products (De Mauri et al., 2013). The results from metagenomic 16S rRNA gene sequencing analysis clearly confirm that spore-forming fermentative bacteria (*C. guangxiense*) were highly cultured and no methanogens were grown in the culture reactor. The biomass samples collected at 30 h, whose VSS concentrations were 726 ± 11 mg/L, were employed as test organisms in the heavy metal toxicity tests.

3.2. Assessment of toxicity of water contaminated with Hg^{2+}

Results from Hg^{2+} toxicity tests are illustrated in Fig. 5. The VSS concentrations of the test bacteria in the toxicity tests were approximately 40 ± 2 mg/L. Overall, as the test organisms were exposed to higher amounts of Hg^{2+}, the volume of gas produced from test kits decreased (Fig. 5A). For example, in the absence of Hg^{2+}, the total volume of produced gas (for 28 h) was, on average, 5.07 ± 0.12 mL, which decreased to 4.13 ± 0.12, 2.53 ± 0.12, 0.57 ± 0.06, and 0 ± 0 mL in the presence of 0.05, 0.1, 0.2, and 0.5 mg/L of Hg^{2+}, respectively. Percentages of H_2 gas in the headspace of test kits also showed a tendency of reduction at increasing doses of Hg^{2+} (Fig. 5B). In the test kits with no Hg^{2+}, the highest H_2 gas percentage of 18.7 ± 1.2% was achieved at 24 h. However, when 0.05, 0.1, 0.2, and 0.5 mg/L of Hg^{2+} were introduced to test kits, the highest H_2 gas percentages declined to 14.3 ± 1.5, 10.4 ± 1.3, 3.7 ± 0.5, and 0 ± 0%, respectively. It is speculated that

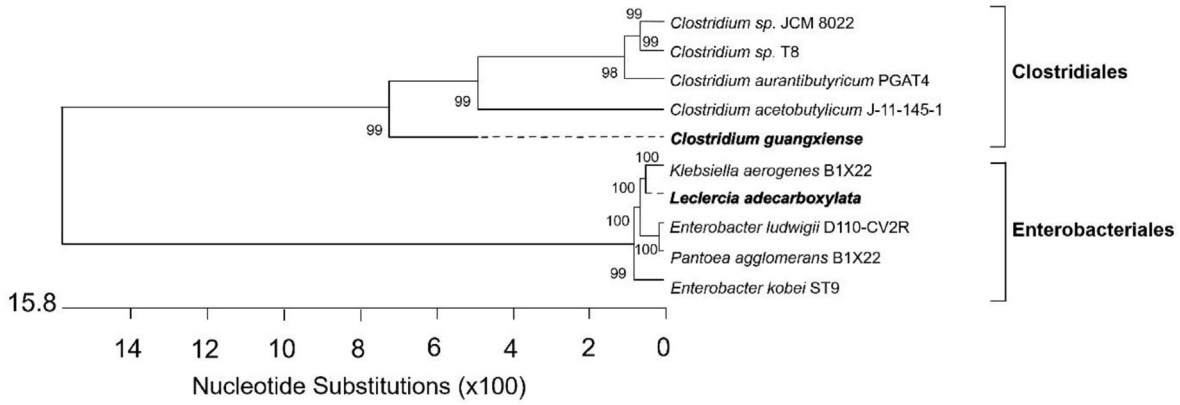

Fig. 4. Phylogenetic tree designed by neighbor-closing analysis of 16S rRNA gene sequence to show position and similarity of *Clostridium guangxiense* and *Leclercia adecarboxylata*.

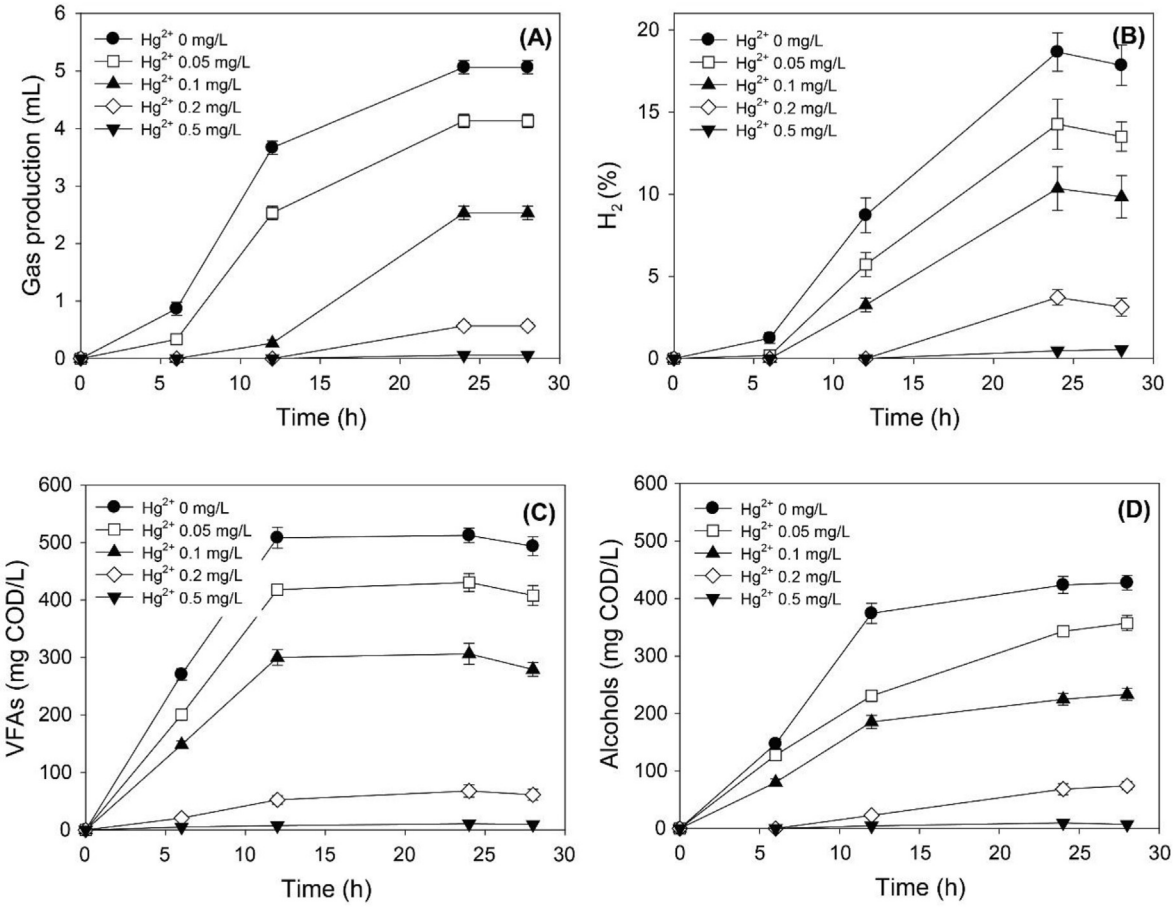

Fig. 5. Fermentative by-products from Hg^{2+} toxicity tests. (A) Gas, (B) Percentages of H_2 gas in head-space, (C) VFAs, (D) Alcoholic compounds. Symbols and error bars represent the average values and standard deviations, respectively, from the triplicate tests.

these relatively lower highest H_2 gas percentages of the test kits, compared to that (70%) of the culture reactor, are attributed to the larger head-space volume (17 mL) of the test kits in comparison with the amounts of fermentative gas production (0.53–6.27 mL) in the test kits. Fig. 5C and D depict fermentative VFAs and alcoholic compounds productions in Hg^{2+} toxicity tests. Similar to the culture reactor, acetic, malic, and lactic acids were major components of VFAs; ethanol was the only element of alcoholic compounds. When no Hg^{2+} was added to the test kits, generations of VFAs and alcoholic compounds for 28 h were 493.6 ± 16.3 and 427.3 ± 12.5 mg COD/L, respectively. However, in the test kits where 0.05, 0.1, 0.2, and 0.5 mg/L of Hg^{2+} were spiked, the generation of VFAs and alcohols for 28 h gradually reduced to 407.9 ± 17.3 and 357.2 ± 13.2 mg COD/L, 279.3 ± 12.3 and 233.2 ± 10.7 mg COD/L, 61.3 ± 9.5 and 74.2 ± 7.1 mg COD/L, and 0 ± 0 and 0 ± 0 mg COD/L, respectively. These findings demonstrate that fermentative VFAs and alcoholic compounds productions were adversely affected with an increase of Hg^{2+} spiked to the test kits. In summary, the test

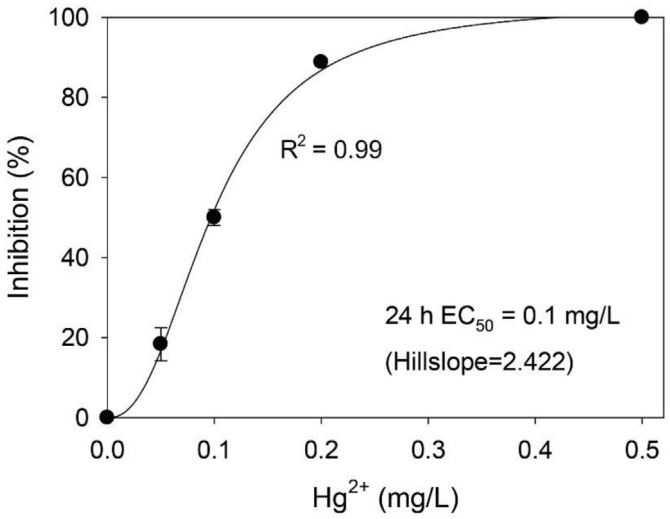

Fig. 6. Inhibition of fermentative gas production in Hg^{2+} toxicity tests. Symbols and error bars represent the average values and standard deviations, respectively, from the triplicate tests.

results indicate that as larger doses of Hg^{2+} were introduced to the test kits, toxicity of water increased, leading to greater inhibition of fermentative activities of the test organisms.

In the present tests, Hg^{2+}-induced water toxicity was assessed by its inhibitory effects on gas production for 24 h (Eq. (1)). Analysis of amounts of gas production is simple and straightforward compared to determining concentrations of H_2 gas, VFAs, and alcoholic compounds. As shown in Fig. 5A, gas production no longer increased after 24 h under the current test conditions, suggesting that fermentative gas production ceased by 24 h; hence, 24 h is sufficient exposure time to evaluate the inhibitory effects of Hg^{2+}. In addition, ANOVA analyses (Table S1) demonstrate that differences among the data (amounts of gas production) at 24 h are statistically significant. As a result, the 24 h inhibition rates of the test organisms by 0.05, 0.1, 0.2, and 0.5 mg/L of Hg^{2+} accounted for 18.4 ± 4.1, 50.0 ± 2.0, 88.8 ± 1.1, and 100 ± 0%, respectively. Based on these values, a dose-response curve is developed in Fig. 6. A dose-response relation is a key concept in ecotoxicology (Wayne, 2004). It represents the magnitude of responses by test organisms as a function of their exposure to doses of contaminants (Wayne, 2004). Fig. 6 satisfactorily describes increased toxicity of water as doses of Hg^{2+} increased and highly corresponds to the Hillslope curve ($R^2 = 0.99$). The 24 h EC_{10} value, which widely serves as the lowest toxicant effect level in biological toxicity tests (Larras et al., 2013; Beasley et al., 2015; Iwasaki et al., 2015), for Hg^{2+} was analyzed to be 0.04 mg/L. This value is higher than generally reported detection limits (0.001–10 μg/L) for heavy metals by advanced physico-chemical analyses based on atomic spectroscopy techniques such as atomic absorption spectrometry (AAS) or inductively coupled plasma emission spectrometry (ICP-ES) (Parsons et al., 1983; Taylor, 2017; Deniz, 2020). However, our kit tests showed more favorable test performance, particularly in terms of test sensitivity, compared to other bacteria-based microbial toxicity tests. As shown in Table 3, our (24 h) EC_{50} value for Hg^{2+} (0.10 mg/L) was lower than published data from earlier microbial toxicity tests. For example, previous bioassay tests using *Vibrio fischeri* and *E. coli*, the most widely employed microorganisms in biological toxicity tests, reported 0.10–1.60 mg/L and 1.50–2.03 mg/L of the EC_{50} values, respectively, for Hg^{2+} with 0.5 min–6 h of exposure times (Gellert, 2000; Bentley et al., 2001; Mowat and Bundy, 2002; Cho et al., 2004; Catterall et al., 2010; Kim and Lee, 2018). A toxicity study employing *Psychrobacter* sp. with determination of electrical current found

Table 3
Comparison of the EC_{50} values of heavy metals from the current and other bacteria-based toxicity tests.

Microorganisms	Exposure time	End-point measurement	EC_{50} (LC_{50}) values (mg/L)						References
			Hg^{2+}	Cu^{2+}	Cr^{6+}	Ni^{2+}	As^{5+} (or As^{3+})	Pb^{2+}	
Fermentative bacteria	24 h	Gas production	0.10	0.51	1.09	3.61	101.33	243.45	Present study
Pseudomonas fluorescens	18 h	Growth	–	17.0	–	8.7	–	14.0	Dutka and Kwan (1981)
Pseudomonas fluorescens	2 h	Growth	–	18.0	2.2	14.0	–	140.0	Paran et al. (1990)
Vibrio fischeri	5 min	Luminescence	–	–	–	10.0–11.0	–	–	Villaescusa et al. (1998)
Vibrio fischeri	6 h	Luminescence	0.12	–	15.9	–	–	272	Gellert (2000)
Escherichia coli PMP 101	0.5 h	Electrical current	1.6	–	–	–	–	–	Bentley et al. (2001)
Escherichia coli PMP 101	0.5 h	Luminescence	1.5–4.6	–	–	–	–	–	Bentley et al. (2001)
Nitrifying bacteria	2 h	Nitrification	–	41.5	35.6	–	–	–	Dalzell et al. (2002)
Activated sludge	3 h	Respiration	–	40.0	36.2	–	–	–	Dalzell et al. (2002)
Activated sludge	0.5 h	ATP luminescence	–	5.5	37.5	–	–	–	Dalzell et al. (2002)
Activated sludge	0.25 h	Enzyme activity	–	10.5	228.5	–	–	–	Dalzell et al. (2002)
Vibrio fischeri	15 min	Luminescence	0.1	2.74	–	–	8.67	0.43	Mowat and Bundy (2002)
Vibrio fischeri	5, 15, 30 min	Luminescence	0.8–1.6	–	17.2–18.9	–	16.5–25.2	–	Cho et al. (2004)
Vibrio fischeri	15 min	Luminescence	–	0.3	–	17.8	–	–	Petala et al. (2005)
Escherichia coli	30 min	Current	1.1	6.5	–	–	–	–	Wang et al. (2008)
Escherichia coli	1 h	Respiration	2.03	–	–	–	–	20.4	Catterall et al. (2010)
Nitrosomonas sp.	21 h	O_2 consumption	–	7.39	–	4.98	–	–	Çeçen et al. (2010)
Nitrosomonas sp.	21 h	CO_2 production	–	10.6	–	5.8	–	–	Çeçen et al. (2010)
Psychrobacter sp.	30 min	Electrical current	0.8	2.6	14.0	–	–	110.1	Wang et al. (2013)
Escherichia coli with GFP	2 min	Fluorescence	–	–	4.5	9.0	–	0.02	Futra et al. (2015)
Vibrio fischeri	5, 15 min	Luminescence	–	2.84–4.20	–	–	–	5.83–6.60	Mansour et al. (2015)
Pseudomonas sp.	30 min	Viability	2.36	–	–	–	–	19.05	Zare et al. (2015)
Aeromonas sp.	30 min	Viability	8.43	–	–	–	–	10.0	Zare et al. (2015)
Enterbacter sp.	30 min	Viability	9.54	–	–	–	–	32.1	Zare et al. (2015)
Bacillus sp.	30 min	Viability	13	–	–	–	–	199.6	Zare et al. (2015)
Escherichia coli with thionine	10 min	Current	–	20.2	–	–	–	34.3	Fang et al. (2016)
Sulfur oxidizing bacteria	2 h	Electrical conductivity	0.21	5.0	1.78	–	–	–	Ahmed and Oh (2018)
Vibrio fischeri	30 min	Luminescence	0.13	12.0	13.7	–	–	1.03	Kim and Lee (2018)
Sulfur oxidizing bacteria	2 h	Electrical conductivity	0.92	–	1.17	–	0.2	–	Eom et al. (2019)
Escherichia coli	1 min	Glucose metabolism	2.8	10.3	–	–	6.9	–	Mi et al. (2019)

0.80 mg/L of the 0.5 h EC_{50} value for Hg^{2+} (Wang et al., 2013). Zare et al. (2015) used various bacterial strains as test organisms and reported consistently higher EC_{50} values for Hg^{2+} than ours: they found that 2.36, 8.43, 9.54, and 13.03 mg/L of the (0.5 h) EC_{50} values by *Pseudomonas*, *Aeromonas*, *Enterobacter*, and *Bacillus* sp., respectively. Furthermore, sulfur oxidizing bacteria (SOB)-based toxicity tests also reported greater (2 h) EC_{50} values for Hg^{2+} (0.21–0.92 mg/L) than our finding (Ahmed and Oh, 2018; Eom et al., 2019). Considering that our detection sensitivity is higher than other microbial toxicity tests, the present test that adopts fermentative gas production as the end-point provides an effective means to assess Hg^{2+}-induced water toxicity.

3.3. Assessment of toxicity of water contaminated with Cu^{2+}, Cr^{6+}, Ni^{2+}, As^{5+}, or Pb^{2+}

Toxicity assessment of Cu^{2+}, Cr^{6+}, Ni^{2+}, As^{5+}, or Pb^{2+}-contaminated water was conducted with the method used in the Hg^{2+} tests. Amounts of gas production after 24 h exposure of the test organisms to heavy metals were determined and displayed in Fig. 7. In all heavy metal tests, the control kits (where no heavy metals were introduced) produced fairly comparable amounts of gas, 5.1–5.3 mL, for 24 h. Tendencies of less gas production with increasing doses of introduced heavy metals were also consistently observed in all heavy metal tests. However, the inhibitory effects of heavy metals on gas production varied depending on the types of metal species. For example, Cu^{2+} caused the highest inhibition of gas production among the tested heavy metals. With introduction of 0.5 and 1 mg/L of Cu^{2+}, the amount of gas produced from the test kits for 24 h decreased to 2.5 and 0.7 mL, respectively; spiking 2 mg/L of Cu^{2+} resulted in no gas production at all. Cr^{6+} also showed high inhibitory effects on gas production. In the presence of 1 and 2 mg/L of Cr^{6+}, 2.7 and 0.9 mL of gas were produced in 24 h, respectively; introducing 5 mg/L of Cr^{6+} led to no gas production. Ni^{2+}, however, created relatively lesser inhibitory effects on gas production, compared to Cu^{2+} and Cr^{6+}. For example, spiking 1 and 5 mg/L of Ni^{2+} resulted in 3.8 and 1.4 mL of gas production, respectively. More than 15 mg/L of Ni^{2+} was required to completely

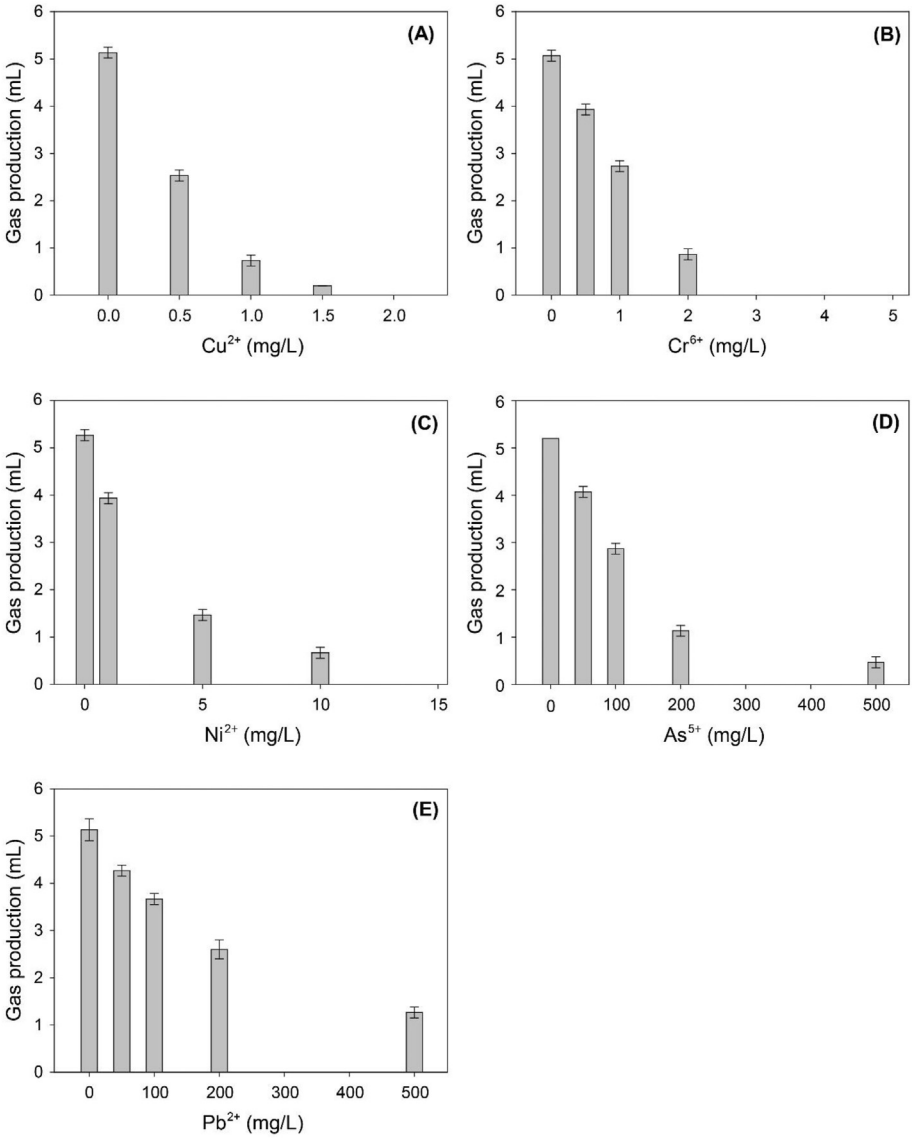

Fig. 7. Fermentative gas production from heavy metal toxicity tests. (A) Cu^{2+}, (B) Cr^{6+}, (C) Ni^{2+}, (D) As^{5+}, (E) Pb^{2+}. Symbols and error bars represent the average values and standard deviations, respectively, from the triplicate tests.

inhibit gas production. Inhibitory effects of As^{5+} and Pb^{2+} were minimal. Even introduction of 100 mg/L of As^{5+} and Pb^{2+} still allowed 2.9 and 3.7 mL of gas production, respectively. In As^{5+} and Pb^{2+} toxicity tests, given our testing ranges, complete inhibition of gas production was not achieved.

Fig. 8 depicts dose-response relations based on inhibition of gas production in Cu^{2+}, Cr^{6+}, Ni^{2+}, As^{5+}, and Pb^{2+} toxicity tests. All these dose-response curves highly correspond to the Hillslope equations with $R^2 > 0.99$. Amounts of gas production at 24 h depending on doses of heavy metals are statistically different in all heavy metal tests (Tables S2–6), suggesting that the dose-response relations based on these values are also statistically significant. As seen with the mercury tests, Fig. 8 supports that the present toxicity tests are able to satisfactorily describe increased water toxicity as levels of introduced heavy metals increased. The 24 h EC_{50} values for Cu^{2+}, Cr^{6+}, Ni^{2+}, As^{5+}, or Pb^{2+} were analyzed to be 0.51, 1.09, 3.61, 101.33, and 243.45 mg/L, respectively. Based on these EC_{50} values, the relative toxicity among heavy metals on the

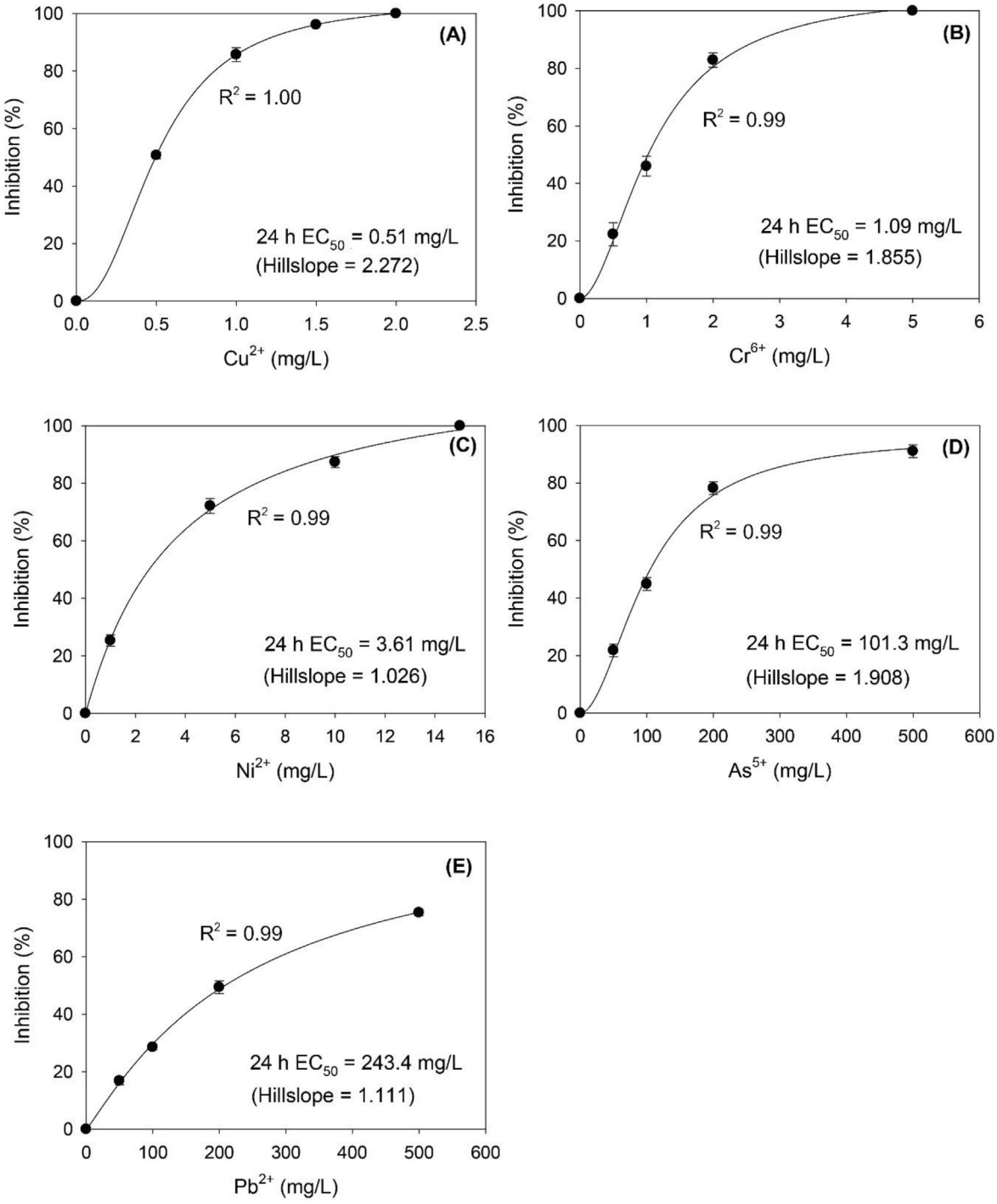

Fig. 8. Inhibition of fermentative gas production in heavy metal toxicity tests. (A) Cu^{2+}, (B) Cr^{6+}, (C) Ni^{2+}, (D) As^{5+}, (E) Pb^{2+}. Symbols and error bars represent the average values and standard deviations, respectively, from the triplicate tests.

fermentative activity of the test organisms is estimated to be as follows: (Hg^{2+})> Cu^{2+}> Cr^{6+}> Ni^{2+} ≫ As^{5+}> Pb^{2+}. It is known that toxicity of heavy metals is associated with disruption of function and structure of enzymes by binding of the metals with some specific functional groups in protein molecules or by replacing essential metals in enzyme prosthetic groups (Vallee and Ulmer, 1972; Tchounwou et al., 2012; Jaishankar et al., 2014). The literature reports that toxicity of heavy metals in water is determined by many factors including not only their species and concentrations but also the characteristics of the test solution such as pH, temperature, and ionic conditions (Tchounwou et al., 2012; Egorova and Ananikov, 2017). Accordingly, inhibitory effects of heavy metal-contaminated water on fermentation can vary depending on test conditions. Nonetheless, there are a number of earlier studies that reported similar results as ours, regarding species-dependent inhibitory effects of heavy metals. For example, Lin (1992) reported that Cu^{2+} is highly toxic but Ni^{2+} and Pb^{2+} show lower inhibitory effects on VFA degradation in anaerobic digestion. Yu and Fang (2001a, 2001b) demonstrated that Cu^{2+} is a more toxic metal element on hydrogen producing fermentation than Cr^{6+}. Yue et al. (2007) also found that Cu^{2+} can inhibit anaerobic digestion more than Cr^{6+}. Studying the fermentation of granular sludge, Li and Fang (2007) investigated inhibitory effects of heavy metals on H_2 production and reported the order of inhibition by heavy metals as follows: Cu^{2+}>Ni^{2+}>Cr^{6+}>Pb^{2+} (Li and Fang, 2007). Although Li and Fang (2007) demonstrated particularly high toxicity of Ni^{2+} compared to our findings, their results still present that Cu^{2+} has greater toxicity on H_2 production than Cr^{6+} and Pb^{2+}, which is comparable to our results. Furthermore, Nguyen et al. (2019) reported similar relative toxicity of heavy metals as our tests. Their study observed that Cu^{2+} is the most toxic metal species regarding the anaerobic co-digestion of waste activated sludge and septic tank sludge, followed by Cr^{6+} and Pb^{2+}.

In spite of our greater 24 h EC_{10} values (0.19 mg/L for Cu^{2+}; 0.34 mg/L for Cr^{6+}; 0.42 mg/L for Ni^{2+}; 32.70 mg/L for As^{5+}; 33.88 mg/L for Pb^{2+}) compared to generally reported detection limits for heavy metals (0.001–10 μg/L) by atomic spectroscopy techniques (Parsons et al., 1983; Taylor, 2017; Deniz, 2020), our test sensitivity particularly for Cu^{2+}, Cr^{6+}, and Ni^{2+} was more favorable than that of other bacteria-based toxicity tests. As shown in Table 3, our EC_{50} values for Cu^{2+}, Cr^{6+}, and Ni^{2+} relatively fall within the lows compared to reports from earlier microbial toxicity tests. For example, microbial tests with *V. fischeri* reported 0.3–12.0, 13.7–18.9, and 10.0–17.8 mg/L of EC_{50} values (with 5 min–6 h of exposure times) for Cu^{2+}, Cr^{6+}, and Ni^{2+}, respectively (Villaescusa et al., 1998; Gellert, 2000; Mowat and Bundy, 2002; Cho et al., 2004; Petala et al., 2005; Mansour et al., 2015; Kim and Lee, 2018). Employment of *E. coli* in toxicity tests resulted in 6.5–20.2, 4.5, and 9.0 mg/L of EC_{50} values (with 1–10 min of exposure times) for Cu^{2+}, Cr^{6+}, and Ni^{2+}, respectively (Wang et al., 2008; Futra et al., 2015; Fang et al., 2016; Mi et al., 2019). The (2–18 h) EC_{50} values for Cu^{2+} and Ni^{2+} from toxicity tests based on *Pseudomonas fluorescens* were 17.0–18.0 and 8.7–14.0 mg/L, respectively (Dutka and Kwan, 1981; Paran et al., 1990). Nitrifying bacteria toxicity tests also reported higher (2–21 h) EC_{50} values for Cu^{2+}, Cr^{6+}, and Ni^{2+}, 7.4–41.5, 35.6, and 4.9–5.8 mg/L, respectively, than ours (Dalzell et al., 2002; Çeçen et al., 2010). Moreover, the (0.25–3 h) EC_{50} values for Cu^{2+} and Cr^{6+} from activated sludge-employed toxicity tests with measurement of respiration, luminescence, and enzymatic activity as the end-points were 5.5–40.0 and 36.2–228.5 mg/L, respectively, which are substantially greater than our results (Dalzell et al., 2002). However, our EC_{50} values for As^{5+} and Pb^{2+} are considerably higher than most other microbial toxicity tests presented in Table 3. One possible explanation for these particularly high EC_{50} values for As^{5+} and Pb^{2+} is associated with the oxidative states of heavy metals. It is generally known that As^{5+} is the ionic form that is much less toxic than other arsenic ionic forms such as As^{3+} (Jang et al., 2016). Moreover, Pb^{2+} might be changed to less toxic ionic forms such as Pb^{4+} during toxicity testing. Another reason would be attributed to complexation with organic matters. During toxicity testing, As^{5+} and Pb^{2+} can form complexes with organic matters, causing less availability of As^{5+} and Pb^{2+} to fermentative bacteria and thus resulting in lower toxicity. Based on the EC_{50} values, one may conclude that our toxicity tests have high sensitivity for detecting toxicity of Cu^{2+}, Cr^{6+}, and Ni^{2+} but not for As^{5+} and Pb^{2+}. Further studies to improve detection sensitivity including optimization of test conditions are necessary.

3.4. Suitability of fermentative bacteria test kits for evaluating heavy metal-induced water toxicity

The present study investigated the suitability of fermentative bacteria-based toxicity tests for assessment of heavy metal-contaminated water. The current test analyzed the toxicity based on inhibition of fermentative gas production. The dose-response relations in all heavy metal tests highly correspond to the Hill-slope curve with $R^2 > 0.99$. As discussed in the introduction, the main merits of the present test are the ease and reliability of end-point measurements. The most widely adopted end-points in microbial toxicity tests include optical density, respiration rate, enzymatic activity, fluorescence, luminescence, and electrical outputs (Tothill and Turner, 1996; Hassan et al., 2016). Determination of these end-points usually requires sophisticated equipment such as optical analytic equipment (fluorometer, luminometer, and spectrophotometer), respirometers, and electrical current measuring devices (Tothill and Turner, 1996; Hassan et al., 2016). In addition, reading of these end-points can be interfered by the conditions of test samples. For example, optical density, fluorescence, and luminescence measurements are possibly altered by turbidity, color, and nanoparticles of test samples (Hassan et al., 2016; Qiu et al., 2017; Rotini et al., 2017). Electrochemical analyses also are potentially affected by the ionic and electrochemical conditions of test samples (Cosio et al., 2012; Hassan et al., 2016). Laboratory analytic techniques such as measuring enzymatic activities are laborious and need lengthy processing times to obtain final results (Moss, 1970; Hassan et al., 2016). In contrast, the current end-point measurement is straightforward, requires only a glass syringe and no special training, and its reading is not interfered by the characteristics of test samples, ensuring high reliability for determination of end-points. Other advantages of the test kits include its high reproducibility and on-site applicability. In the present study, we performed our experiments in triplicate – the results of gas production were fairly comparable, leading to minor standard deviations in all heavy metal tests. We speculate that the simple and easy test protocol will enable less experimental error and high reproducibility. Moreover, since the present test is based on kit-type bioassays with no additional laboratory analyses, our toxicity test is suitable for field on-site toxicity tests. Given its advantages, a fermentative bacteria-based toxicity test represents an improvement over other existing toxicity tests, particularly as a simple and reliable toxicity screening tool.

The current study provides a fundamental framework for fermentative bacteria-based toxicity tests. Our future study will investigate actual application of fermentative bacteria-based test kits to real field situations. For this purpose, we will perform toxicity tests with diverse toxicants and test samples. We expect that these studies will provide information to establish thresholds of criteria for toxicity assessment under various field conditions. In addition, we will study the impact of characteristics of test samples on the microbial activity of fermentative bacteria. As discussed

above, reading our end-point is rarely interfered by the conditions of test samples. However, characteristics of test samples (including ionic conditions and organic substances) can affect activity of the test organisms in our kit tests, which is unavoidable and inherent in all biological toxicity tests. To control and minimize these effects, we will investigate pre-treatments of test samples, such as filtration (simple screening for excessive organic matters) or buffering. Calibration of test results based on the characteristics of test samples would also be helpful to offset these undesirable effects. We believe that these future studies will facilitate extending real field application of fermentative bacteria-based toxicity kits under various conditions.

4. Conclusions

Fermentative bacteria-based test kits were developed to assess toxicity of heavy metal-contaminated water. Toxicity was evaluated based on inhibition of fermentative gas production, which was analyzed via a syringe method. Dose-response relations in all heavy metal tests satisfactorily identified increased toxicity of water with increasing amounts of heavy metals. Hg^{2+}, Cu^{2+}, Cr^{6+}, Ni^{2+} showed considerably greater inhibitory effects on fermentative gas production than As^{5+} and Pb^{2+}. The 24 h EC_{50} values for Hg^{2+}, Cu^{2+}, Cr^{6+}, Ni^{2+}, As^{5+}, and Pb^{2+} were found to be 0.10, 0.51, 1.09, 3.61, 101.33, and 243.45 mg/L, respectively. Detection sensitivity of our test kits for Hg^{2+}, Cu^{2+}, Cr^{6+}, Ni^{2+}, but not As^{5+} and Pb^{2+}, compared favorably to other bacteria-based toxicity tests. The main advantages of the current test protocol include ease and reliability of end-point measurement, making the fermentative bacteria-based test kits suitable for simple screening tests for heavy metal-contaminated water.

CRediT authorship contribution statement

Heonseop Eom: Conceptualization, Writing - original draft. **Woochang Kang:** Methodology, Investigation. **Seunggyu Kim:** Methodology, Data curation. **Kangmin Chon:** Data curation. **Yong-Gu Lee:** Data curation. **Sang-Eun Oh:** Supervision, Writing - review & editing.

Declaration of competing interest

None.

Acknowledgments

This research was supported by Basic Science Research Program of the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2019R1A6A3A01096287), and partially funded by Korean Ministry of Environment (grant no. 2018002270002).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2020.127412.

References

Ahmed, N., Oh, S.-E., 2018. Toxicity assessment of selected heavy metals in water using a seven-chambered sulfur-oxidizing bacterial (SOB) bioassay reactor. Sensor. Actuator. B Chem. 258, 1008–1014. https://doi.org/10.1016/j.snb.2017.11.149.

APHA, 2005. Standard Methods for the Examination of Water and Wastewater, 21th ed. American Public Health Association, Washington, DC.

Bae, M.J., Park, Y.S., 2014. Biological early warning system based on the responses of aquatic organisms to disturbances: a review. Sci. Total Environ. 466–467, 635–649. https://doi.org/10.1016/j.scitotenv.2013.07.075.

Barata, C., Alañon, P., Gutierrez-Alonso, S., Riva, M.C., Fernández, C., Tarazona, J.V., 2008. A *Daphnia magna* feeding bioassay as a cost effective and ecological relevant sublethal toxicity test for environmental risk assessment of toxic effluents. Sci. Total Environ. 405, 78–86. https://doi.org/10.1016/j.scitotenv.2008.06.028.

Beasley, A., Belanger, S.E., Brill, J.L., Otter, R.R., 2015. Evaluation and comparison of the relationship between NOEC and EC10 or EC20 values in chronic *Daphnia* toxicity testing. Environ. Toxicol. Chem. 34, 2378–2384. https://doi.org/10.1002/etc.3086.

Bentley, A., Atkinson, A., Jezek, J., Rawson, D.M., 2001. Whole cell biosensors — electrochemical and optical approaches to ecotoxicity testing. Toxicol. In. Vitro. 15, 469–475. https://doi.org/10.1016/S0887-2333(01)00052-2.

Brayner, R., Couté, A., Livage, J., Perrette, C., Sicard, C., 2011. Micro-algal biosensors. Anal. Bioanal. Chem. 401, 581–597. https://doi.org/10.1007/s00216-011-5107-z.

Catterall, K., Robertson, D., Hudson, S., Teasdale, P.R., Welsh, D.T., John, R., 2010. A sensitive, rapid ferricyanide-mediated toxicity bioassay developed using *Escherichia coli*. Talanta 82, 751–757. https://doi.org/10.1016/j.talanta.2010.05.046.

Çeçen, F., Semerci, N., Geyik, A.G., 2010. Inhibitory effects of Cu, Zn, Ni and Co on nitrification and relevance of speciation. J. Chem. Technol. Biotechnol. 85, 520–528. https://doi.org/10.1002/jctb.2321.

Cho, J.-C., Park, K.-J., Ihm, H.-S., Park, J.-E., Kim, S.-Y., Kang, I., Lee, K.-H., Jahng, D., Lee, D.-H., Kim, S.-J., 2004. A novel continuous toxicity test system using a luminously modified freshwater bacterium. Biosens. Bioelectron. 20, 338–344. https://doi.org/10.1016/j.bios.2004.02.001.

Cosio, M.S., Scampicchio, M., Benedetti, S., 2012. Electronic noses and tongues. In: Picó, Y. (Ed.), Chemical Analysis of Food: Technique and Applications. Academic Press, Cambridge, pp. 219–247. https://doi.org/10.1016/C2010-0-64808-5.

Dalzell, D.J.B., Alte, S., Aspichueta, E., De la Sota, A., Etxebarria, J., Gutierrez, M., Hoffmann, C.C., Sales, D., Obst, U., Christofi, N., 2002. A comparison of five rapid direct toxicity assessment methods to determine toxicity of pollutants to activated sludge. Chemosphere 47, 535–545. https://doi.org/10.1016/s0045-6535(01)00331-9.

Deniz, S., 2020. Atomic spectroscopy. In: Khan, M., Nascimento, G.M.D., El-azazy, M. (Eds.), Modern Spectroscopic Techniques and Applications, pp. 1–11. https://doi.org/10.5772/intechopen.89269.

De Mauri, A., Chiarinotti, D., Andreoni, S., Molinari, G.L., Conti, N., De Leo, M., 2013. *Leclercia adecarboxylata* and catheter-related bacteraemia: review of the literature and outcome with regard to catheters and patients. J. Med. Microbiol. 62, 1620–1623. https://doi.org/10.1099/jmm.0.059535-0.

Dutka, B.J., Kwan, K.K., 1981. Comparison of three microbial toxicity screening tests with the microtox test. Bull. Environ. Contam. Toxicol. 27, 753–757. https://doi.org/10.1007/bf01611091.

Egorova, K.S., Ananikov, V.P., 2017. Toxicity of metal compounds: knowledge and myths. Organometallics 36, 4071–4090. https://doi.org/10.1021/acs.organomet.7b00605.

Eilersen, A.M., Arvin, E., Henze, M., 2004. Monitoring toxicity of industrial wastewater and specific chemicals to a green alga, nitrifying bacteria and an aquatic bacterium. Water Sci. Technol. 50, 277–283. https://doi.org/10.2166/wst.2004.0386.

Eom, H., Hwang, J.-H., Hassan, S.H.A., Joo, J.H., Hur, J.H., Chon, K., Jeon, B.-H., Song, Y.-C., Chae, K.-J., Oh, S.-E., 2019. Rapid detection of heavy metal-induced toxicity in water using a fed-batch sulfur-oxidizing bacteria (SOB) bioreactor. J. Microbiol. Methods 161, 35–42. https://doi.org/10.1016/j.mimet.2019.04.007.

Fang, D., Gao, G., Shen, J., Yu, Y., Zhi, J., 2016. A reagentless electrochemical biosensor based on thionine wrapped *E. coli* and chitosan-entrapped carbon nanodots film modified glassy carbon electrode for wastewater toxicity assessment. Electrochim. Acta 222, 303–311. https://doi.org/10.1016/j.electacta.2016.10.174.

Farré, M., Barceló, D., 2003. Toxicity testing of wastewater and sewage sludge by biosensors, bioassays and chemical analysis. Trac. Trends Anal. Chem. 22, 299–310. https://doi.org/10.1016/S0165-9936(03)00504-1.

Futra, D., Heng, L.Y., Ahmad, A., Surif, S., Ling, T.L., 2015. An optical biosensor from green fluorescent *Escherichia coli* for the evaluation of single and combined heavy metal toxicities. Sensors-basel 15, 12668–12681. https://doi.org/10.3390/s150612668.

Gellert, G., 2000. Sensitivity and significance of luminescent bacteria in chronic toxicity testing based on growth and bioluminescence. Ecotoxicol. Environ. Saf. 45, 87–91. https://doi.org/10.1006/eesa.1999.1849.

Gottschalk, G., 1986. Bacterial Metabolism, second ed. Springer-Verlag, New York. https://doi.org/10.1007/978-1-4684-0465-4.

Gunjan, D., Gargi, B., 2015. Toxicity tests to check water quality. Int. Res. J. Environ. Sci. 4, 87–90.

Hassan, S.H.A., Van Ginkel, S.W., Oh, S.-E., 2012. Detection of Cr^{6+} by the sulfur oxidizing bacteria biosensor: effect of different physical factors. Environ. Sci. Technol. 46, 7844–7848. https://doi.org/10.1021/es301360a.

Hassan, S.H.A., Van Ginkel, S.W., Hussein, M.A.M., Abskharon, R., Oh, S.-E., 2016. Toxicity assessment using different bioassays and microbial biosensors. Environ. Int. 92–93, 106–118. https://doi.org/10.1016/j.envint.2016.03.003.

Hassan, S.H.A., Van Ginkel, S.W., Oh, S.-E., 2013. Effect of organics and alkalinity on the sulfur oxidizing bacteria (SOB) biosensor. Chemosphere 90, 965–970. https://doi.org/10.1016/j.chemosphere.2012.06.040.

Hernando, M.D., Fernández-Alba, A.R., Tauler, R., Barceló, D., 2005. Toxicity assays applied to wastewater treatment. Talanta 65, 358–366. https://doi.org/10.1016/

j.talanta.2004.07.012.
Iwasaki, Y., Kotani, K., Kashiwada, S., Masunaga, S., 2015. Does the choice of NOEC or EC10 affect the hazardous concentration for 5% of the species? Environ. Sci. Technol. 49, 9325–9330. https://doi.org/10.1021/acs.est.5b02069.
Iyer, P., Bruns, M.A., Zhang, H., Van Ginkel, S., Logan, B.E., 2004. H_2-producing bacterial communities from a heat-treated soil inoculum. Appl. Microbiol. Biotechnol. 66, 166–173. https://doi.org/10.1007/s00253-004-1666-7.
Jaishankar, M., Tseten, T., Anbalagan, N., Mathew, B.B., Beeregowda, K.N., 2014. Toxicity, mechanism and health effects of some heavy metals. Interdiscipl. Toxicol. 7, 60–72. https://doi.org/10.2478/intox-2014-0009.
Jang, Y.-C., Somanna, Y., Kim, H., 2016. Source, distribution, toxicity and remediation of arsenic in the environment – a review. Int. J. Appl. Environ. Sci. 11, 559–581.
Kim, B.C., Park, K.S., Kim, S.D., Gu, M.B., 2003. Evaluation of a high throughput toxicity biosensor and comparison with a *Daphnia magna* bioassay. Biosens. Bioelectron. 18, 821–826. https://doi.org/10.1016/s0956-5663(03)00027-7.
Kim, I., Lee, J., 2018. Bio toxicity assessment and kinetic model of 6 heavy metals using luminous Bacteria. J. Korean Soc. Urban Environ. 18, 547–555. https://doi.org/10.33768/ksue.2018.18.4.547.
Larras, F., Montuelle, B., Bouchez, A., 2013. Assessment of toxicity thresholds in aquatic environments: does benthic growth of diatoms affect their exposure and sensitivity to herbicides? Sci. Total Environ. 1, 469–477. https://doi.org/10.1016/j.scitotenv.2013.06.06.
Lay, J.-J., Lee, Y.-J., Noike, T., 1999. Feasibility of biological hydrogen production from organic fraction of municipal solid waste. Water Res. 33, 2579–2586. https://doi.org/10.1016/S0043-1354(98)00483-7.
Li, C., Fang, H.H., 2007. Inhibition of heavy metals on fermentative hydrogen production by granular sludge. Chemosphere 67, 668–673. https://doi.org/10.1016/j.chemosphere.2006.11.005.
Lin, C.-Y., 1992. Effect of heavy metals on volatile fatty acid degradation in anaerobic digestion. Water Res. 26, 177–183. https://doi.org/10.1016/0043-1354(92)90217-R.
Liu, D., Chau, Y.K., Dutka, B.J., 1989. Rapid toxicity assessment of water-soluble and water-insoluble chemicals using a modified agar plate method. Water Res. 23, 333–339. https://doi.org/10.1016/0043-1354(89)90099-7.
Logan, B.E., Oh, S.-E., Kim, I.S., Van Ginkel, S., 2002. Biological hydrogen production measured in batch anaerobic respirometers. Environ. Sci. Technol. 36, 2530–2535. https://doi.org/10.1021/es015783i.
Lors, C., Ponge, J.-F., Martínez Aldaya, M.M., Damidot, D., 2011. Comparison of solid and liquid-phase bioassays using ecoscores to assess contaminated soils. Environ. Pollut. 159, 2974–2981. https://doi.org/10.1016/j.envpol.2011.04.028.
Mansour, S.A., Abdel-Hamid, A.A., Ibrahim, A.W., Mahmoud, N.H., Moselhy, W.A., 2015. Toxicity of some pesticides, heavy metals and their mixtures to *Vibrio fischeri* bacteria and *Daphnia magna*: comparative Study. J. Biol. Life Sci. 6, 221–240. https://doi.org/10.5296/jbls.v6i2.8174.
Medina, R., 2019. Types of fermentations technology: aerobic and anaerobic fermentation. In: Medina, R. (Ed.), Fermentation Technology. Tech Press, Waltham Abbey, pp. 170–194.
Mi, Y., Tao, X., Zhang, X., Si, Y., 2019. Acute biotoxicity assessment of heavy metals in sewage sludge based on the glucose consumption of *Escherichia coli*. R. Soc. Open. Sci. 6, 181769. https://doi.org/10.1098/rsos.181769.
Mishra, S., Barik, S.K., Ayyappan, S., Mohapatra, B.C., 2000. Fish bioassays for evaluation of raw and bioremediated dairy effluent. Bioresour. Technol. 72, 213–218. https://doi.org/10.1016/S0960-8524(99)00124-8.
Moreira-Santos, M., Soares, A.M.V.M., Ribeiro, R., 2004. An in situ bioassay for freshwater environments with the microalga *Pseudokirchneriella subcapitata*. Ecotoxicol. Environ. Saf. 59, 164–173. https://doi.org/10.1016/j.ecoenv.2003.07.004.
Moss, D.W., 1970. Accuracy, precision, and quality control of enzyme assays. J. Clin. Pathol. Suppl. 4, 22–30. https://doi.org/10.1136/jcp.s1-4.1.22.
Mowat, F.S., Bundy, K.J., 2002. Experimental and mathematical/computational assessment of the acute toxicity of chemical mixtures from the Microtox® assay. Adv. Environ. Res. 6, 547–558. https://doi.org/10.1016/S1093-0191(01)00099-5.
Munawar, M., Munawar, I.F., Leppard, G.G., 1989. Early warning assays: an overview of toxicity testing with phytoplankton in the North American Great Lakes. Hydrobiologia 188, 237–246. https://doi.org/10.1007/BF00027789.
Muratoğlu, H., Kati, H., Demirbag, Z., Sezen, K., 2009. High insecticidal activity of *Leclercia adecarboxylata* isolated from *Leptinotarsa decemlineata*(Col.: Chrysomelidae). Afr. J. Biotechnol. 8, 7111–7115.
National Research Council, 2014. A Framework to Guide Selection of Chemical Alternatives, first ed. The National Academies Press, Washington D.C. https://doi.org/10.17226/18872.
Nguyen, Q.-M., Bui, D.-C., Phuong, T., Doan, V.-H., Nguyen, T.-N., Nguyen, M.-V., Tran, T.-H., Do, Q.-T., 2019. Investigation of heavy metal effects on the anaerobic co-digestion process of waste activated sludge and septic tank sludge. Int. J. Chem. Eng. 2019, 5138060. https://doi.org/10.1155/2019/5138060.
Niemirycz, E., Nichthauser, J., Staniszewska, M., Nałęcz-Jawecki, G., Bolalek, J., 2007. The Microtox® biological test: application in toxicity evaluation of surface waters and sediments in Poland. Oceanol. Hydrobiol. Stud. 36, 151–163. https://doi.org/10.2478/v10009-007-0030-5.
Oh, S.-E., Hassan, S.H.A., Van Ginkel, S.W., 2011. A novel biosensor for detecting toxicity in water using sulfur-oxidizing bacteria. Sensor. Actuator. B Chem. 154, 17–21. https://doi.org/10.1016/j.snb.2010.01.052.
Oh, S.-E., Iyer, P., Bruns, M.A., Logan, B.E., 2004. Biological hydrogen production using a membrane bioreactor. Biotechnol. Bioeng. 87, 119–127. https://doi.org/10.1002/bit.20127.
Oh, S.-E., Van Ginkel, S., Logan, B.E., 2003. The relative effectiveness of pH control and heat treatment for enhancing biohydrogen gas production. Environ. Sci. Technol. 37, 5186–5190. https://doi.org/10.1021/es034291y.
Owen, W.F., Stuckey, D.C., Healy Jr., J.B., Young, L.Y., McCarty, P.L., 1979. Bioassay for monitoring biochemical methane potential and anaerobic toxicity. Water Res. 13, 485–492. https://doi.org/10.1016/0043-1354(79)90043-5.
Palma, P., Palma, V.L., Matos, C., Fernandes, R.M., Bohn, A., Soares, A.M.B.M., Barbosa, I.R., 2008. Effects of Atrazine and Endosulfan sulphate on the ecdysteroid system of *Daphnia magna*. Chemosphere 74, 676–681. https://doi.org/10.1016/j.chemosphere.2008.10.021.
Paran, J.H., Sharma, S., Qureshi, A.A., 1990. A rapid and simple toxicity assay based on growth rate inhibition of *Pseudomonas fluorescens*. Environ. Toxic. Water 5, 351–365. https://doi.org/10.1002/tox.2540050404.
Park, W., Hyun, S.H., Oh, S.-E., Logan, B.E., Kim, I.S., 2005. Removal of headspace CO_2 increases biological hydrogen production. Environ. Sci. Technol. 39, 4416–4420. https://doi.org/10.1021/es048569d.
Parsons, M.L., Major, S., Forster, A.R., 1983. Trace element determination by atomic spectroscopic methods—state of the art. Appl. Spectrosc. 37, 411–418. https://doi.org/10.1366/0003702834634857.
Petala, M., Tsiridis, V., Kyriazis, S., Samaras, P., Kungolos, A., Sakellaropoulos, G.P., 2005. Evaluation of toxic response of heavy metals and organic pollutants using microtox acute toxicity test. Proceedings of the 9th Conference on Environmental Science and Technology. 1-3 September 2005. Rhodes island, Greece, pp. 1200–1205.
Pritchard, J.B., 1993. Aquatic toxicology: past, present, and prospects. Environ. Health Perspect. 100, 249–257. https://doi.org/10.1289/ehp.93100249.
Qiu, T.A., Nguyen, T.H.T., Hudson-Smith, N.V., Clement, P.L., Forester, D.-C., Frew, H., Hang, M.N., Murphy, C.J., Hamers, R.J., Feng, Z.V., Haynes, C.L., 2017. Growth-based bacterial viability assay for interference-free and high-throughput toxicity screening of nanomaterials. Anal. Chem. 89, 2057–2064. https://doi.org/10.1021/acs.analchem.6b04652.
Rahmanian, N., Ali, S.H.B., Homayoonfard, M., Ali, N.J., Rehan, M., Sadef, Y., Nizami, A.S., 2015. Analysis of physiochemical parameters to evaluate the drinking water quality in the State of Perak, Malaysia. J. Chem. 2015, 716125. https://doi.org/10.1155/2015/716125.
Rönnpagel, K., Liss, W., Ahlf, W., 1995. Microbial bioassays to assess the toxicity of solid-associated contaminants. Ecotoxicol. Environ. Saf. 31, 99–103. https://doi.org/10.1006/eesa.1995.1048.
Rotini, A., Manfra, L., Spanu, F., Pisapia, M., Cicero, A.M., Migliore, L., 2017. Ecotoxicological method with marine bacteria *Vibrio anguillarum* to evaluate the acute toxicity of environmental contaminants. JoVE 123, 55211. https://doi.org/10.3791/55211.
Sponza, D.T., 2002. Incorporation of toxicity tests into the Turkish industrial discharge monitoring systems. Arch. Environ. Contam. Toxicol. 43, 186–197. https://doi.org/10.1007/s00244-002-1150-2.
Tada, Y., Kobata, T., Nakaoka, C., 2001. A simple and easy method for the monitoring of environmental pollutants using oligotrophic bacteria. Lett. Appl. Microbiol. 32, 12–15. https://doi.org/10.1111/j.1472-765X.2001.00850.x.
Taylor, A., 2017. Atomic spectroscopy, biomedical applications. In: Lindon, J.C., Tranter, G.E., Koppenaal, D.W. (Eds.), Encyclopedia of Spectroscopy and Spectrometry. Academic press, Cambridge, pp. 76–80.
Tchounwou, P.B., Yedjou, C.G., Patlolla, A.K., Sutton, D.J., 2012. Heavy metal toxicity and the environment. Exper. Suppl. (Basel) 101, 133–164. https://doi.org/10.1007/978-3-7643-8340-4_6.
Tencaliec, A.M., Laschi, S., Magearu, V., Mascini, M., 2006. A comparison study between a disposable electrochemical DNA biosensor and a *Vibrio fischeri*-based luminescent sensor for the detection of toxicants in water samples. Talanta 69, 365–369. https://doi.org/10.1016/j.talanta.2005.09.042.
Tothill, I.E., Turner, A.P.F., 1996. Developments in bioassay methods for toxicity testing in water treatment. Trac. Trends Anal. Chem. 15, 178–188. https://doi.org/10.1016/0165-9936(96)80640-6.
Tyagi, S., Sharma, B., Singh, P., 2013. Water quality assessment in terms of water quality index. Am. J. Water Resour. 1, 34–38.
Vallee, B.L., Ulmer, D.D., 1972. Biochemical effects of mercury, cadmium, and lead. Annu. Rev. Biochem. 41, 91–128. https://doi.org/10.1146/annurev.bi.41.070172.000515.
Van Ginkel, S.W., Oh, S.-E., Logan, B.E., 2005. Biohydrogen gas production from food processing and domestic wastewaters. Int. J. Hydrogen Energy 30, 1535–1542. https://doi.org/10.1016/j.ijhydene.2004.09.017.
Van Ginkel, S., Sung, S., Lay, J.-J., 2001. Biohydrogen production as a function of pH and substrate concentration. Environ. Sci. Technol. 35, 4726–4730. https://doi.org/10.1021/es001979r.
Villaescusa, I., Matas, C., Hosta, C., Martinez, M., Murat, J.C., 1998. Evaluation of lead(II) and nickel(II) toxicity in NaCl and $NaClO_4$ solutions by using Microtox® bioassay. Fresenius' J. Anal. Chem. 361, 355–358. https://doi.org/10.1007/s002160050903.
Wang, H., Wang, X.J., Zhao, J.F., Chen, L., 2008. Toxicity assessment of heavy metals and organic compounds using CellSense biosensor with *E.coli*. Chin. Chem. Lett. 19, 211–214. https://doi.org/10.1016/j.cclet.2007.10.053.
Wang, X., Liu, M., Wang, X., Wu, Z., Yang, L., Xia, S., Chen, L., Zhao, J., 2013. P-benzoquinone-mediated amperometric biosensor developed with *Psychrobacter sp.* for toxicity testing of heavy metals. Biosens. Bioelectron. 41, 557–562. https://doi.org/10.1016/j.bios.2012.09.020.
Wayne, S., 2004. Concepts and terminology. In: Plumlee, K.H. (Ed.), Clinical

Veterinary Toxicology. Mosby Inc., St. Louis, pp. 2–7. https://doi.org/10.1016/B0-32-301125-X/50004-2.

Yu, H.Q., Fang, H.H.P., 2001a. Inhibition on acidogenesis of dairy wastewater by zinc and copper. Environ. Technol. 22, 1459–1465. https://doi.org/10.1080/09593332208618183.

Yu, H.Q., Fang, H.H.P., 2001b. Inhibition by chromium and cadmium of anaerobic acidogenesis. Water Sci. Technol. 43, 267–274. https://doi.org/10.2166/wst.2001.0691.

Yue, Z.-B., Yu, H.-Q., Wang, Z.-L., 2007. Anaerobic digestion of cattail with rumen culture in the presence of heavy metals. Bioresour. Technol. 98, 781–786. https://doi.org/10.1016/j.biortech.2006.03.017.

Zare, M.-R., Amin, M.-M., Nikaeen, M., Bina, B., Rahmani, A., Hemmati-Borji, S., Rahmani, H., 2015. Acute toxicity of Hg, Cd, and Pb towards dominant bacterial strains of sequencing batch reactor (SBR). Environ. Monit. Assess. 187 https://doi.org/10.1007/s10661-015-4457-y. Article no 263.

Zhao, X., Li, D., Xu, S., Guo, Z., Zhang, Y., Man, L., Jiang, B., Hu, X., 2017. *Clostridium guangxiense sp.* nov. and *Clostridium neuense sp.* nov., two phylogenetically closely related hydrogen-producing species isolated from lake sediment. Int. J. Syst. Evol. Microbiol. 67, 710–715. https://doi.org/10.1099/ijsem.0.001702.

Zlatev, R., Magnin, J.-P., Ozil, P., Stoytcheva, M., 2006. Bacterial sensors based on *Acidithiobacillus ferrooxidans*: Part I. Fe^{2+} and $S_2O_3^{2-}$ determination. Biosens. Bioelectron. 21, 1493–1500. https://doi.org/10.1016/j.bios.2005.07.007.