DOCTORAL THESIS

Development of Micro-droplet Hydrodynamic Voltammetric Techniques Based on Enzyme Inhibition and Its Application for the Toxicity Assessment of Environmental Water Pollutants

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Dedication

To my beloved parents, my faithful wife and our adorable daughter

Abstract

Environmental water pollution has gained global attention because of the hostile effects it made toward the ecological balance and human health. There are varieties of toxicants, including chemicals, detergents, coloring agents, heavy metals, pesticides, endocrine disruptors, antibiotics, carcinogens, and toxins are releasing to the environmental water through various natural and anthropogenic processes which poses serious threats to the survival of aquatic plants and animals. Contaminated water contains a mixture of pollutants that together create a complex toxicity which is difficult to assess using conventional chemical analysis techniques. Most of the current monitoring levels are articulated on the basis of single pollutants which might lead to under or over estimation of toxicity. Therefore, to evaluate the true hazard posed by these pollutants, analytical quantification must be supplemented with toxicological studies.

The main objectives of this study evaluate the rightness of electrochemical hydrodynamic technique using the rotating disk electrode (RDE) in a microdroplet for the determination of aquatic toxicity arising from heavy metals and microcystins. We have developed electrochemical enzyme inhibition assay for the toxicity determination of heavy metals, a representative of inorganic pollutants and microcystin, a toxin produced by cyanobacteria. The RDE can effectively mix the enzyme-substrate mixture and at the same time performs as electrochemical detection device. The rapid determination of enzymatic activity was achieved using hydrodynamic voltammetry in a 50 μL micro-droplet with a RDE. In both case, *p*-aminophenyl phosphate (PAPP) has been used as a substrate. The chronoamperometric response were obtained from the electrochemical oxidation of *p*-aminophenol (PAP) following the enzymatic

conversion of PAPP. Enzymatic activity over a PAPP substrate is affected by these toxicants, and this phenomenon decreases the chronoamperometric current signal.

For the determination of heavy metal toxicity in water, we developed microalgal bioassay on the basis of the alkaline phosphatase (ALP) enzyme inhibition of *Chlamydomonas reinhardtii*. The induced ALP activity of *C. reinhardtii* was inhibited using the phosphate starvation method. Five heavy metals were chosen as toxicants: Hg, Cd, Pb, Zn, and Cu. 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 system was demonstrated to be capable of detecting enzymatic activity by using a small amount of regent, and a detection limit of 5.4×10^{-7} U. The results were compared with those from a micro-scaled algal growth inhibition (μ-AGI) test and an electrochemical enzyme inhibition test using purified ALP on the basis of micro-droplet hydrodynamic voltammetry.

Protein phosphatase 2A (PP2A) inhibition has been used for the determination of microcystin-LR (MC-LR), one of the most frequent and most lethal cyanobacterial toxins and a vital environmental pollutant due to its toxicity and persistence. It is hepatotoxins and have been shown to be potent tumor-promoters which pose a serious threat to human health in the form of chronic exposure through drinking water. The parameters for measurement of PP2A inhibition by MC-LR were optimized in this study. The results were compared with other electrochemical and colorimetric measurements. Comparison among these studies revealed that the PP2A inhibition assay has a sensitive response to MC-LR. The IC_{50} value was calculated as 0.08 μg L^{-1} which is well below the World Health Organization (WHO) provisional guideline value for total MC-LR of 1 μg L^{-1} in drinking water.

We have studied the effectiveness of RDE for the electrochemical determination of enzyme activity inhibition to determine the toxicity of heavy metals and microcystin using ALP and PP2A, respectively. Both the assays were discovered as sensitive in comparison with other electrochemical and spectrophotometric methods in terms of EC_{50} and IC_{50} values. This is due to the effective mixing of enzymes and substrates by RDE which decreases the incubation time and reduces the nonenzymatic hydrolysis of the enzyme substrate. The microdroplet reaction vessel reduces diffusional distance, resulting faster detection. It also reduces the dilution of enzymatic product which results in lower detection limits. The use of enzyme inhibition with minimum instrumentation requirements make these essays really convenient. Thus, it can be suggested that enzyme inhibition assay using RDE is a suitable candidate for measuring the toxicity of aquatic environmental pollutants using fewer chemicals in a rapid manner.

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Chapter 1

General introduction

1.1. Background

Water, covering more than two thirds of Earth's surface is the most indispensable element for the very existence of all life forms in Earth including human being. It provides the lifeblood of the planet linking all ecosystems. It helps to transport all the necessary minerals, nutrients and waste products to and from the cells. Water is also vital for the household uses, agriculture, industry, fishery and other economic development program. The water quality is deteriorated by the industrialization and urbanization activities as a result of rapid population growth and their ever-increasing pressures on water resources. The consequential water pollution is one of the greatest challenges faced by human kind today which poses serious threats to the ecological balance. It has increased in both developed and developing countries. Water is polluted when a wide variety of toxicants, including chemicals, detergents, coloring agents, heavy metals, pesticides, endocrine disruptors, antibiotics and carcinogens are discharging directly or indirectly to the environmental water through various natural and anthropogenic processes. Untreated industrial wastewater, sewage waste, agricultural drainage containing pesticides and fertilizers, and urban runoff dump large quantities of organic and inorganic pollutants into aquatic ecosystems. Environmental water is also being polluted by the leachate of solid wastes as rainwater sweeps through the open dumping ground. They are causing reductions in wildlife numbers, degrading ecosystem functions and threatening human health with their toxic impacts. Industries, agriculture and human settlements are considering as the main sources for water pollution (Mateo-Sagasta et al., 2017). According to the United Nations World Water Development Report 2017, 80% of the global municipal wastewater is releasing into the surface water without any treatment, where, tonnes of solvents, toxic sludges, heavy metals and other wastes are discharged by several industries (WWAP, 2017). Industries

are influencing water quality through direct discharge of wastewater, emitting toxic gases which can cause acid rain, and changing the water temperature with their disposals into waterways. Large amount of pesticides, fertilizers, nutrients, and other organic matters are reaching to the water bodies from agricultural lands and the subsequent water pollution threatening the balance of aquatic ecosystem (UNEP, 2016). Sewage disposal into surface water is another source of water pollution with billions of people on the planet Earth which leads water-related human health problem. The underprivileged admittance to basic sanitation and water services in the developing countries are triggering intense effects. According to WHO, around 850,000 diarrheal deaths in low- and middle-income countries are resulting from poor drinking water, sanitation access and hand washing practices (WHO, 2014). The United Nations Environment Programme reported the death of approximately 3.4 million people annually from diseases associated with pathogens in water (UNEP, 2016). The fatalities are largely connected with the use of polluted water sources. Therefore, to provide the safe environment for the people, toxic pollution monitoring is very crucial. It is also important to select the right approach for environmental risk assessment.

1.2. Water pollution by heavy metals

Heavy metal contamination is potentially a significant problem in several community and agricultural areas (Rattan et al., 2005). These are the trace metals having at least five times higher density than that of water and include lead, mercury, cadmium, chromium, iron, copper, zinc etc. (Tchounwou, 2012). However, the official term toxic metal is more valid for the group of metals such as cadmium, copper, chromium, lead, and mercury etc. They can occur naturally in the ecosystem with various concentrations. In recent time, the discharges of heavy metals are gradually increasing due to the rapid

growth of industrial activities and urbanization which have led to huge increases in the amount of various waste comprising heavy metals inputs (Wang et al., 2016). 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). Although some heavy metals, such as cobalt, copper, zinc, nickel, manganese, molybdenum, vanadium, strontium, and Iron are necessary for life activities, beyond a certain threshold, all the metal elements can be deadly (Gadd, 1992). Besides, some of the heavy metals are classified as highly toxic having no known cellular roles. The list includes arsenic (As), silver, antimony, cadmium, mercury, lead etc. (Nies, 1999). Many of these metals are found to be carcinogens that has been reported to be closely related to several diseases such as Alzheimer, Parkinson, multiple sclerosis, osteoporosis, developmental disorders and organ failures (Mates et al., 2010; Jomova and Valko, 2011). Cadmium can be entered into human body through ingestion and inhalation. The resultant symptoms including gastrointestinal tract irritation, nausea, vomiting, respiratory difficulties, cramps, loss of consciousness, cardiovascular diseases, renal problems, and hypertension etc. (Halstead et al., 2019). Long term of lead exposure in humans can cause hypertension, anemia, nervous system breakdown, kidney damages and impairment of intellectual functions in affected patients. Copper is an essential substance to human life, but in high doses it can cause anemia, liver and kidney damage, and stomach and intestinal irritation (Gibson et al., 1992). Chromium is used in metal alloys and pigments for paints, cement, paper, rubber, and other materials. Low-level exposure can irritate the skin and cause ulceration (Koropatnick

et al., 2000; Kennish, M. J.,1992). Long-term exposure can cause kidney, liver, circulatory and nerve tissue damages. Chromium often accumulates in aquatic fishes which have been exposed to high levels of chromium, makes it poisonous to consume (Gupta et al., 1999; Grass et al., 2001; Hamlett et al., 1992). Mercury is a very toxic and exceedingly bio accumulative which adversely affects the aquatic environment. The primary target organ for this toxic metal is the brain. Besides, it can damage other organ organs and causes the failure of muscles, kidneys and nervous systems (Jaishankar et al., 2014).

1.3. Water pollution by cyanotoxins

Dangerous toxins are produced sometimes as a result of biological pollution. Cyanotoxins are produced by cyanobacteria particularly in freshwater and marine ecosystem where, they are capable to form visible blooms under definite circumstances. The bloom formations become a global environmental issue having serious threat to ecological balance and human health problem due to their associated toxicity (Lin et al., 2013; Catanante et al., 2015). Generally, cyanotoxins comprised of cyclic peptides (microcystins (MCs) and nodularins) and alkaloids (anatoxin-a, anatoxin-a(S), cylindrospermopsin, saxitoxins (STXs), aplysiatoxins and lyngbyatoxin). Cyanotoxins were discovered to be responsible for many intoxication cases involving animals, such as cows, pigs, dogs, fishes, birds and bats (Alonso-Andicoberry et al., 2002; Apeldoorn et al., 2007; Cox et al., 2003; Krienitz et al., 2003). According to their mode of action, cyanotoxins are classified into hepatotoxins (e.g. MCs), neurotoxins (e.g. anatoxins), skin irritants, and other toxins. Commonly found cyanobacteria in surface water can produce both hepatotoxins and neurotoxins and, therefore have strong influence of the water quality.

Among the various types of cyanotoxins, MCs produced in fresh and brackish waters ecosystem around the globe are the most common. More than 50 congeners of MCs are known most of which produced during bloom (WHO, 2003). MCs were first isolated from the cyanobacteria *Microcystis aeruginosa* and were named after this organism (WHO, 1998). Several types of MCs including MC-LR, MC-RR and MC-YR are known to be produced by common bloom forming cyanobacteria. It is a potent hepatotoxins and very stable in the natural environment due to the presence of double bonds and ring structure in the molecule (Lin et al., 2013; Li et al., 2016). At near neutral pH, MCs are resistant to oxidation or chemical hydrolysis. They are also found resistant to degrade by boiling for several hours (Harada et al., 1996). MCs are known for their specific inhibition of protein phosphatases type 1 and 2A (PP1 and PP2A). These enzymes inhibition produces hyperphosphorylation of the structural proteins, lead to tumor promotions and liver cancer (Eriksson et al., 1990; Campas et al., 2007). There is not much information available regarding the mode of action of other MC varieties than MC-LR. It is assumed that they could have similar effects on protein phosphatase as all MC contains unusual amino acid ADDA (Fischer et al., 2001).

1.4. Water pollution scenario in Bangladesh

Bangladesh located in south Asia is a deltaic floodplain created by the sediment flow by several rivers. These rivers are providing major inland surface water sources for domestic, agricultural and industrial purposes in Bangladesh. In recent times, these rivers have become more polluted as a consequence of rapid population growth, unplanned industrialization and urbanization, and agricultural operations. Among various sources of water pollution, industries such as tanneries, steel plants, battery producers, engineering, and textiles are considered as the prime emitters due to their

excessive use of water and releasing of unprocessed wastewater into the surface water system during different steps of product manufacturing. The poor quality of the water is resulted from inconsiderate disposal of untreated wastewater and solid waste to the water system. Among the industries listed above, tanneries and textiles are contributing significantly over the others and the discharged wastewater alters physicochemical and biological properties of aquatic ecosystem. Fecal pollution also contributing surface water pollution extensively along with industrial sources when sanitation is generally poor (Mizan, 2006).

Dhaka City, the capital of Bangladesh, with a population of more than 16 million stands one of the most overcrowded cities in the world. It is located on the northern bank of the Buriganga River and is surrounded by some other rivers: the Turag, Dhaleshwari, Tongi canal, Balu, and Shitalakkhya. The majority of the industries and factories in Dhaka are situated on the banks of these rivers or close to the river system. It has been found that, more than 7,000 industries located beside these rivers mostly in three main areas of the Dhaka-Narayanganj-Demra dam, Hazaribagh, and Tejgaon (Roy, 2009). These rivers are gradually being polluted by massive dischrge of toxic wastes from industrial areas and sewage lines (Islam et al., 2006) and also petroleum discharge from ships, launches, cargoes, boats, etc. (Khan et al., 2007). The river system around Dhaka city receives about 60,000 m^3/day of toxic wastes discharged mainly from nine major industrial clusters (Tongi, Hazaribagh, Tejgaon, Tarabo, Narayanganj, Savar, Gazipur, DEPZ, Ghorashal) (RPMC, 2008). Bangladesh Poribesh Andolon (BAPA) reported that a total of 6,000 tons of liquid waste is dumped into the Buriganga every day, half of which comes from Hazaribagh tanneries (The Daily Star, 2010). Another study carried out by Blacksmith Institute (2007) revealed that Hazaribagh tanneries daily generate 7.7 million L of liquid waste and 88 million tons

of solid waste. Moreover, at least 7000 tons of solid wastes are generated in and around the Dhaka City Corporation area every day (The Daily Star, 2009). The pollution load increases with annual increases in the population.

The water quality parameters in the three main rivers around Dhaka city during both the dry and monsoon seasons have been found exceeding the recommended levels set by the Department of Environment (DoE), Bangladesh in our previous study, indicating a high level of water pollution. The study results are given in Table 1.1.

Table 1.1. General water quality of the three rivers around Dhaka City, Bangladesh (Islam et al., 2015).

	DoE*	Turag		Buriganga		Shitalakkhya	
	Standard	Mean	Range	Mean	Range	Mean	Range
pH	6-9	7.9	6.9-9.1	8.0	7.1-9.8	7.7	6.5-8.3
EC ($\mu S\ cm^{-1}$)	1200	1807	790-2850	1209	830-1990	1150	720-1920
TDS ($mg\ L^{-1}$)	2100	1003	650-1510	999	620-1260	820	475-1180
DO ($mg\ L^{-1}$)	4.5-8.0	1.2	0.45-3.2	1.7	0.45-3.5	2.1	0.6-3.8
BOD ($mg\ L^{-1}$)	50	110	56-179	93.7	41-151	86.7	44-146
COD ($mg\ L^{-1}$)	200	97.7	5-177	100	17-173	87	14-172

*DoE: Department of Environment, Ministry of Environment and Forest, Bangladesh (DoE, 1997).

The higher value beyond threshold level can characterize potential health and environmental risks to the people living around these rivers. During the dry season the pollution level becomes even higher due to the reduced mean daily discharges of these rivers. Alternatively, during the rainy season the concentration was diluted from the higher water flow. Therefore, river pollution is largely dependent on the upstream river

flow, which indicates that the control of different upstream rivers in India and China indirectly controls the river pollution in Bangladesh. In order to determine the actual level and severity of the pollution, it is important to measure the organic matter content together with the humic substances in the river water. Microbial status is another important aspect of Bangladesh's river pollution.

1.5. Bioassays for toxicity determination

For the determination of toxic chemicals in the aquatic environment, analytical techniques available are organized into two main groups: techniques based on chemical analysis, and biological methods (Hassan et al., 2016) and given in Figure 1.1.

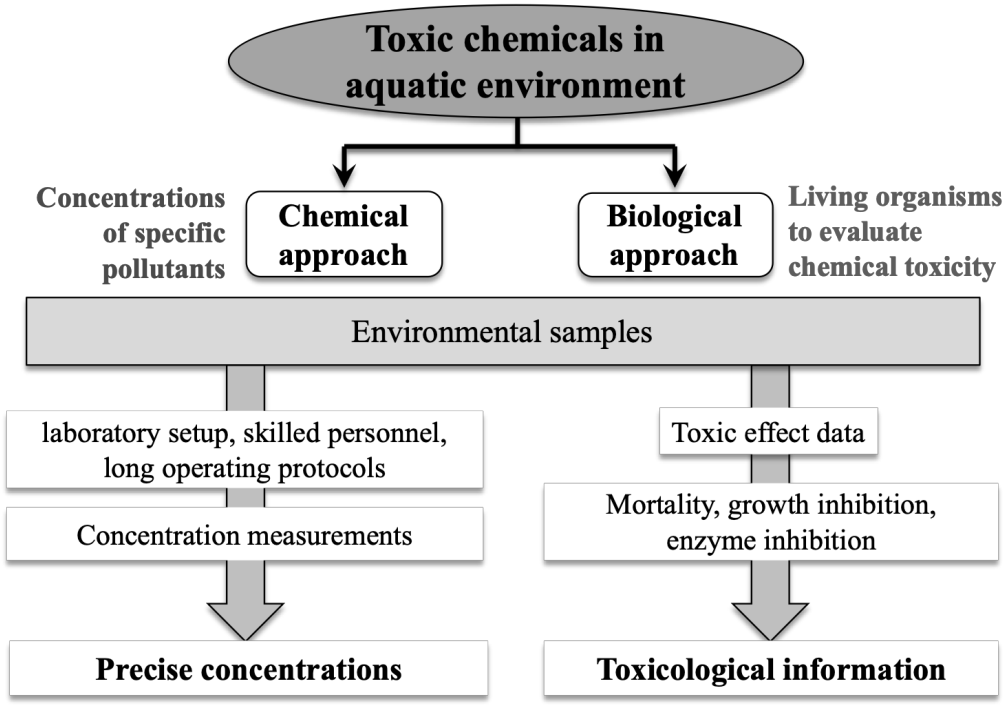


Figure 1.1. Aquatic toxic chemicals determination techniques.

The conventional chemical analysis techniques are very suitable to detect specific pollutants. However, the requirement of compound laboratory setup, skilled personnel, long operating protocols, sample pretreatment make them incompatible for monitoring

purpose. In addition, the evaluation of complex toxicity exhibited from the mixture of pollutants is very difficult using these techniques (Hassan et al., 2016). For the evaluation of true toxic impacts poses by these toxicants, analysis technique must be accompanied with toxicological studies of ecologically relevant organisms (Smolders et al., 2004; van der Grinten et al., 2010). Biological methods, i.e. bioassay provides the toxicological information of the pollutants present in the sample and possible to detect the complex toxicity.

Bioassays are able to deliver information concerning toxic effects on certain test organisms during a specified period of time made by chemicals present in the target sample. They are considered as one of the most useful methods for the determination of complex toxicity in environmental water and can be used for pollution monitoring where traditional methods are unable to apply (Shitanda et al., 2009, Hassan et al., 2016). Bioassays can be carried out either *in vitro* or *in vivo* where the former can be done with the use of laboratory-based cell culture techniques and latter can be done either in the laboratory or in the field. *In vitro* bioassays are usually conducted for the assessment of specific mechanisms of action, such as cytotoxicity, genotoxicity or estrogenicity of chemicals and *in vivo* bioassays are conducting for a more integrated organism response (Ghosh et al., 2017). Bioassays are also recommended by several environment protection agencies where it has been suggested to apply the assay using aquatic organism during different stages of their life cycle (Vosylienė, 2007). Several standard techniques have been designed and suggested by the U.S. Environmental Protection Agency (USEPA), the Organization for Economic Cooperation and Development (OECD), the International Standardization Organization (ISO) and some other organizations. They are focusing on the consequences including growth inhibition, abnormal development, physiological parameters, and reproduction etc.

Recent bioassay-based environmental assessments have used different organisms including 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 (Juardo et al., 2012; Marugan et al., 2012; Vazquez 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) etc. and summarizes in Table 1.2. Some of these organisms are difficult to handle (fish larvae) and some of them maybe ethically offensive (animals).

Table 1.2. The list of organisms commonly used for toxicity bioassays (Hassan et al., 2016, Pujol Vila, 2017).

Type of organism	Organisms	Endpoints	Test time (days)
Algae	*Chlamydomonas* sp.	Mortality	3-4
	Chlorella sp.	Photosynthesis activity	
	Chlorella vulgaris	Algal growth	
	Monoraphidium sp.	Enzymatic activity	
	Scenedesmus subspicatus	Cell counts	
Bacteria	Aerobic bacteria	Respiration inhibition	5
	Vibrio fischeri	Bioluminescence inhibition	1-2
	Anaerobic activated sludge	Denitrification inhibition	2-5
	Soil bacteria	Tensile strength	
Plants	*Brassica campestris*	Growth, biomass,	4, 14-30, 21
	Avena sativa	germination rate	
	Vicia faba		
	Allium cepa		
Invertebrate	*Daphnia magna*	Mortality, motility	1-2 (acute),
	Brachionus calyciflorus	Survival and reproduction	21 (chronic)
	Artemia salina	Proliferation	3-4
	Shrimps	Speed variation	0.5, 1, 2
	Bivalves, mussels	Open/closing of shell	
Fish	Zebra fish embryos	Larval growth and survival	1-2 to 7
	Fathead minnow	Mortality, motility	180
	Rainbow trout	ATP levels in white muscle	1-2 to 7

1.5.1. Algal bioassay

Among the organisms using for bioassays, microalgae are extensively used in aquatic toxicological testing due to their reproducibility and high sensitivity to various chemicals including organic pollutants, herbicides, oil dispersants, effluents, solid

waste leachates etc. (Peterson and Stauber, 1996, Adachi et al., 2004; 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 most common algal species used are *Chlamydomonas variabilis, C. reinhardtii, Chlorella vulgaris, C. pyrenoidosa, C. kessleri, Pseudokirchneriella subcapitata, Monoraphidium pusillum, Scenedesmus quadricauda, S. subspicatus,* and *Selenastrum capricornutum* (Harris, 1989; Harris, 2001; Hassan et al., 2016).

In case of algal bioassay, endpoints including growth inhibition, motility inhibition, chlorophyll fluorescence, enzyme (esterase) inhibition, ATP energy losses are usually assessed. 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 aim of this test is to evaluate the toxic impacts of a pollutants on algal growth. The organisms in their exponential growth stages are exposed to test chemicals in batch cultures over a specific period of time usually 72-96 hours. The response is evaluated as a function of the exposure concentration in comparison with the average growth of control cultures. The endpoints growth inhibition calculated as EC_{50} expressing the test concentration corresponding to 50% inhibition. The test can assess the toxic impacts over several generations instead of relatively brief test duration (OECD, 1984). The AGI test is a widely used technique and provides ecologically relevant results. However, this test takes a relatively long time to perform difficult to test substances that are colored, having a low solubility in water, volatile, strongly adsorbing, or substances that may affect the availability of nutrients in the test medium.

1.5.2. Bacterial bioassays

Bacterial bioassays are also very popular techniques for the toxicity determination using various mechanism including CNS (carbon, nitrogen, and sulfur) transformation, enzyme activity inhibition, growth inhibition, mortality and mobility, respiration inhibition, glucose uptake, and luminescence output (Tothill and Turner, 1996). ISO and OECD have established activated sludge, respiration inhibition test as an effective method for evaluating the toxicity of chemicals on wastewater treatment systems (ISO, 2007; OECD, 2014). The measurement of oxygen uptake rate is the most common method using bacterial respiration to detect pollutants toxicity. Luminescent bacteria such as *Vibrio fischeri* or *Photobacterium phosphoreum* have been used in various toxicity test devices. These tests are measuring the bioluminescence inhibition when exposed to certain toxicants. For acute toxicity evaluation, the specific strain of *V. fischeri* (*V. fischeri* NRRL B11177) has been used in various commercial test kits, i.e. Microtox, LUMIStox, and ToxAlert (Ren, 2004). OECD standardized the bioluminescence inhibition of *V. fischeri* with short analysis time and simple operation procedures (OECD, 2014).

1.5.3. Plant bioassays

Plant bioassays are providing good options for toxicity determination with endpoint assessments of germination rate, biomass weight, enzyme activity, etc. The advantages of these assays include rapid test activation and low maintenance cost. Besides, these are applicable to measure the solid waste toxicity (Hassan et al., 2016). The duration of the assay period is quite long comparing to algal bioassays. This is a big drawback for the application of plant bioassays.

1.5.4. Invertebrate bioassays

The use of invertebrates for aquatic toxicity determination is well accepted due to their feasibility and practicality with respect to labor demand and other requirements (Hassan et al., 2016). Water flea (*Daphnia magna*) is the most common invertebrate for the aquatic toxicity test and has been well established and standardized by ISO and USEPA for acute lethality test (ISO, 2007; USEPA, 2002). There are various advantages including simple operation, short exposure period, short reproductive phase, and high sensitivity to pollutants make this species ideal for routine toxicity test (Tothill and Turner, 1996). Besides daphnia, rotifer (*Brachionus calyciflorus* and *Brachionus plicatilis*) are also used in toxicity tests (Janssen et al., 1994; Snell et al., 1991). Survival and reproduction data are obtained by exposing isolated rotifer neonates to the toxicant and obtaining life history characteristics at regular intervals. In addition, brine shrimp (*Artemia salina*) based bioassays are also available for the screening of bioactive compounds in natural products, cyanobacterial toxicity detection, and anthropogenic chemicals detection (Ruebhart et al., 2008). They are very attractive due to various reasons, i.e. commercial availability, quick and simple assay, low cost, small sample volume and ethical acceptance in many countries (Rosenfeldt et al., 2007). There are other assays available based on growth and survival of mayflies, amphipods, stoneflies, or oysters (Price et al., 1990; Sarakinos et al., 2000; Suter Ii and Rosen, 1988).

1.5.5. Fish bioassays

Fish bioassays are the oldest form of bioassays for toxicity assessment and have been used for many decades. These are mainly carried out using larval growth and survival as endpoint indicator where fishes are exposed to certain range of contaminants for a period of time. The mostly used types for acute lethality tests are zebra fish

(*Brachydanio rerio*), fathead minnow (*Pimephales promelas*), bluegill (*Lepomis macrochirus*), rainbow trout (*Oncorhynchus mykiss*), and common carp (*Cyprinus carpio*) (Munkittrick et al., 1991). The advantages of fish bioassays are their presence in almost all aquatic ecosystems, easy identification, and long-life cycles (suitable indicators for chronic toxicity determination) etc. The main drawbacks of those assays are longer test time, specialized arrangements, and lower sensitivity towards low concentrated pollutants (Farré and Barceló, 2003).

1.6. Enzyme activity inhibition assays for toxicity determination

Enzyme activity inhibition assays are widely used in recent time and can be applied for the toxicity determination of a wide range of pollutants including heavy metals, pesticides, and insecticide derivatives (Alvarado-Gamez et al., 2014). Enzyme inhibition assays determine the enzyme-catalyzed reaction rate and measure the physical response which yields a quantity related to this rate. Assays are depending on the quantitative measurement of the enzymatic activity difference before and after exposure to toxicants (Figure 1.2).

The enzymes targeted in these studies included glucose oxidase, urease, acetylcholinesterase, alkaline phosphatase (ALP), protein phosphatases 1 and 2A, and esterase etc.

Glucose oxidase (GOx), an oxido-reductase enzyme mainly catalyzes the oxidation of glucose into hydrogen peroxide and glucono-lactone have been widely used. GOx is readily available, inexpensive and stable enzyme and mostly used in the biosensors for industrial processes and highly specific for β-D-glucose.

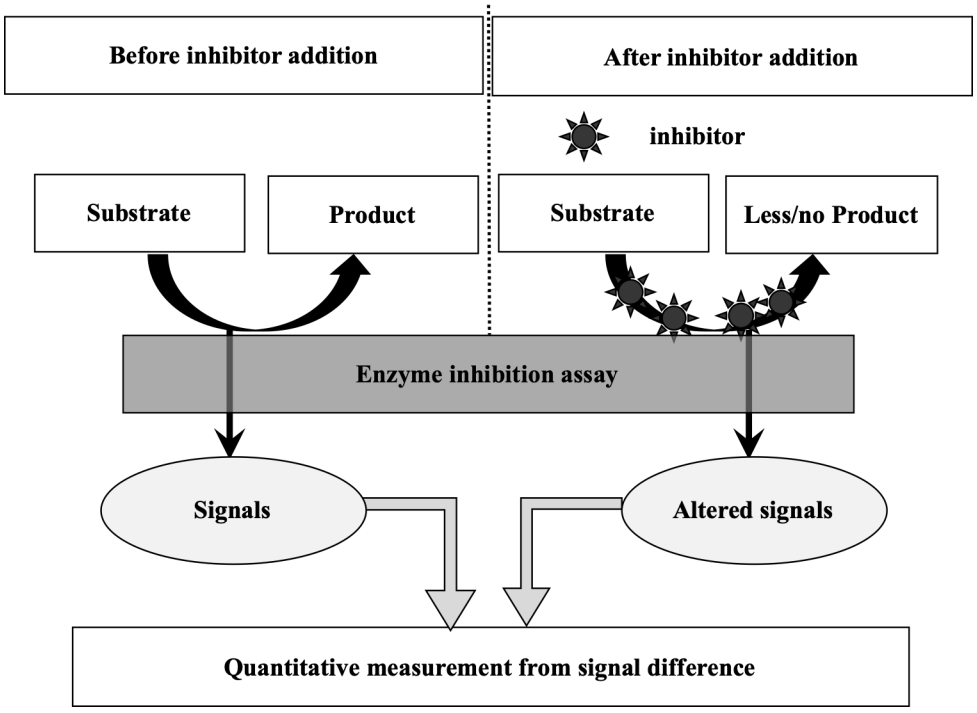


Figure 1.2. Illustrative representation of the working principle of enzyme inhibition assay (modified from Bachan Upadhyay and Verma, 2013).

Urease was the first enzyme to be crystallized out from jack bean and their inhibition was mostly used for the determination of heavy metals like copper, cadmium, chromium, lead and mercury (Balasubramanian and Ponnuraj, 2010; Bachan and Verma, 2013).

Cholinesterase is a family of enzymes that breaks apart the neurotransmitter acetylcholine, which is vital for nerve impulses transmission. This enzyme has been widely used in the electrochemical detection of organophosphorus compounds (Amine et al., 2006).

ALP is probably the most commonly used enzyme because of its rapid turnover, broad substrate specificity, and ease of application (Alvarado-Gamez et al., 2014). This enzyme has been used for indirect monitoring of heavy metals that inhibit biocatalytic

properties. The determination of ALP activity has been carried out using various substrates such as phenyl phosphate, naphthyl phosphate, ascorbic acid 2-phosphate, p-nitrophenyl phosphate, *p*-aminophenyl phosphate etc. among which *p*-aminophenyl phosphate (PAPP) is an appropriate substrate since the enzymatically produced *p*-aminophenyl (PAP) can be detected electrochemically (Alvarado-Gámez et al., 2014). Besides these enzymes, some other enzymes are also reported in literature including choline oxidase, polyphenol oxidase, nitrate reductase, invertase, and horseradish peroxidase/catalase. These enzymes are not a part of extensive research for the development of inhibition-based assays.

Several parameters including solution pH, temperature, substrate concentration, and enzyme concentrations are generally affecting the performance of enzyme activity inhibition assays. Enzymes, like all-natural proteins, have pH sensitive structures and can be denaturized at extreme pH (Amine et al., 2006). Therefore, it is highly recommended to optimize the pH for any kind of enzymatic assays. Temperature is also very important parameters for enzymatic assays. Enzymes have their specific temperatures to get the maximum performance, i.e. ALP activity is best obtained at 37˚C (Copeland et al., 1985). The degree of enzyme inhibition can be varied upon substrate concentration. A study carried out for pesticides determination concluded that inhibition level rises with substrate concentration increases (Kok et al., 2002).

The determination of enzyme activity has been conducted using both electrochemical (Gayet et al., 1993; Zhang et al., 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 et al., 1998; Franklin et al., 2001a; Franklin et al., 2001b; Durrieu at al., 2002; Durrieu at 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).

1.7. Electrochemical detection using RDE

The electrochemical detection of enzymatic activity using rotating disk electrode (RDE) developed by Levich has become a sensitive and popular hydrodynamic technique due to its ability to mix the sample solution effectively and at the same time acts as an electrochemical detection device (Levich, 1962). It is a classical hydrodynamic technique which can limit the diffusion layer thickness (Denualt et al., 2007). The centrifugal forces create hydrodynamic flows at the RDE that move the solution horizontally away from the electrode surface, while fresh solution flows vertically to the surface of the electrode. The rate constants can be calculated from the limiting current changes with rotation rate (Kuramitz, 2009). The hydrodynamic flow achieves a highly sensitive determination for enzymatic activity that decreases the incubation time for enzyme with substrate. RDE voltammetry is a useful tool now a days in the area of neurochemistry, neuropharmacology, and environmental analysis (Burgess et al., 1999).

In RDE measurement, continuous oxidation current is generating corresponds to the concentrations of substrates present in the reaction vessel. The usual electrochemical cell uses as reaction vessels containing 10 to 125 mL of sample solution which is a relatively large volume. Significant dilution of enzyme product can be happened due to this large sample volume which is considered as a big disadvantage

of this technique. In order to minimize the product dilution, smaller cells can be used. The solution spillover from the cells and high turbulence proved smaller cells challenging to prevent dilution (Wijayawardhana et al., 1999). The problem can be avoided using microdroplet as reaction vessel sandwiched between RDE and hydrophobic surface.

The small volume microdroplet can be formed from the high surface tension. The reference and counter electrodes are placed inside the microdroplet (20 to 150 μL) for the electrochemical determination. The equilibrium of the microdroplet can be retained at electrode rotation rates up to 3000 rpm. The decrease in sample solution volume effectively reduces the diffusional distances, results in a shorter period of incubation time approximately 20 s (commonly 30–60 min) and, consequently rapid detection, and therefore, speedy assays. Another advantage of microdroplet is the reduction of enzymatic product dilution, which was a major disadvantage in case of large sample volume. The reduced dilution of enzymatic product helps to achieve low detection limits, thus increasing sensitivity, speeding up detection, and minimizing the nonenzymatic hydrolysis of the substrate and product.

Wijayawardhana et al. (1999a, 1999b) developed a magnetic microbead-based immunoassay with rapid amperometric detection by using RDE and microliter droplets as the reaction vessel. 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; Kuramitz et al., 2012b; Sazawa and Kuramitz, 2015).

1.8. Aim and objectives

The preceding sections have given an overview of the environmental water pollutants along with their toxic impacts and the use of electrochemical enzyme inhibition assay for toxicity determination. It is understandable that these pollutants are contributing significantly for the aquatic environmental pollution. Furthermore, their presence in the drinking water is very dangerous for public health. The conventional techniques are capable to measure the accurate concentration of these pollutants effectively, however, lack of providing toxic impacts on living organisms. So, this study was attempted to develop bioassay to measure the toxicity of these pollutants. The hydrodynamic electrochemical determination using RDE in a microliter of droplet have been used for the measurements aiming the rightness evaluation of this techniques for the determination of aquatic toxicity arising from heavy metals and microcystins. The main objective of this thesis work is to establish electrochemical RDE technique as a suitable, rapid and sensitive for the toxicity determination of aquatic pollutants as the conventional techniques are lacking the advantages provided by RDE detection.

The specific objectives are as follows:

- To develop micro-droplet hydrodynamic voltammetric method for toxicity testing on the basis of the inhibition of ALP enzyme activity in microalgae *Chlamydomonas reinhardtii*. The algal species used for this assay is not a recommended species by OECD for aquatic toxicity determination and no research work has been done previously using ALP enzyme with RDE determination. This study is the foremost research for determining heavy metal toxicity on the basis of the ALP inhibition activity in microalgae. *C. reinhardtii* using micro-droplet hydrodynamic voltammetry.

- To develop toxicity test based on the inhibition of protein phosphatase enzyme activity using the micro-droplet hydrodynamic voltammetry for the determination of Microcystin-LR. The protein phosphatase inhibition assays are widely used to determine the MC-LR, mostly spectrophotometric determination, largely influenced by the presence of colored or suspended materials and requirement of longer time period for the determination. Therefore, we targeted this analyte to measure with electrochemical RDE technique as this is a rapid and sensitive technique able to detect sample with colored materials and suspended solids within very short time.

The thesis consists of total four chapters. A detailed description of these chapters is given for the well understanding of this thesis.

Chapter 1 serves as a foundation for understanding the background, purpose, significance and structure of the research plan for the remainder of the project. This chapter portrays the water pollution scenario, pollution from various sources, techniques for the pollutant's concentration measurements, their advantages and drawbacks etc. This chapter also provides the fundamentals of enzyme inhibition assays, electrochemical determination using RDE, the usefulness and the suitability to use for heavy metals and microcystin toxicity determination.

Chapter 2 describes the application of novel micro-droplet hydrodynamic voltammetric technique for toxicity testing on the basis of the inhibition of alkaline phosphatase (ALP) enzyme activity in microalgae *C. reinhardtii*. The ALP enzyme has been induced by the phosphate starvation method. The study is the first to apply the enzyme inhibition of *C. reinhardtii* for toxicity determination. 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 (m-AGI) test and an electrochemical enzyme inhibition test using purified ALP on the basis of micro-droplet hydrodynamic voltammetry.

Chapter 3 presents the development of toxicity test based on the inhibition of protein phosphatase enzyme activity using the micro-droplet hydrodynamic voltammetry for the determination of MC-LR. The electrochemical hydrodynamic voltammetry was previously discovered effective for the determination of enzymatic activity. We have applied the method for the determination MC-LR, the most frequent and most lethal MCs produced by cyanobacteria. The comparative analysis with other methods has also been presented.

Chapter 4 covers the conclusions and future scope of works.

Chapter 2

Micro-droplet hydrodynamic voltammetry for microalgal bioassay based on alkaline phosphatase for the determination of heavy metal toxicity

2.1. Introduction

Heavy metal pollution is considered a serious global environmental problem now a days due to the advancement of urbanization and industrialization activities. Heavy metals can cause DNA damages and exert cancer to humans. They are responsible to produce potential detrimental consequences on the aquatic ecosystem. Thus, it is essential to monitor heavy metal contamination in environmental water. The classical methods are unable to provide satisfactory understanding of their toxic impacts to living organisms and therefore, simple, sensitive and effective approach is needed to assess the biological impacts.

A number of techniques for the determination of heavy metal toxicity using enzyme inhibition have been reported because of their rapid and economic approach (Gayet et al., 1993; Kamtekar et al., 1995; Zhang et al., 2000; Konckiet al., 2006; Szydłowska et al. 2006; Berezhetskyy et al., 2008; Park and Kim, 2013). Durrieu et al., 2002; Durrieu et al., 2003; Chouteau et al., 2005; Tekaya et al., 2013). The enzymes targeted in these studies included alkaline phosphatase (ALP) (Durrieu et al., 2002; Durrieu et al., 2003; Chouteau et al., 2005; Tekaya et al., 2013), acetylcholinesterase (Chouteau et al., 2005), and esterase (Blaise et al., 1998; Franklin et al., 2001a; Franklin et al., 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).

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 (Figure 2.1). 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 research group of Heineman pioneered the use of PAPP substrate for ALP in immunoassays with electrochemical detection (Tang et al., 1988). 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 (Wijayawardhana et al., 1999a). 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, an electrochemical enzyme inhibition test using purified ALP on the basis of micro-droplet hydrodynamic voltammetry, and the results from other published articles.

2.2. Materials and methods

2.2.1. 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 (1,000 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 microfiber filter (5 mm in diameter with a pore size of 0.7 μm) was purchased from Whatman™. All reagents were of analytical grade, and the solutions were

prepared using Milli-Q water (resistivity ≥ 18.2 MΩ cm^{-1}, Direct-Q 3 UV).

Hydrodynamic amperometry was performed using RDE with an electrochemical analyzer (ALS-1200, Bioanalytical Systems Inc., IN, USA). A glassy carbon (GC) disk electrode (6 mm outer diameter, 3 mm inner diameter) (Bioanalytical Systems Inc.), a silver wire (0.7 mm diameter), and a platinum wire (0.5 mm diameter) were used as the working, reference, and counter electrodes, respectively. Prior to use, the GC disk electrode was polished sequentially with 0.3 and 0.05 μm alumina paste and then rinsed well with distilled water.

2.2.2. Test algae

A wild-type green algae strain of *C. reinhardtii* (C-239: UTEX-90, mt+) was used in these experiments. The stock cultures of algae were stored at room temperature on an agar medium. Subsamples were transferred to a flask containing 100 mL of Sager and Granick 1 (SG1) medium. The cell suspensions were incubated for 72 h and maintained at a temperature of 24 ± 2 °C under fluorescent lamps (light intensity of 64 μmol photon m^{-2} s^{-1}) (FL20SS-D/18, Mitsubishi, Tokyo, Japan). After pre-cultivation, the cells were transferred to a phosphate-free SG1 medium (m-SG1) at an initial density of 1.0×10^4 cells mL^{-1} and incubated for a further 72 h under the same conditions to induce the ALP. ALP activity is negatively correlated with phosphate concentration (Huang and Hong, 1999).

2.2.3. Electrochemical determination of ALP activity

A schematic diagram of the detection system is given in Figure 1, which shows the RDE and enzymatic/electrochemical reactions. A total of 4 mL of *C. reinhardtii*

suspension-induced ALP were passed through a filter placed on Parafilm® (Pechiney Plastic Packaging Co., IL, USA) covering a glass slide located on a thermostatically controlled warm plate (KM-1, Kitazato Science Co. Ltd., Shizuoka, Japan). The temperature of the plate was maintained at 37 °C to encourage ALP activity (Copeland et al., 1985). Forty μL of 0.1 M Tris-HCl buffer solution were then placed on the filter as the reaction medium. A droplet was sandwiched between the RDE and Parafilm surface. The micro-droplet formed by the strong surface tension was used as the reaction vessel for electrochemical detection.

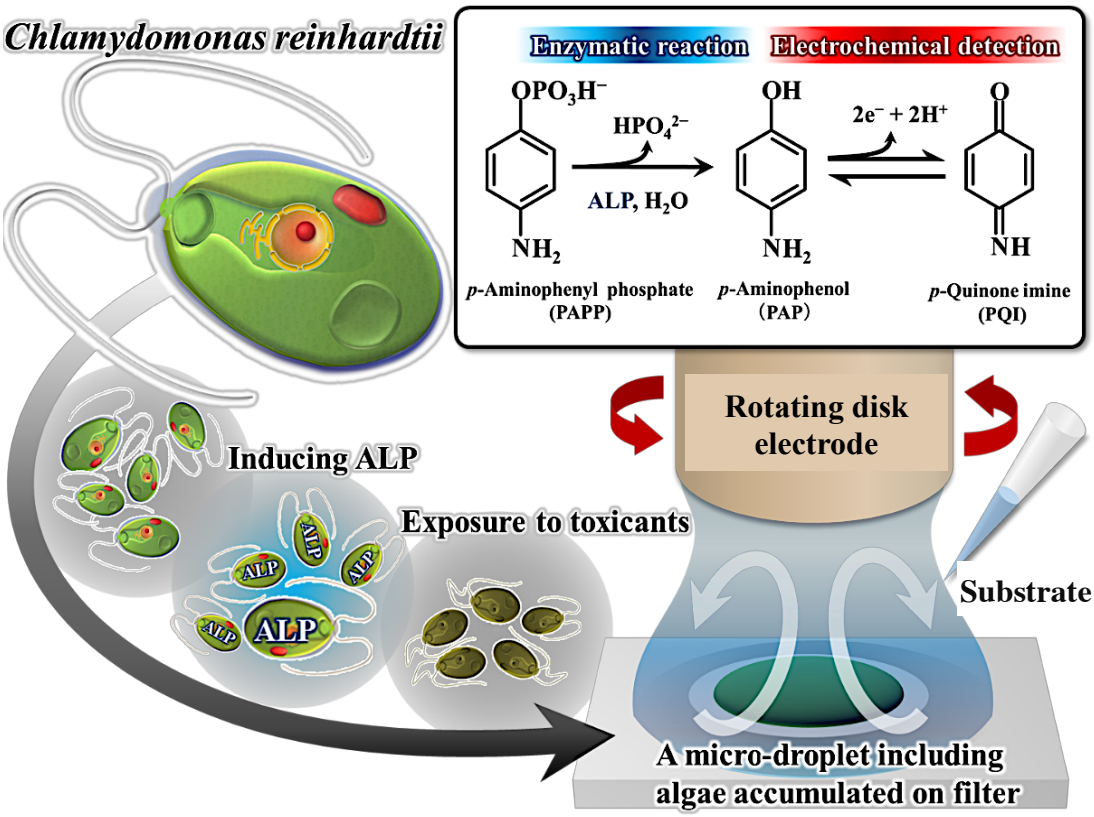


Figure 2.1. Schematic diagram of the microalgal bioassay based on ALP with hydrodynamic electrochemical detection using RDE in a micro-droplet.

Hydrodynamic chronoamperometry was performed by applying a potential of 350 mV at a rotation rate of 3,000 rpm. After an equilibration time of 5 s, the current

was measured to establish a baseline. After 20 s, 10 μL of 5 mM PAPP solution were injected into the droplet by using a micropipette, and the current was recorded for a further 60 s. To calculate the reaction rate, the slope recorded between 50 and 60 s was corrected by subtracting the background slope taken between 10 and 20 s.

2.2.4. Toxicant exposure procedure with test algae

To determine the enzyme inhibition produced by heavy metals, *C. reinhardtii*-induced ALP (1×10^4 cells mL^{-1}) was exposed to m-SG1 medium dosed with metal at concentrations ranging from 1×10^{-8} g L^{-1} to 1×10^{-2} g L^{-1}. After incubation for 24 h under the same conditions as those used to induce the enzyme, the rate of enzyme inhibition was compared with that from a blank test by using the hydrodynamic chronoamperometry data described in the previous section.

2.2.5. Electrochemical enzyme inhibition test using purified ALP

To strengthen the comparison, the inhibition of purified ALP by heavy metals was also examined using micro-droplet hydrodynamic voltammetry based on RDE. Prior to amperometric detection, which was performed as described in Section 2.3, the purified ALP (0.016 U mL^{-1}) was incubated with the metal solution for 24 h.

2.2.6. Procedure for the μ-AGI test

The μ-AGI tests were performed following our previous study (Sazawa et al., 2013). The initial cell density of *C. reinhardtii* was 1×10^4 cells mL^{-1}. The algae cells were counted using a hemocytometer (Fuchs-Rosenthal, Sunlead Glass Co., Tokyo, Japan). Two-fold dilution series of heavy metals to 100 mg L^{-1} were prepared in the wells of a 96-well round-bottom polystyrene micro-plate (Falcon, Franklin Lakes, NJ, USA). In

each well, 100 μL of the heavy metal solution, 50 μL of the five-fold concentrated SG1 medium, 50 μL of sterilized Milli-Q water, and 50 μL of 5×10^4 cells mL^{-1} *C. reinhardtii* suspension were mixed. The absorbance of the *C. reinhardtii* was measured using a spectrophotometric micro-plate reader (Model 550, Bio-Rad, Hercules, CA, USA) at 655 nm before and after incubation at 24 ± 2 °C under fluorescent lamps for 96 h. The relative growth amount of the algae was calculated using the following formula:

$$RGA\ (\%) = \frac{\{Abs._{655}(96h) - Abs._{655}(0h)\}}{\{Control\ Abs._{655}(96h) - Control\ Abs._{655}(0h)\}} \times 100$$

The values of the half-maximal effective concentration (EC_{50}) were calculated using EcoTox-Statics version 2.6 d (Yoshioka, 2001), which is distributed by the Japan Society for Environmental Toxicology.

2.3. Results and discussion

2.3.1. Investigation of electrochemical behavior of PAP and PAPP

The hydrodynamic electrochemical behavior of PAP and PAPP in a micro-droplet was investigated. Within the droplet, the substrate PAPP was converted into PAP by the enzymatic reaction and then further oxidized to PQI. The electrochemical oxidation of PAPP should have no effect on the oxidation of PAP because the PAPP molecules are at a much higher concentration than the PAP molecules. Hydrodynamic linear sweep voltammograms were obtained from measurements performed at a scan rate of 100 mV s^{-1} with a rotation rate of 3,000 rpm in 50 μL of 0.1 M Tris-HCl, including 1 mM PAP or PAPP (Figure 2.2 (A)).

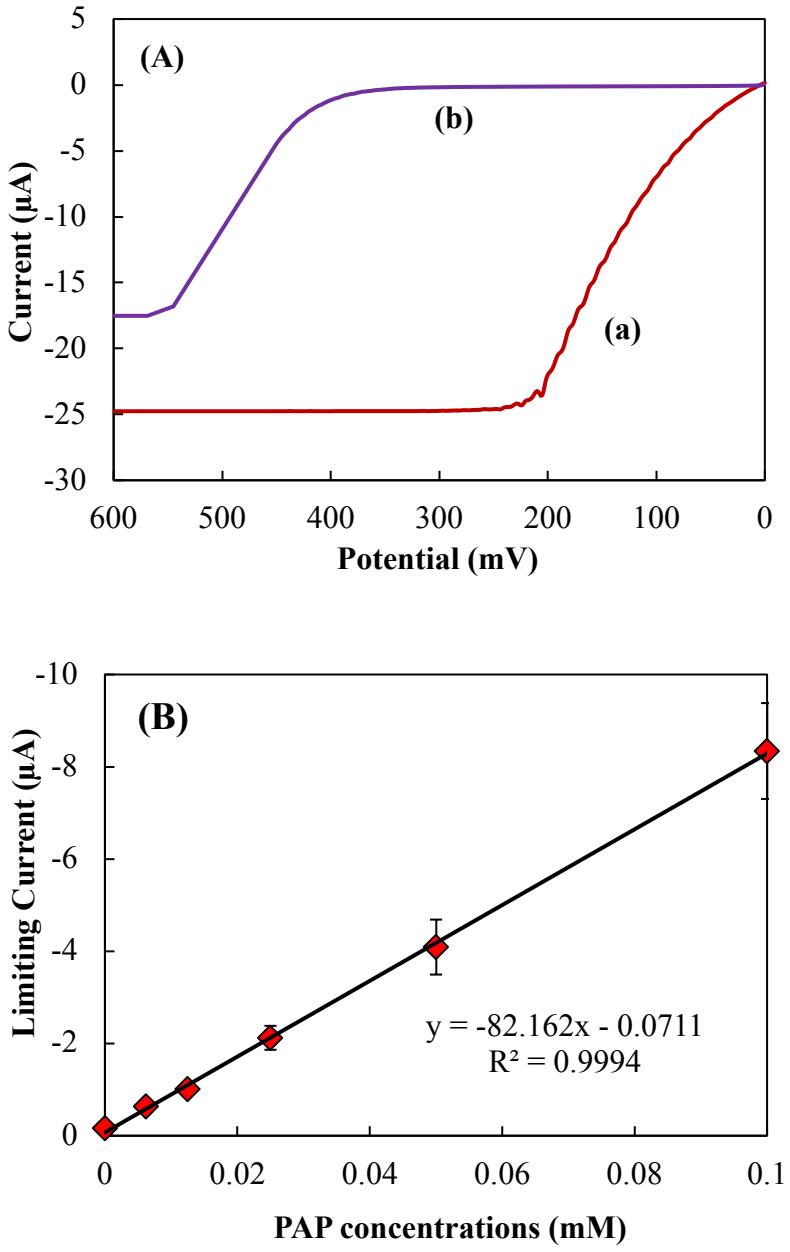


Figure 2.2. (A) Hydrodynamic linear sweep voltammograms of (a) 1 mM PAP and (b) 1 mM PAPP in 0.1 M Tris-HCl buffer solution (pH 8.4). (B) The limiting current of PAP measured by hydrodynamic linear sweep voltammograms at concentrations ranging from 0 to 0.1 mM. The measurements were performed at a scan rate of 100 mV s^{-1} and a rotation rate of 3,000 rpm in 50 µL of 0.1 M Tris-HCl droplet. Error bar represents standard deviation (n=3).

The mass-transfer limited current, i.e., the Levich current, for the oxidation of PAP and PAPP were observed at potentials greater than 200 and 550 mV, respectively. These results suggested that the oxidation current of between 250 and 400 mV for PAP was not influenced by the presence of PAPP. Therefore, a potential of 350 mV was chosen for the hydrodynamic chronoamperometric measurements used to determine the enzymatic activity.

Figure 2.2 (B) shows the results of PAP electrochemical response obtained by hydrodynamic linear sweep voltammetry at concentrations ranging from 0 to 0.1 mM. A linear relationship exists between the mass-transfer limiting current and the corresponding PAP concentrations. The limit of detection (LOD) (3σ) was estimated at 2.0×10^{-7} M ($R^2 = 0.999$). This value is lower than that found when detection is conducted without rotation (10.4×10^{-7} M). Thus, it can be said that the application of RDE system based on hydrodynamic voltammetry is useful strategy for sensitive measuring a small volume of PAP solution.

2.3.2. Growth behavior of C. reinhardtii in different culture medium

In order to obtain the best possible enzymatic activity from *C. reinhardtii*, the optimum growth time for algae was determined. The growth behavior of algae incubating in both SG1 and mSG1 medium was measured. A minute volume of algal cells was added with these medium from the stock cultures. Algal growth curves were measured up to 80 h at an interval of 8 h by counting the number of algae using hemocytometer. A 10 μL aliquot of algal solutions with glutaraldehyde were placed on the hemocytometer chambers and covered with thin cover glass, and finally positioned on microscope for counting. The results are showing in the Figure 2.3.

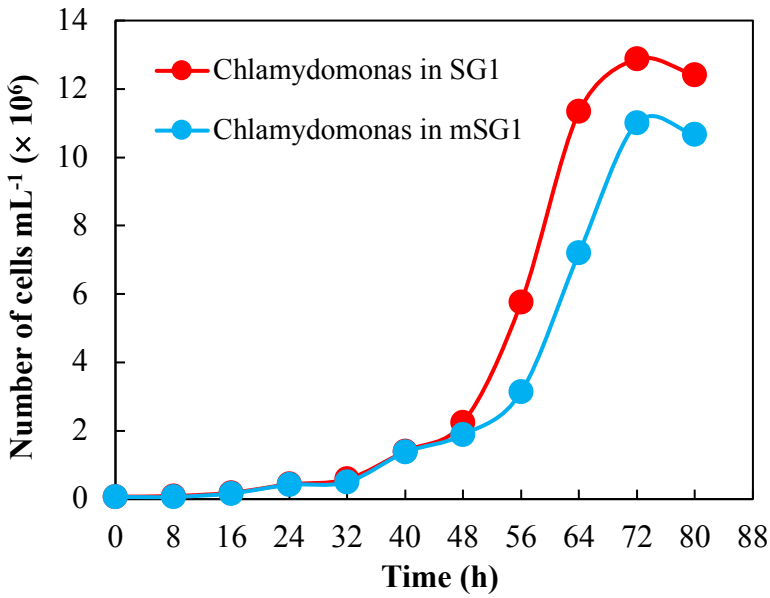


Figure 2.3. Growth behavior of *C. reinhardtii* in SG1 and mSG1 medium. The incubating time was ranges from 0 to 80 h.

The results illustrate that the algal growth in both medium up to 48 h was not very rapid expressing the lag phase of growth. After this time interval, a sharp increase of cell number was observed in the logarithmic phase and at 72 h, it reached the stationary phase. In this phase algae reached their maximum number and after that it will start decreasing. Therefore, in our study, 72 h was chosen as an optimum time for *C. reinhardtii* incubation.

The turbidity of algal cell suspensions was measured along with cell number counting to establish the relationship between cells mL^{-1} and turbidity. This relationship will help to know the cell count by measuring only the turbidity. The same cell suspension was used to measure both the turbidity and cell counting. Six mL of algal solution incubated in SG1 medium was taken for the measurement. The calibration curve of cell density vs. turbidity (Figure 2.4) was very close to a straight line with R^2

value 0.998 showing a very good linear relationship between cell density and turbidity of *C. reinhardtii.*

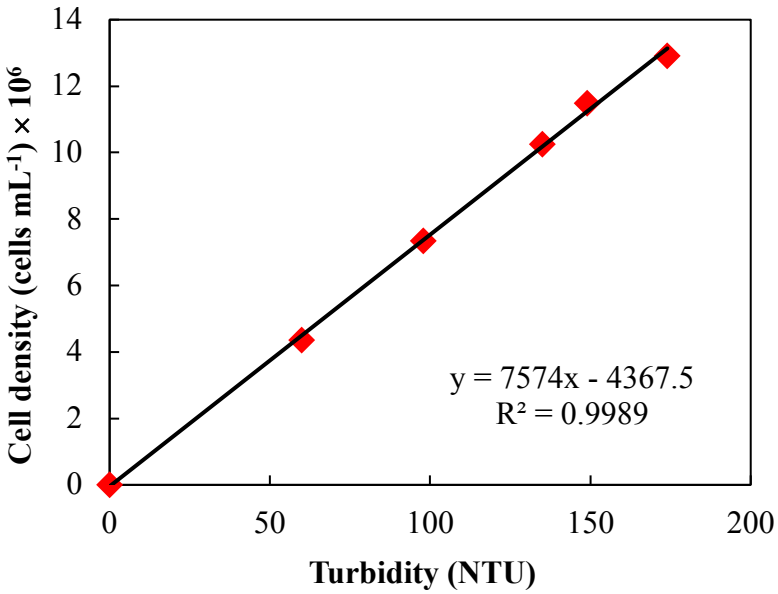


Figure 2.4. The relationship between cell density and turbidity of *C. reinhardtii.*

2.3.3. Inducing ALP activity of C. reinhardtii

Several previous studies have reported the presence of various phosphatases in *C. reinhardtii* (Lien and Knutsen, 1972; Lien and Knutsen, 1973a; Lien and Knutsen, 1973b; Loppes and Matagne, 1973; Loppes, 1976; Matagne et al., 1976; Patni and Dhawale, 1977). There have been discrepancies in the number and type of phosphatases reported, but all reports have indicated the presence of ALPs. The ALP activity of the *C. reinhardtii* after incubation in the SG1 and m-SG1 media was evaluated using RDE-based hydrodynamic chronoamperometry, with droplets containing 3.46×10^6 cells of *C. reinhardtii*. The results are shown in Figure 2.5. No enzymatic activity was observed when *C. reinhardtii* was incubated in the SG1 medium (curve a in Figure 2.5 (A)), whereas significant responses were obtained from algae cultivated in the m-SG1

medium. These results were attributed to the electrochemical oxidation of PAP produced from PAPP by ALPs. In the m-SG1 medium, an increase in the current value was found with increasing cultivation time. This increase continued up to 72 h (curve d), followed by a decrease (curve f).

The ALP activity of *C. reinhardtii* with the inducing period in m-SG1 was estimated from the rate of PAP production. This activity was obtained by the velocity values and by applying Levich's equation:

$$I_l = 0.620 n F A C D^{2/3} \nu^{-1/6} \omega^{1/2}$$

where I_l is the mass-transfer limited current, n is the number of electrons involved in the reaction, C is the analyte concentration, ν is the kinematic viscosity of the fluid, and ω is the angular velocity of the disk ($2\pi \times$ rpm). The results are presented in Figure 2.5 (B) and suggest that constant enzymatic activity was occurring in the inducing period from 72 h to 96 h. The average induced ALP activity per cell was calculated as 1.3×10^{-9} U. Therefore, the incubation time for the induction of ALP was set to 72 h in the following experiments.

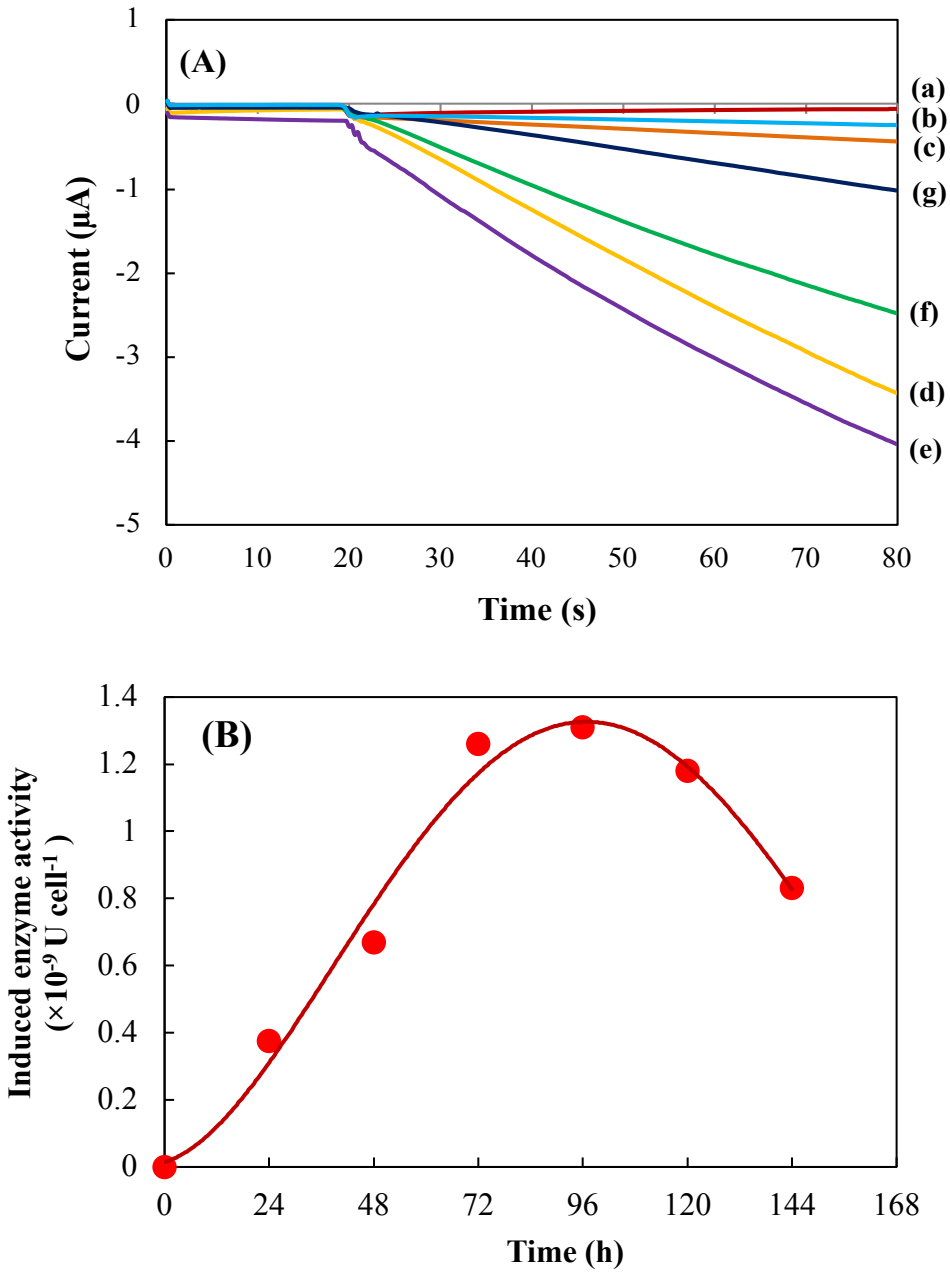


Figure 2.5. The optimal inducing period for ALP activity of *C. reinhardtii*. (A) Chronoamperograms obtained from 3.46 × 10^6 cells of *C. reinhardtii* in 50 μL of 0.1 M Tris-HCl buffer solution including 1 mM PAPG. The measurements were performed by the RDE system at 350 mV applied potential with a rotation rate of 3,000 rpm. *C. reinhardtii* was cultivated in (a) SG1 medium and m-SG1 for (b) 0, (c) 48, (d) 72, (e) 96, (f) 120, and (g) 144 h. (B) The relationship between ALP activity per cell and the cultivation time in m-SG1.

2.3.4. Evaluation of the ALP activity of C. reinhardtii using RDE

The optimal number of algal cells for determining ALP activity using the micro-droplet RDE system was investigated. Different volumes of algae solution with a density of 8.65×10^5 cells mL^{-1} were filtered, and the enzymatic activity was measured. Figure 2.6 shows phosphatase activity by cell numbers.

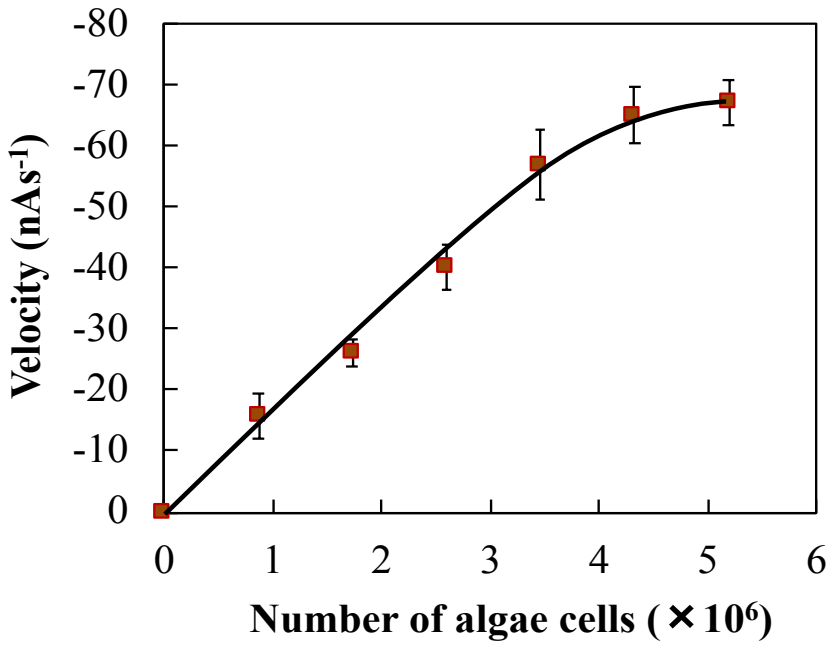


Figure 2.6. The optimal algal cell number for ALP activity. Measurements were conducted in a 50 μL micro-droplet using the RDE system at 350 mV applied potential with a rotation rate of 3,000 rpm.

The velocity values calculated from the chronoamperograms increased in line with the cell numbers, reflecting ALP activity. The increase in enzymatic activity showed a good linear relationship with the number of algae cells up to 3.46×10^6 cells (4 mL of algae solution) and then leveled off. This result was attributed to the high

concentration of algal cells on the filter surface, which prevented the effective mixing of the substrate and restricted its reaction with the enzyme. On the basis of the results, 3.46×10^6 cells (4 mL of algae solution) were chosen as the optimal number of algal cells for use in the subsequent experiments. The LOD of the algae cell number was calculated as 4.3×10^2 cells, with an enzyme activity of 5.4×10^{-7} U.

In RDE measurements, the optimization of electrochemical detection parameters such as rotation rate and substrate concentration are important. Therefore, the role of rotation rate and substrate concentration on detection of enzyme activity by using RDE were investigated. The hydrodynamic amperometric responses of 3.46×10^6 *C. reinhardtii* cells with different concentrations of PAPP were measured at rotation rates of 0, 1000, 2000, and 3000 rpm. The results confirmed that the rotation rate played a clear role in determining the ALP activity (Figure 2.7 (A)). This result demonstrated the benefits of rotation. High rotation rate gives high current value and above 3,000 rpm the droplet sometimes was broken. Based on that result, 3,000 rpm was considered as the optimum for the whole measurement process.

The responses were fitted using the Michaelis–Menten equation ($v = v_{max}$ [PAPP]/ (K_M + [PAPP])). At all rotation rates, the velocity became close to steady-state above a PAPP concentration of 1 mM. Therefore, the optimal PAPP concentration for hydrodynamic chronoamperometry was determined to be 1 mM. The Michaelis–Menten constant (K_M) and the maximum velocity (v_{max}) obtained at 3000 rpm from the Hanes–Woolf plots (Figure 2.7 (B)) were 0.26 mM and 0.66 μmol min^{-1}, respectively (Table 2.1). Without rotation, a K_M of 0.34 mM and a v_{max} of 0.24 μmol min^{-1} were obtained. The findings suggest that the hydrodynamic amperometry with an RDE

system using micro-droplet has shown promising potential to be an effective technique in evaluating the enzyme activity of algae.

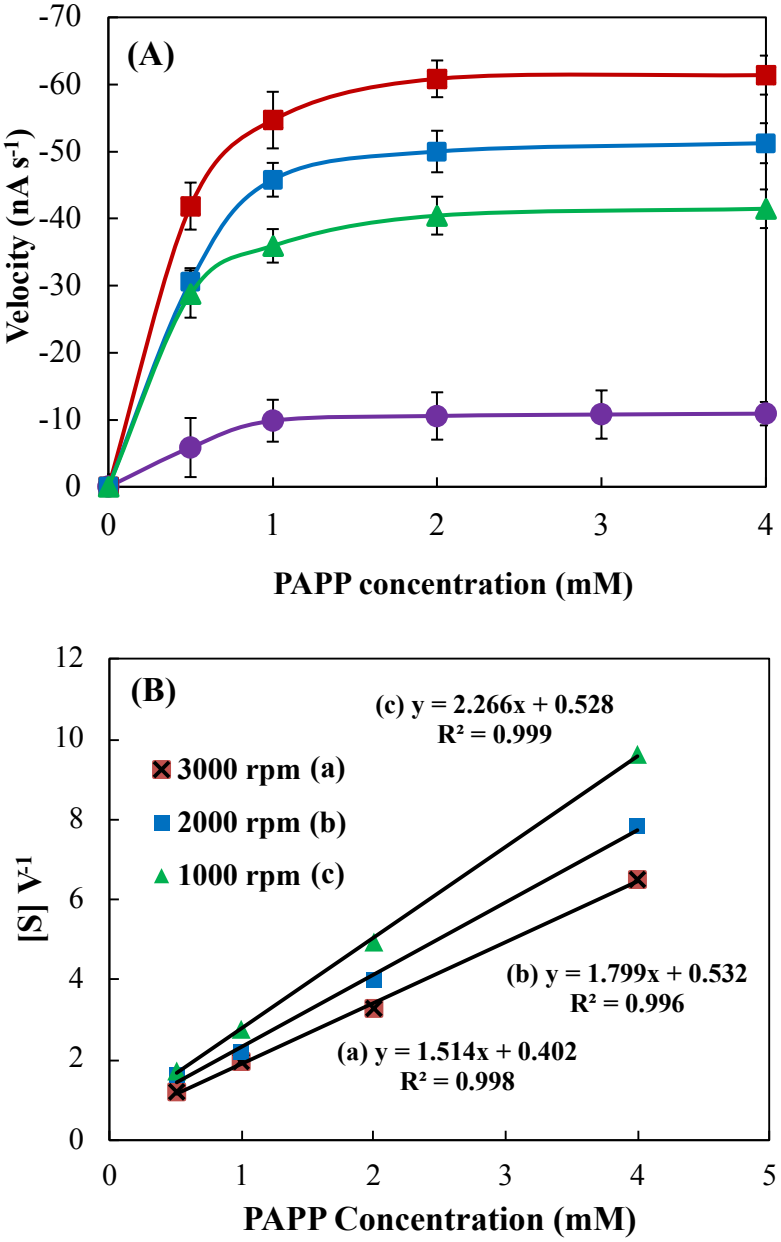


Figure 2.7. (A) Effect of rotation rate and substrate concentration on RDE measurement. Measurements were conducted for 3.46×10^6 cells with different PAPP concentrations at an applied potential of 350 mV and rotation rates of 0 (○), 1000 (▲), 2000 (□), and 3000 rpm (×). (B) Hanes-Woolf plots of alkaline phosphatase in *C. reinhardtii.*

Table 2.1. The maximum velocity (v_{max}) and Michaelis-Menten constant (K_M) in the case of electrode rotated from 1000-3000 rpm.

Rotation rate (rpm)	V_{max}	K_M (nM)
1000	0.44	0.23
2000	0.56	0.29
3000	0.66	0.26

2.3.5. Optimization of exposure time of C. reinhardtii to toxicant

We then investigated the effect of the length of exposure to toxicants on the ALP activity induced in *C. reinhardtii*. This investigation was conducted using different concentrations of Cu(II) solution with exposures of up to 72 h (Figure 2.8).

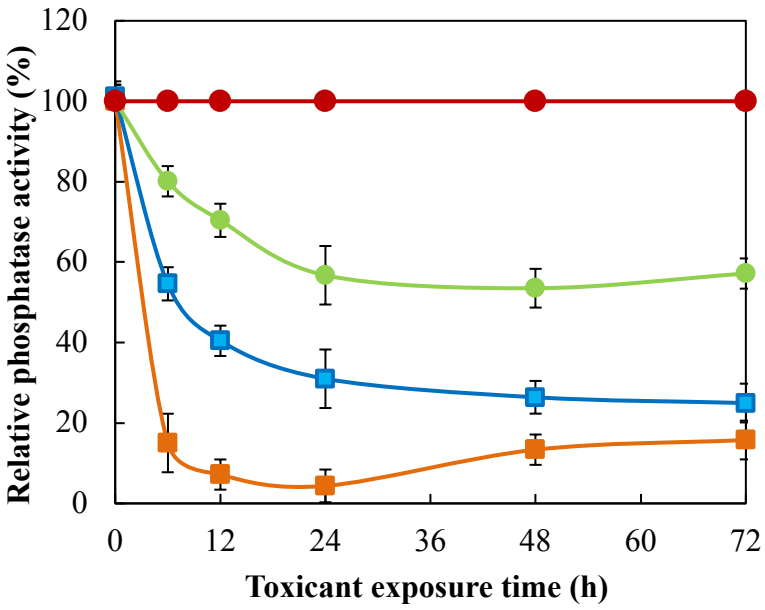


Figure 2.8. Effect of toxicant exposure time on the inhibition of ALP activity. *C. reinhardtii* was exposed to SG1 medium containing 0 (●), 1 (○), 10 (■), and 100 mg L^{-1} (□) Cu(II) for different time periods. Hydrodynamic chronoamperometric measurements were performed for 3.46 × 10^6 cells and 1 mM PAPP at an applied potential of 350 mV and a rotation rate of 3000 rpm.

At all three concentrations, the phosphatase activity decreased with increasing incubation time. The degree of inhibition corresponded to the Cu(II) concentration. At all concentrations, an exposure time of 24 h was shown to maximize inhibition, with steady states appearing beyond this time. This optimum exposure time of 24 h was lower than the 72–96 h required for the conventional AGI test (OECD, 1984). Durrieu et al. (2003) used an exposure of 24 h in their investigation of the effect of heavy metals on the ALP activity of *Chlorella vulgaris*.

2.3.6. Assay for heavy metal toxicity

Toxicity assay based on inhibition of ALP activity was demonstrated for Cu, Pb, Cd, Hg, and Zn. The enzyme activity of 3.46×10^6 cells of *C. reinhardtii* was measured using hydrodynamic chronoamperometry. Figure 2.9 shows the dose–response curves for the heavy metals tested.

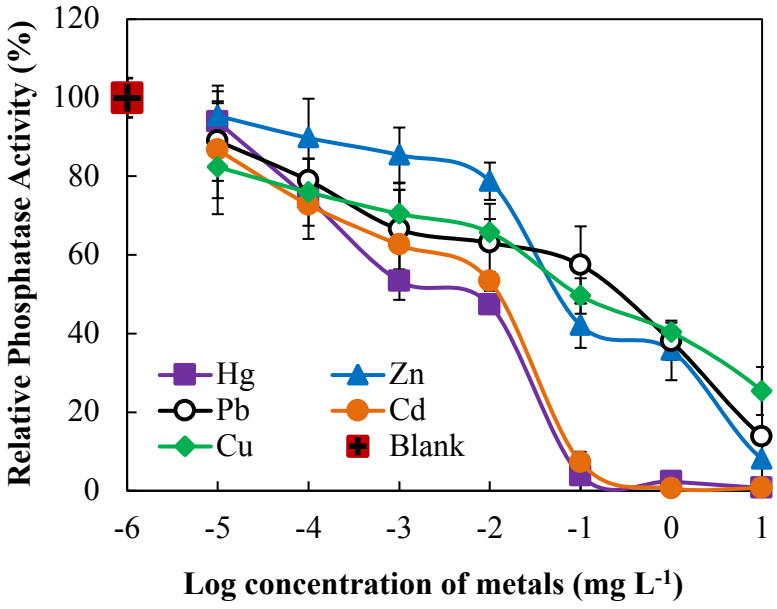


Figure 2.9. Dose–response curves for heavy metals Zn, Cu, Pb, Cd, and Hg at concentrations ranging from 1×10^{-5} to 10 mg L^{-1} using *C. reinhardtii*. The error bars represent standard errors (n = 3).

At low concentrations, the presence of heavy metals triggers the production of stress promoters in the cells, increasing enzyme activity (Mazorra et al., 2002). The EC_{50} (the metal concentration at which ALP activity was half that of the control) for Hg(II), Cd(II), Pb(II), Zn(II), and Cu(II) were calculated as 0.017 (0.0035 mg L^{-1}), 0.021 (0.0024 mg L^{-1}), 0.27 (0.056 mg L^{-1}), 1.30 (0.085 mg L^{-1}), and 1.36 (0.0865 mg L^{-1}) μM, respectively. The toxicity gradient was Hg > Cd > Pb > Cu > Zn. Durrieu et al. (2003) reported a similar order of toxicity from bioassays in which *Chlorella vulgaris* was used to detect the effect of heavy metals on ALP activity.

2.3.7. Comparison with other methods

The metal concentrations wherein ALP activity was half of that of the control were calculated, and the results were compared with those from a μ-AGI test, pure ALP enzyme inhibition, and a published study (Durrieu et al., 2003). The results are presented in Table 2.2. The first three measurements were those from the present study. The μ-AGI test used spectrophotometric detection to determine the growth inhibition of *C. reinhardtii*. The reference method was the inhibition of the ALP activity of *Chlorella vulgaris* with *p*-nitrophenyl phosphate as the substrate. Comparison of the EC_{50} values obtained from the two methods clearly showed that the proposed test has a sensitive response to heavy metals. The proposed assay and the pure ALP inhibition test both measured ALP activity inhibition. The lower EC_{50} values obtained by our proposed method established its greater sensitivity than the pure ALP assay. The wide variety of metabolic reactions to the heavy metals in the cell may have increased sensitivity to changes in the reaction medium (Teo and Wong, 2014). The present study and the research of Durrieu et al. (2003) both measured the inhibition of ALP activity by using two different species of algae. The lower EC_{50} values in our study suggested

that *C. reinhardtii* is more sensitive to heavy metals than *Chlorella vulgaris.* Previous algal toxicity bioassay studies have reported similar results for the two species (Juneau et al., 2002; Ferro et al., 2012; Pinheiro et al., 2013). Our experiments demonstrated that the proposed electrochemical bioassay method has significant advantages over existing methods in terms of both sensitivity and measurement time.

Table 2.2. Comparison of the present electrochemical bioassay using *C. reinhardtii* and other toxicity detection techniques.

Detection method	EC_{50} (μM)					References
	Hg	Cd	Pb	Zn	Cu	
Proposed bioassay	0.017	0.021	0.27	1.30	1.36	Present method
Purified ALP inhibition	0.41	0.25	1.02	1.53	14.39	Present study
μ-AGI test	2.64	8.54	7.53	29.20	36.35	Present study
Bioassay using ALP inhibition	0.95	1.51	1.11	6.73	7.39	Durrieu et al., 2003

2.4. Summary

A novel microalgal bioassay for determining heavy metal toxicity was proposed on the basis of ALP enzyme activity inhibition. *C. reinhardtii* was used as the test species, and the determination was conducted using RDE hydrodynamic electrochemical detection in a microliter droplet. The PAPP substrate was converted by the enzymatic reaction to PAP, which was in turn oxidized to PQI. The oxidation current of the PAP was measured using hydrodynamic chronoamperometry. To sustain elevated enzymatic activity, ALP was successfully induced by culturing *C. reinhardtii* in a phosphate-free

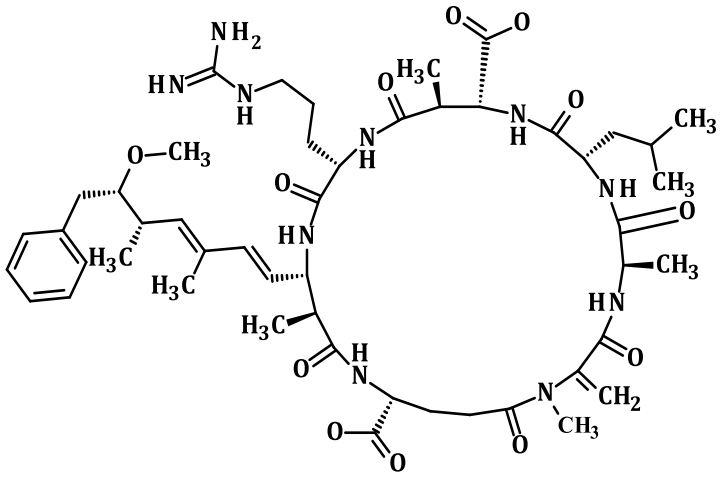
medium. The algae activated by this procedure were mounted through a filter. The enzymatic reaction was conducted in a 50 μL micro-droplet. The effective mixing produced by the RDE was shown to increase the speed of reaction with the substrate, thus allowing the procedure to be conducted with high sensitivity using few chemicals and within a 60 s reaction time. On the basis of EC_{50} values, comparative tests were conducted for the proposed method, AGI test, and ALP enzyme inhibition based on electrochemical and spectrophotometric detection. The results demonstrated the superior detection limits of the proposed technique. This assay is sensitive to heavy metals and reflects the toxic inhibition; therefore, it has toxicological significance. The electrochemical detection used in this assay allows it to be used for samples containing colored or suspended solids, with little or no sample preparation. The use of C. *reinhardtii* with this electrochemical technique offers a tool for heavy metal toxicity monitoring that is superior to existing methods in cost, testing speed, and sensitivity.

Chapter 3

Micro-droplet hydrodynamic voltammetry for the determination of microcystin-LR based on protein phosphatase

3.1. Introduction

Cyanotoxins are produced by cyanobacteria particularly in freshwater and marine ecosystem where, they are capable to form visible blooms under definite circumstances. The bloom formations become a global environmental issue having serious threat to ecological balance and human health problem due to their associated toxicity (Lin et al., 2013; Catante et al., 2015). Among the various types of cyanotoxins, microcystins (MCs) produced in freshwater ecosystem are the most common. More than 80 variants have been discovered with different structure and toxicity, where Microcystin-LR (MC-LR) is the most frequent and most lethal toxicants having LD_{50} by intraperitoneal and oral route of approximately 25-150 μg kg^{-1} and 5 mg kg^{-1} of body weight in mice, respectively (WHO, 1998). It is a potent hepatotoxins and tumor-promoters which pose a serious threat to human health in the form of chronic exposure through drinking water. (Lin et al., 2013; Tsutsumi et al., 2000). MC-LR binds with protein phosphatase type 2A (PP2A) in an irreversible approach and inhibiting their enzymatic activity, results in tumor promotions and liver cancer (Erikson et al., 1990; Campas et al., 2007).



Chemical structure of Microcystin-LR.

These potential risks directed the World Health Organization (WHO) to ascertain a guideline value for MC-LR in drinking water of 1 μg L^{-1} (equivalent to 1.0 × 10^{-9} mol L^{-1}) (WHO, 1998). Therefore, rapid, sensitive and reliable technique for MC-LR detection and quantification at a low concentration become very important issues to minimize the potential human health risk.

There are various techniques available for the determination of MCs in environmental water where no single technique can provide an accurate measure of the toxin concentration of microcystin mixture (Adamovsky, 2010). Therefore, the use of combining approach with screening and advanced quantification method is the best way for MC monitoring (WHO, 2003). Despite the potential risk of other variants of MC, toxicity tests are usually conducted based on MC-LR. The evaluation has been suggested to all the other congeners and expressing as MC-LR equivalents ($MC\text{-}LR_{eq}$). $MC\text{-}LR_{eq}$ are usually used for the expression of total MC varieties concentration in absence of others congeners information (Faassen and Lurling, 2013). Mouse bioassay was the first screening method to detect MC-LR (Campbell et al., 1994; Falconer, 1993). The commonly used methods are enzyme linked immunosorbent assays (ELISA) (Liu et al., 2012), whole cell bioassays (Sangolkar et al., 2006), protein phosphatase inhibition assays (PPIA) (Dawson, 1998; Sassolas et al., 2011a and 2011b; Covaci et al., 2012), high-performance liquid chromatography (HPLC) (Aguete et al., 2003), and liquid chromatography-mass spectrometry (LC-MS) (Rios et al., 2013) etc. where HPLC, ELISA, and LC-MS are probably the three most used methods. All of them are routinely used for MCs determination, however, varies intensely in their limit of detection (LOD). Mouse bioassay determines the lowest quantity of toxin required to exterminate a mouse and is suitable to get the sample toxicity indication. However, it was not widely accepted due to its low sensitivity, lack of consistency, and moral

allegations (Erikson et al., 1990). Lack of specific toxin identification is another disadvantage for this method. Significant research works have been carried out to find alternative techniques of mouse bioassay since last two decades for the routine monitoring of MCs and many novel and sensitive methods have become available in recent years. However, none of them are singly able to replace the mouse for the detection of all MCs congeners using a single assay, and further validation and comparison of methods is needed before general recommendations on their application can be given (Agrawal, 2012). High-performance liquid chromatography has three folds higher LOD and been extensively used for the determination of MCs in cyanobacterial biomass compare to ELISA and LC-MS (Adamovsky, 2010). The high acquisition cost is the main limitations of HPLC. Enzyme linked immunosorbent assays are very sensitive and good screening method for MCs determination in difficult matrices such as animal or plant tissue and sediment. However, researchers are concerned about the unspecific matrix influences which may prevent to get the actual information (Ernst et al., 2005; Orr et al., 2003). Besides these techniques, various number of bioassay method has been developed so far for MCs determination the bioactivity of toxins, such as potent hepatotoxicity, cytotoxicity, enzymatic activity and immunological interactions. The protein phosphatase inhibition assays are suitable substitutions for rapid detection of MC-LR because of its potency to inhibit the activities of protein phosphatases 1 and 2A (PPl, PP2A) as a similar manner to okadic acid (Loing et al., 2009; Singh et al., 2012). These assays are very sensitive up to sub-nanogram levels of MCs and allowing rapid analysis of many samples within few hours. They are sensitive than ELISA due to the provision of insight on toxicology (Catanante et al., 2015). Like MC-LR, other MC variants are also involving the potent inhibition of protein phosphatases and, therefore, PPIA has been used for the determination of

MC contamination (David et al., 2018). These assays are considered as the ideal toxicity assays for MC determination in the environmental samples (Honkanen et al, 1990; Honkanen et al, 1994; Moore et al., 2016). Protein phosphatase 2A (PP2A) was discovered as more sensitive towards MC compare to PP1 and have higher prospective to detect MC (Liang et al., 2011). The method has also been successfully used for MCs determination in drinking water before and after water treatment (Lambert et al., 1994). In addition to these techniques, colorimetric enzyme inhibition assays using colorless *p*-nitrophenyl phosphate (*p*-NPP) substrate to produce yellow colored *p*-nitrophenol (*p*-NP) are also developed by some researchers (Rivasseau et al., 1999; Bouaicha et al., 2002; Campas et al., 2005). However, these techniques require expensive instrumentation, very complex sample preparation, skilled operators, and are also time consuming. Additionally, they are not suitable for determining samples have suspended materials or coloring substances.

In this research work, we are proposing the development of toxicity test based on the inhibition of protein phosphatase enzyme activity using the micro-droplet hydrodynamic voltammetry for the determination of MC-LR. The electrochemical detection has been performed according to our previous study described in Chapter 2. The parameters for measurement of PP2A inhibition by MC-LR were optimized in this study. The results were compared with other electrochemical and colorimetric measurements.

3.2. Materials and Methods

3.2.1. Reagents

MC-LR was purchased from Sigma-Aldrich (MO, USA). MC-LR stock solution was prepared in methanol at a concentration of 25 μg mL^{-1} and subsequently diluted in a buffer solution containing 30 mM Tris–HCl, 2 mM ethylenediaminetetraacetic acid (EDTA) and 20 mM $MgCl_2$ at pH 8.4. PP2A isolated from bovine kidney was obtained from EMD Millipore (Billerica, MA, USA). The activity of the purchased PP2A solution was 800 U mL^{-1}, one unit is defined as the amount of enzyme that will release 1.0 nmol of [^{32}P] Pi from ^{32}P-labeled phosphorylase per min at 30°C. The PP2A solution was prepared immediately before using in the same Tris-HCl buffer described earlier. The enzyme is usually stored at -20°C and aliquot has been taken for solution preparation following initial thaw.

3.2.2. Apparatus

All apparatus and the electrodes are already described in the section 2.2.1 in chapter 2.

3.2.3. Electrochemical protein phosphatase assay

The electrochemical measurements were performed according to the description given in section 2.2.3. A schematic diagram of the detection system is given in Figure 3.1. A micro-droplet (40 μL) of 0.05 U mL^{-1} PP2A solution was sandwiched between the RDE and the Parafilm® covered glass slide as the reaction medium on a thermostatically controlled warm plate. The temperature of the micro-warm plate was always maintained at 37˚C.

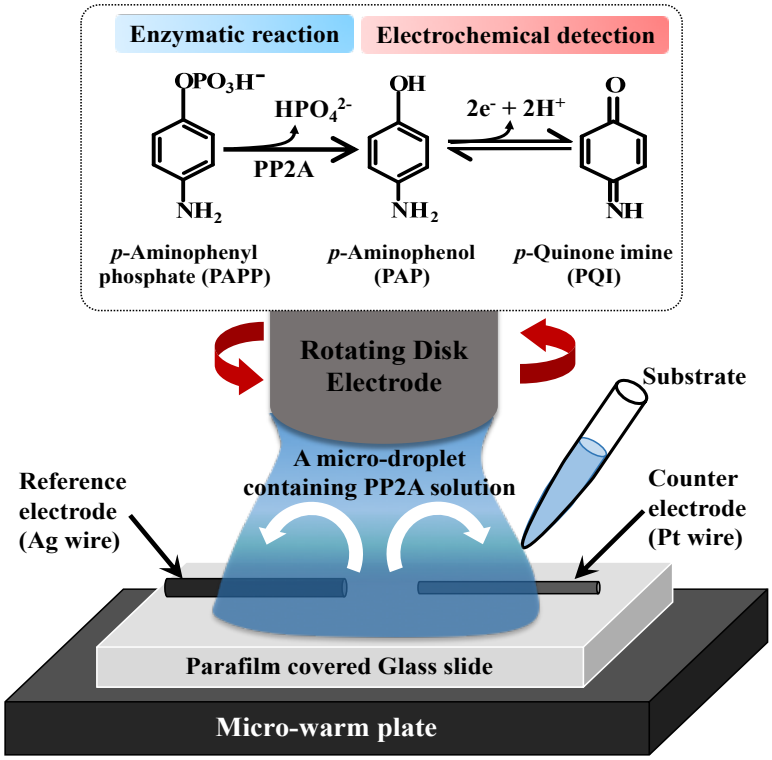


Figure 3.1. Schematic diagram of the PP2A inhibition bioassay with electrochemical hydrodynamic detection using RDE in a micro-droplet.

Chronoamperometric detection was performed by applying a potential of 350 mV at a rotation rate of 3,000 rpm. After an equilibration time of 5 s, the current was measured to establish a baseline. At 20 s, 10 µL of 12.5 mM PAPP solution were added to the droplet via micropipette, and the current recording was continued for an additional 120 s. The slope between 80 s and 90 s was corrected by deducting the background slope, taken between 10 and 20 s, for calculating the reaction rate.

3.2.4. Optimization of electrochemical parameters

For the optimization of substrate concentration, hydrodynamic amperometric responses of 0.016 U mL^{-1} PP2A with PAPP concentrations of 0 to 5.0 mM were investigated at

a rotation rate of 3000 rpm. The electrochemical measurements were done according to the previously described procedure.

3.2.5. Effect of methanol on quantitation of MC-LR

Since methanol was used to solubilize the MC-LR, the effect of different concentrations of methanol on the PP2A activity was studied. Methanol was added with the enzymatic reaction buffer (30 mM Tris/HC1, 20 mM $MgCl_2$, 2 mM EDTA, pH 8.4). The electrochemical measurements were done according to the previously described procedure.

3.2.6. MC-LR exposure procedure with PP2A

To investigate the enzyme inhibition from MC-LR, 0.05 U mL^{-1} PP2A (final concentration) was exposed with MC-LR solutions at concentrations ranging from 0 to 500 µg L^{-1}. Hundred µL of PP2A and MC-LR solution was mixed at 50:50 ratio (vol./vol.) by gentle shaking at room temperature (23 ± 3ºC) for 30 minutes to ensure that the enzyme is fully mixed with toxicant. Subsequently, the rate of enzyme inhibition was compared with that from a blank test by using the hydrodynamic chronoamperometry data described in the previous section.

3.3.7. Generation of dose–response curve for MC-LR

Standard curve for the PP2A inhibition by MC-LR was generated with 8 different concentrations in the range 0 to 500 µg L^{-1}. The half maximal inhibitory concentration (IC_{50}) against the reference value was determined from dose–response activity curves versus the log concentration.

3.3. Results and Discussion

3.3.1. PAP detection by micro-droplet hydrodynamic voltammetry

The electrochemical behavior of PAP was investigated according to the measures explained in section 2.3.1.

3.3.2. Optimization of electrochemical detection parameters for RDE measurements

The hydrodynamic amperometric responses of 0.016 U mL^{-1} PP2A with different concentrations of PAPP ranges from 0 to 5 mM were investigated for the optimization of substrate concentration and shown in Figure 3.2(A). The responses were fitted according to the Michaelis-Menten equation ($v = v_{max}$ [PAPP]/ (K_M + [PAPP])). The result shows that velocity become close to steady-state above a PAPP concentration of 2.5 mM. Therefore, the optimal PAPP concentration for hydrodynamic chronoamperometry was determined to be 2.5 mM.

Figure 3.2(B) illustrates the Hanes-Woolf plot of PP2A obtained by the hydrodynamic voltammetry. The Michaelis-Menten constant (K_M) and the maximum velocity (v_{max}) obtained from the Hanes-Woolf plots were 0.78 mM and 2.02 nA/s, respectively. The findings suggest that the hydrodynamic amperometry with an RDE system using micro-droplet has shown promising potential to be an effective technique in evaluating the enzyme activity inhibition.

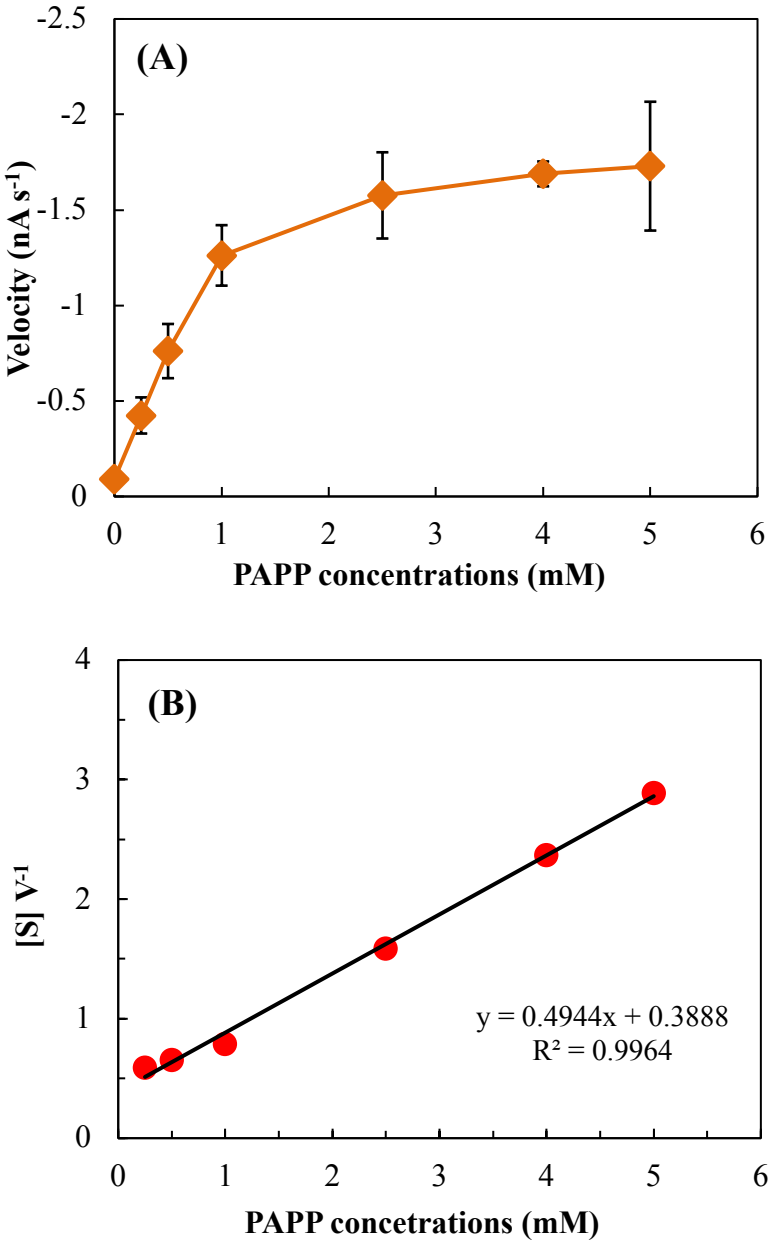


Figure 3.2. (A) Optimization of substrate concentration for RDE measurement (0, 0.25, 0.5, 1, 2.5, 4, and 5 mM PAPP), (B) Hanes-Woolf plots of PP2A. The measurements were done at 350 mV applied potential with a rotation rate of 3000 rpm. Error bar represents standard deviation (n=3).

3.3.3. Optimization of exposure time to MC-LR

To elucidate the optimal exposure time, we then investigated the effect of the length of exposure to MC-LR on the PP2A activity. This investigation was conducted using 1 μg mL^{-1} of MC-LR and 0.016 U mL^{-1} of PP2A. The solutions were mixing at 50:50 ratio (vol./vol.) by gentle shaking at room temperature (23 ± 3 ºC) for up to 50 min.

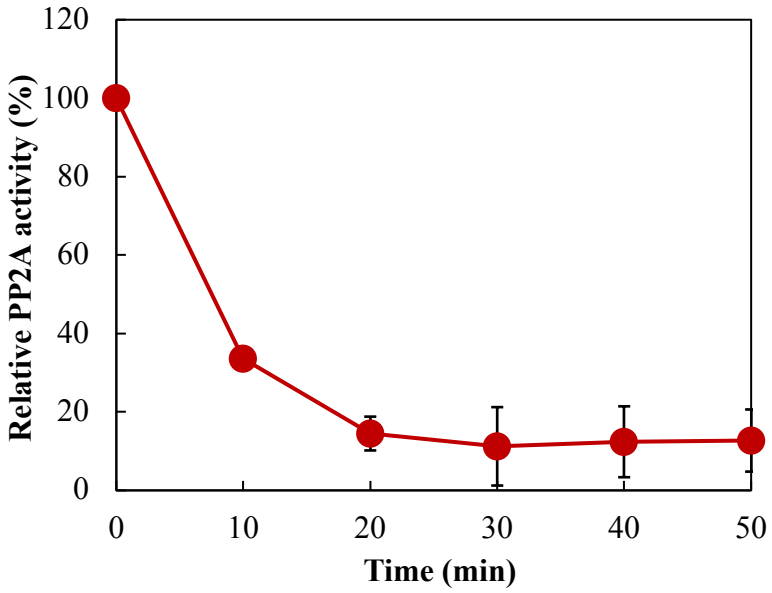


Figure 3.3. Effect of MC-LR exposure time on the inhibition of enzyme activity. PP2A was exposed to MC-LR at a concentration of 1 μg ml^{-1} for different time periods. Hydrodynamic chronoamperometric measurements were performed for 0.008 U mL^{-1} of PP2A at an applied potential of 350 mV and a rotation rate of 3000 rpm with 2.5 mM PAPP. Error bar represents standard deviation (n=3).

The electrochemical measurements were done according to the procedure described in the section of electrochemical protein phosphatase assay, and the relative PP2A activity was calculated. As presented in Figure 3.3, PP2A activity was inhibited by the added MC-LR with increasing incubation time. For the given concentration, a

steady-state inhibition value was obtained above 20 min and the time might be required for MC-LR and PP2A binding. Campàs and coworkers have used an incubation time of 30 min in their research for the detection of MC based on PP2A inhibition (Campàs et al., 2005 and 2007).

3.3.4. Effect of methanol content in the MC-LR quantitation

The effect of methanol on the PP2A activity was studied since methanol was used to solubilize the MC-LR. Methanol was added with the enzymatic reaction buffer (30 mM Tris/HC1, 20 mM $MgCI_2$, 2 mM EDTA, pH 8.4) and electrochemical measurements have been carried out.

Table 3.1. Effect of methanol content in the MC-LR concentration on the activity of PP2A.

Methanol (%)	PP2A activity	
	Mean	SD
0	100	0.19
0.5	98.19	0.28

In a study conducted by Tubaro et al. (1996) for the determination of Okadic acid using PP2A obtained almost no impact up to the ethanol concentration of 0.5%. In this study, we have tested the effect of 0.5% methanol on PP2A and the results are presented in the Table 3.1. The experiments have been carried out thrice. The results revealed that the mean PP2A inhibition for 0.5% of methanol concentration was 1.81% which is considerable and therefore, in all succeeding measurements the final methanol concentration was maintained below 0.5%.

3.3.5. MC-LR assay

Electrochemical MC-LR assay based on protein phosphatase inhibition was demonstrated. The activity inhibition of 0.05 U mL^{-1} PP2A was measured using hydrodynamic chronoamperometry. The dose-response curve exhibits a typical sigmoidal response, is given in Figure 3.4. The IC_{50} and the limit of detection (LOD) was calculated as 0.08 µg L^{-1} and 0.045 µg L^{-1}, respectively. Those values are lower than the values (1.40 µg L^{-1} and 0.90 µg L^{-1}, respectively) obtained by Campàs et al. (2005) using colorimetric inhibition assay.

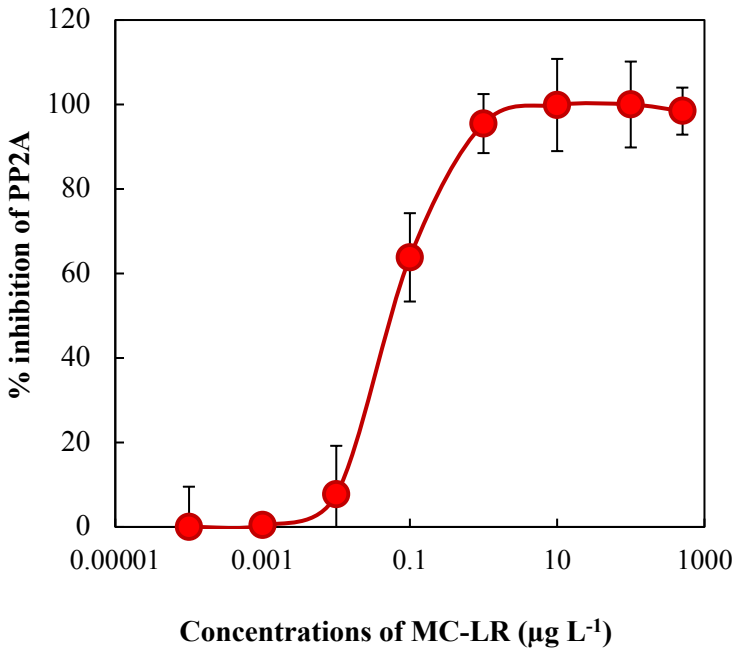


Figure 3.4. Dose–response curve for MC-LR. Inhibition is expressed as percentage of the control (no microcystin). Error bar represents standard deviation (n=3).

The value is well correlated with the LD_{50} value measured by mouse bioassays (Yao et al., 1998). Thus, the PP2A inhibition assay could be a suitable alternative of mouse bioassay to evaluate MC-LR toxicity in water samples. The IC_{50} value was

calculated and compared with other electrochemical and colorimetric determinations are given in Table 3.2. Comparison among these studies revealed that the PP2A inhibition assay has a sensitive response to MC-LR. The present assay and the study conducted by Campàs et al. both measured PP2A activity inhibition (Campàs et al., 2007). The lower IC_{50} values for our study settled its greater sensitivity than the immobilized PP2A. This might be due to the reduction in reaction rates for immobilized enzyme when enzymes cannot mix properly with the substrate. In case of RDE, rotation causes effective mixing of enzymes and substrates which produces sensitive response. The colorimetric determinations are showing high sensitivity towards MC-LR. However, the lower IC_{50} value obtained in the proposed study established it as more sensitive method than those colorimetric determination. Our experiments demonstrated that the proposed enzyme inhibition assay using hydrodynamic chronoamperometry has advantages over the reported methods in terms of both sensitivity and measurement time.

Table 3.2. Comparison of the proposed assay with other electrochemical and colorimetric techniques.

Techniques	Detection method	IC_{50} (μg L^{-1})	References
PP2A inhibition assay	Hydrodynamic chronoamperometry	0.08	Proposed method
PP2A inhibition biosensor	Cyclic voltammetry, chronoamperometry	83.0	Campàs et al., 2007
PP2A inhibition biosensor	Absorbance measurement	1.40	Campàs et al., 2005
PP2A inhibition assay	Measuring color production of *p*-NP	0.25	Bouaïcha et al., 2002

In our previous study, the effects of the conditions commonly found in drinking water supply systems on the immunoassay for ovalbumin based on the hydrodynamic amperometric detection of PAP were investigated (Kuramitz et al., 2012). The assay was unaffected for pH 6.5 and 9.5, water hardness (558 mg L^{-1} Ca^{2+} and 550 mg L^{-1} Mg^{2+}), 10 mg mL^{-1} total iron, 14 mg L^{-1} phosphate, and total organic content (20 mg L^{-1} fulvic acid and humic acid) in water. Therefore, the MC-LR assay might be applicable for both environmental waters and drinking water.

3.4. Summary

We have developed electrochemical protein phosphatase 2A enzyme inhibition assay to quantify low levels of MC-LR in water. The electrochemical determination was conducted by hydrodynamic chronoamperometry in a 50 μL micro-droplet using rotating disk electrode. The PAPP substrate used in the study was converted into PAP by the enzymatic activity of PP2A. Chronoamperometric detection was used to measure the oxidation current of PAP. The results confirmed that the proposed method is suitable to detect very low concentration of MC-LR. The IC_{50} calculated from the study is 0.08 μg L^{-1} which is well below the WHO standard for drinking water. The comparative study with other colorimetric and electrochemical techniques demonstrated the superior detection limits of the proposed method. The assay is sensitive to MC-LR and reflects the toxic inhibition. The effective mixing by RDE increases the reaction speed, consequently achieving the procedure to be conducted with high sensitivity using fewer chemicals. Therefore, the method can be attractive for microcystin contaminated drinking water routine assessment.

Chapter 4

Conclusions

The classical chemical analytical techniques for environmental risk assessment measure the absolute concentrations of pollutants present in the environmental water. However, these techniques are unable to provide toxicity level of the pollutants. Bioassays are useful technique for assessing the acute and chronic effects of hazardous chemicals. Enzyme activity inhibition assays using electrochemical RDE detection is advantageous over optical methods in terms of simplicity, sensitivity, and speedy determination. It was the main objective of this study to develop new enzyme activity inhibition-based assay using electrochemical RDE detection that are capable to detect toxicity of various pollutants present in the environmental water, independent from the influence of colored components and suspended solids, and can be used for pollution monitoring. Establishing RDE detection as an effective determination method appeared most important to me in order to provide a technical means to study the enzyme activity for toxicity measurements.

In this thesis, I have established two different enzyme inhibition assays to quantify aquatic toxicity generated by various pollutants. In the first part of the research, I have presented the inhibition of ALP enzyme activity in microalgae *C. reinhardtii* using micro-droplet hydrodynamic voltammetric determination for heavy metals toxicity testing. ALP activity was induced in *C. reinhardtii* by phosphate starvation method. Enzyme activity was determined using RDE on 50 µL of droplet containing *C. reinhardtii* accumulated through filtration before and after the heavy metal exposures. A clear diminution of the enzyme activity was found for the samples exposed with heavy metals, an expression of inhibitor effect. The toxicity of five different heavy metals including Cu, Zn, Pb, Cd, and Hg was successfully detected. The results demonstrated that this assay is sensitive to heavy metals and reflects the toxic inhibition and can be applicable for other heavy metals. In the second part of my thesis, I presented

the determination the development of toxicity test based on the inhibition of protein phosphatase enzyme activity using the micro-droplet hydrodynamic voltammetry for the determination of MC-LR. The electrochemical detection has been performed according to the method described in the first part.

Thus, this is the first time, RDE as a simple, rapid, and sensitive technique has been applied for the toxicity determination using enzyme activity inhibition. The hydrodynamic voltammetry using RDE allows to evaluate the toxicity within very short time, thereby significantly reducing the assay time, as compared with the conventional methods. The determination was done with fewer chemicals and little or no sample pretreatment. These approaches also have great potential for the investigation of pesticides, organic pollutants, and antibiotics etc. I expect that the developed methods might be useful for environmental monitoring and drinking water routine analysis. The work presented thus far has provided a foundation for the use of RDE for toxicity evaluation of pollutants present in environmental water.

The use of ALP enzyme in microalgae was successfully used for heavy metals toxicity determination. However, the experiments using the mixed pollutants and real samples were not carried out. Before using in critical sensing applications, validation of their performance capabilities is required. There are various issues including the reproducibility, the ability to discriminate different types and concentrations of chemical agents, study using mixed pollutants, and the practicality in realistic applications need to be addressed.

Protein phosphatase 2A enzyme inhibition assay determines the MC-LR level in the sample. However, a variety of other MC analogs including MC-RR, MC-YR, and MC-LA, which also inhibit PP2A, are co-present in the environmental water,

leading to positive error of the MC-LR concentration. Therefore, further experiments should be conducted with MC analogs as well as okadaic acid as other PP2A inhibitor. The real samples, such as lake and river water, should also be tested to verify the applicability of the established method. The development of appropriate pretreatment procedures may be required for real sample analysis.

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