Chemosphere 188 (2017) 337–344

Contents lists available at ScienceDirect

Chemosphere

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

# Determination of heavy metal toxicity by using a micro-droplet hydrodynamic voltammetry for microalgal bioassay based on alkaline phosphatase

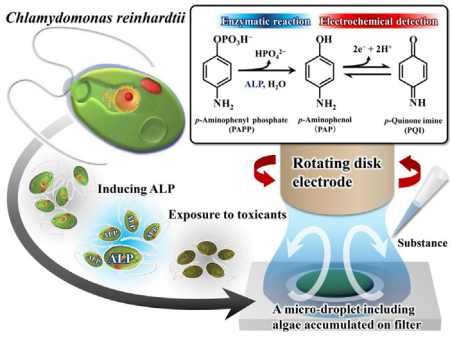
Md. Saiful Islam [a], Kazuto Sazawa [a], Noriko Hata [a], Kazuharu Sugawara [b], Hideki Kuramitz [a, *]

[a] Department of Environmental Biology and Chemistry, Graduate School of Science and Engineering for Research, University of Toyama, Gofuku 3190, Toyama, 930-8555, Japan
[b] Maebashi Institute of Technology, Maebashi, 371-0816, Gunma, Japan

## HIGHLIGHTS

- Alkaline phosphatase enzyme inhibition bioassay using *Chlamydomonas reinhardtii* was developed.
- The induction of alkaline phosphatase activity was successfully carried out.
- A 50 μL micro-droplet was used as reaction medium.
- Electrochemical measurements were carried out using Rotating disk electrode (RDE).
- The enzyme inhibition test using RDE shows the most sensitive response to heavy metals than some common methods.

## GRAPHICAL ABSTRACT




## ARTICLE INFO

*Article history:*
Received 18 May 2017
Received in revised form
29 August 2017
Accepted 2 September 2017
Available online 4 September 2017

Handling Editor: Frederic Leusch

*Keywords:*
Microalgal bioassay
*Chlamydomonas reinhardtii*
Alkaline phosphatase
Hydrodynamic voltammetry
Droplet
Rotating disk electrode

## ABSTRACT

We developed an electrochemical microalgal bioassay for the determination of heavy metal toxicity in water on the basis of the alkaline phosphatase (ALP) enzyme inhibition of *Chlamydomonas reinhardtii*. Five heavy metals were chosen as toxicants: Hg, Cd, Pb, Zn, and Cu. The induced ALP activity of *C. reinhardtii* was inhibited using the phosphate starvation method, and the results were evaluated by measuring the electrochemical oxidation of *p*-aminophenol (PAP) following the enzymatic conversion of *p*-aminophenyl phosphate (PAPP) as a substrate. The rapid determination of enzymatic activity was achieved using hydrodynamic voltammetry in a 50 μL micro-droplet with a rotating disk electrode (RDE). Enzymatic activity over a PAPP substrate is affected by heavy metal ions, and this phenomenon decreases the chronoamperometric current signal. The concentrations of Hg, Cd, Pb, Zn, and Cu in which the ALP activity was half that of the control ($EC_{50}$) were found to be 0.017, 0.021, 0.27, 1.30, and 1.36 μM, respectively. The RDE system was demonstrated to be capable of detecting enzymatic activity by using a small amount of regent, a reaction time of only 60 s, and a detection limit of $5.4 \times 10^{-7}$ U.



* Corresponding author.
*E-mail address:* kuramitz@sci.u-toyama.ac.jp (H. Kuramitz).

http://dx.doi.org/10.1016/j.chemosphere.2017.09.008
0045-6535/

## 1. Introduction

Aquatic environment pollution by a range of inorganic and organic chemicals poses serious threats to the survival of plants and animals. Untreated industrial wastewater, agricultural drainage containing pesticides and fertilizers, and urban runoff dump large quantities of organic and inorganic pollutants into aquatic ecosystems. Among these pollutants, heavy metals are a significant problem in many communities and agricultural areas (Rattan et al., 2005). The toxicity, persistence and bioaccumulation of heavy metals in surface and groundwater make them a severe threat to aquatic organisms, as well as posing a risk to humans and other ecosystems (Liang et al., 2011). Heavy metals may enter the human body through direct and indirect intakes at levels that may adversely affect human health (Rashed, 2010; Chotpantarat and Sutthirat, 2011; Chotpantarat et al., 2011; Taboada-Castro et al., 2012). Therefore, rapid and accurate methods for determining heavy metal contamination are needed.

Environmental risk assessment uses conventional chemical analysis techniques. Contaminated water contains a mixture of pollutants that together create a complex toxicity risk. To evaluate the true hazard posed by these pollutants, analytical quantification must be supplemented with toxicological studies of ecologically relevant organisms (Smolders et al., 2004; van der Grinten et al., 2010). Bioassay is a useful technique for assessing the acute and chronic effects of hazardous chemical releases. Recent bioassay-based environmental assessments have used algae (Peterson and Stauber, 1996; Huang and Hong, 1999; Durrieu et al., 2003; Fanjul-Bolado et al., 2006; Choi et al., 2012; Jurado et al., 2012; Prado et al., 2015; Gissi et al., 2015), bacteria (Jurado et al., 2012; Marugán et al., 2012; Vázquez and Rial, 2014; Ma et al., 2014), plant tissue (Saltzman and Heuer, 1985), animal cells (Slabbert et al., 1984), and fish (Zhu et al., 2011). Microalgae, which are highly sensitive to environmental pollutants, have been widely used in aquatic toxicological testing (Shitanda et al., 2009). Algae are known to be a dominant primary producer in the aquatic food chain and constitute most of the mass balance in fresh water ecosystems. They are easy to grow, and their short generation time makes them appropriate for use in transgenerational assessment of toxicity. The standard algal bioassay uses an algal growth inhibition (AGI) test of over 72–96 h exposure to a toxicant recognized in the standards of USEPA (USEPA, 2002), ISO (ISO 8692, 2012), and OECD (OECD, 1984). The green algae *Pseudokirchneriella subcapitata* and *Selenastrum capricornutum* are widely used as model species. The freshwater microalgae *Chlamydomonas reinhardtii* has also been extensively used in biological research (Harris, 1989, 2001). The AGI test is a widely used technique and provides ecologically relevant results. However, this test takes a relatively long time to perform.

Enzyme activity inhibition can be applied to the toxicity determination of a wide range of pollutants, including heavy metals, pesticides, and insecticide derivatives (Alvarado-Gámez et al., 2014). A number of techniques for the determination of heavy metal toxicity have been reported (Gayet et al., 1993; Kamtekar et al., 1995; Zhang et al., 2000; Koncki et al., 2006; Szydłowska et al., 2006; Berezhetskyy et al., 2008; Park and Kim, 2013). Some of these techniques are based on the inhibition of the enzyme activity of algae caused by heavy metals (Blaise and Menard, 1998; Franklin et al., 2001a, 2001b; Durrieu and Tran-Minh, 2002; Durrieu et al., 2003; Chouteau et al., 2005; Tekaya et al., 2013). The enzymes targeted in these studies included alkaline phosphatase (ALP) (Durrieu and Tran-Minh, 2002, 2003; Chouteau et al., 2005; Tekaya et al., 2013), acetylcholinesterase (Chouteau et al., 2005), and esterase (Blaise and Menard, 1998; Franklin et al., 2001a, 2001b). The enzyme inhibition method using microalgae is rapid and sensitive and is becoming a well-accepted indicator of environmental stress (Franklin et al., 2001b). ALP is the enzyme that is most commonly used in these assays because of its rapid turnover, broad substrate specificity, and ease of application (Alvarado-Gámez et al., 2014).

The determination of enzyme activity has been conducted using both electrochemical (Gayet et al., 1993; Zhang and Cass, 2000; Chouteau et al., 2005; Szydłowska et al., 2006; Koncki et al., 2006; Berezhetskyy et al., 2008; Tekaya et al., 2013) and optical techniques (Kamtekar et al., 1995; Blaise and Menard, 1998; Franklin et al., 2001a, 2001b; Durrieu and Tran-Minh, 2002; Durrieu et al., 2003; Park and Kim, 2013). The advantages of electrochemical detection include its relative simplicity, sensitivity, speed, and ability to be used with samples containing colored components or suspended solids. Electrochemical detection can be used with small volume samples without losing sensitivity, in contrast to optical techniques such as UV/vis spectrometry or fluorometry (Kuramitz et al., 2012a). Electrochemical detection using a rotating disk electrode (RDE), which was developed by Levich (1962), is a sensitive and popular hydrodynamic technique. The rotating disk simultaneously mixes the sample solution and performs electrochemical detection. The centrifugal forces create hydrodynamic flows at the RDE, increasing sensitivity to enzymatic activity and significantly decreasing the time needed for incubation of the enzyme with the substrate. Wijayawardhana et al. (1999a, 1999b) developed a magnetic micro-bead based immunoassay with rapid amperometric detection by using RDE and microliter droplets as the reaction vessel. The major advantage of this technique is that it reduces the dilution product of the enzymatic reaction, thus increasing sensitivity, speeding up detection, and minimizing the nonenzymatic hydrolysis of the substrate and product. Our research group also reported a rapid and simple method based on micro-droplet hydrodynamic voltammetry and demonstrated its use in evaluating genotoxicity and enzyme activity in soil (Kuramitz et al., 2012a, 2012b; Sazawa and Kuramitz, 2015).

This study is the first to apply micro-droplet hydrodynamic voltammetry for toxicity testing on the basis of the inhibition of ALP enzyme activity in microalgae. *C. reinhardtii*, which induces ALP activity by phosphate starvation, was used in the study. Electrochemical detection was performed by chronoamperometry by using RDE on 50 μL of droplet containing *C. reinhardtii* accumulated through filtration. *p*-Aminophenyl phosphate (PAPP) was chosen as the substrate for the ALP. The enzymatic reaction product *p*-aminophenol (PAP) is known to have excellent electrochemical properties, including a low oxidation potential, negligible electrode fouling, and reversible electrochemical behavior (Tang et al., 1988). The method of microalgal bioassay with hydrodynamic electrochemical detection used in the study successfully detected Cu, Zn, Pb, Cd, and Hg toxicity. The results were compared with those from a micro-scaled AGI (μ-AGI) test and an electrochemical enzyme inhibition test using purified ALP on the basis of micro-droplet hydrodynamic voltammetry.

## 2. Materials and methods

### 2.1. Reagents and instrumentation

ALP from *Escherichia coli* and PAP was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan), and PAPP from LKT Laboratories Inc. (St. Paul, MN, USA). The substrate solution was used immediately after preparation. Standard solutions of Cu, Pb, Cd, Hg, and Zn at the target concentrations were prepared by dilution of stock standard solutions (1000 mg $L^{-1}$) from Wako Pure Chemical Industry Ltd. Tris-HCl buffer solution (0.1 M Tris with 0.1 M hydrochloric acid and 0.01 mM $MgCl_2$ at pH 8.4) was used for the incubation of the algal cells with substrate. A GF/F glass