electrochem

MDPI

Article

Rotating Droplet Hydrodynamic Electrochemistry for Water Toxicity Bioassay Based on Electron-Transfer Mediator

Kazuto Sazawa [1], Yeasna Shanjana [1,2], Kazuharu Sugawara [3] and Hideki Kuramitz [1,*]

[1] Department of Natural and Environmental Sciences, Faculty of Science, Academic Assembly, University of Toyama, Gofuku 3190, Toyama 930-8555, Japan; sazawa@sci.u-toyama.ac.jp (K.S.); yeasna101@gmail.com (Y.S.)
[2] Department of Education, Wilkes University, Wilkes Barre, PA 18766, USA
[3] Department of Life Engineering, Maebashi Institute of Technology, Maebashi 371-0816, Japan; kzsuga@maebashi-it.ac.jp
* Correspondence: kuramitz@sci.u-toyama.ac.jp; Tel.: +81-76-445-6669

Abstract: An electrochemical bioassay based on rotating droplet electrochemistry by using an electron-transfer mediator was developed for the evaluation of a wide variety of pollutants such as antibiotics, heavy metals, and pesticides in the water environment. Ferricyanide was used as an electron-transfer mediator for obtaining the catalytic response of *Escherichia coli*. The electrochemical response of *E. coli* was measured via hydrodynamic chronoamperometry in a microdroplet on a screen-printed carbon electrode (SPCE). The constructed electrode system successfully evaluates the catalytic response of *E. coli* solution in the presence of ferricyanide. An assay for antibiotic toxicity on *E. coli* was carried out. The EC_{50} for ampicillin, sulfamonomethoxine, chlorotetracycline, tetracycline, and oxytetracycline evaluated by the pre-incubation method were 0.26, 0.77, 5.25, 18.5, and 19.0 μM, respectively. The toxicity order was ampicillin > sulfamonomethoxine > chlorotetracycline > tetracycline > oxytetracycline. The proposed method can be used to evaluate the antibiotic toxicities in different real samples, such as pond water, powder, and raw milk. Recoveries were found in the range of 90 and 99%. The developed methods do not require additional incubation time to evaluate toxicity.

Keywords: electrochemical bioassay; mediator; *Escherichia coli*; hydrodynamic voltammetry; antibiotics

Citation: Sazawa, K.; Shanjana, Y.; Sugawara, K.; Kuramitz, H. Rotating Droplet Hydrodynamic Electrochemistry for Water Toxicity Bioassay Based on Electron-Transfer Mediator. *Electrochem* **2024**, *5*, 370–379. https://doi.org/10.3390/electrochem5030024

Academic Editor: Masato Sone

Received: 17 June 2024
Revised: 9 August 2024
Accepted: 26 August 2024
Published: 19 September 2024

1. Introduction

Antibiotics are biologically active substances used extensively in human and veterinary medicine as therapeutics or growth promoters [1]. A large proportion of antibiotics (50–90%) are excreted from the body in an unchanged form in urine, feces, and manure, eventually reaching the aquatic environment via sewage treatment plants, soil amendments, or organic fertilizers used in agricultural fields [2,3]. In addition, antibiotics may directly reach the water environment through the wastes of pharmaceutical plants and hospitals [4]. Thus, antibiotic contamination has been commonly detected in wastewater, surface water, groundwater, seawater, and living organisms [5–9]. This causes the development of antibiotic resistance genotypes of bacteria [10] and the fatal risk to the indigenous microorganisms [11] and threatens the ecosystem and human life. In addition, their sustained release to the various environmental compartments produces complex toxicities after mixing with other chemical substances in the environment. Therefore, there is a great concern in monitoring the overall antibiotic toxicities in the water environment by using appropriate analytical methods. Chemical analysis methods are used to determine the absolute concentration of a known single pollutant. However, these methods are not suitable for evaluating the overall toxicities in the water environment.

Consequently, a bioassay has been developed to determine the overall toxicities in the environment using fish [12], animal cells, luminescent bacteria [13], algae [14,15], and microorganisms [16–21]. Microorganisms have been extensively used in bioassays because

of their high sensitivity towards toxins, and easy manipulation [22–29]. Furthermore, microorganisms are primary producers of the ecosystem and exhibit similar physiological responses to higher organisms [30]; thus, the toxic impact analysis on microorganisms helps us to know the influence on higher organisms by illuminating any small changes in water quality [31,32].

There are many benefits of electrochemical detection techniques in bioassays, such as relatively simple, sensitive, rapid, and inexpensive methods. In addition, it is applicable to evaluate the toxicities in color samples with suspended solids containing analytes. In several recent works, the electrochemical bioassay using redox mediators has been developed to evaluate toxicity [33–37]. The mediators are involved in microbial respiration and accept electrons from the respiratory chains [38]. Hence, the currents of reduced mediators reflect the microbial activity. In response to a toxicant, microbial activity is disturbed which can be easily evaluated as a deviation away from the currents produced by healthy cells. This approach enables a rapid and reliable screening for the evaluation of toxicity. Liu et al. proposed a direct toxicity assessment based on chronoamperometry using *E. coli* cultured with ferric cyanide, and evaluated the toxicity of 3,5-dichlorophenol (IC50 8.0 mg L^{-1}). Moreover, the findings suggest that this technique can be extended to assess the toxicity of other compounds, including KCN and As_2O_3, with IC_{50} values of 4.9 mg L^{-1} and 18.3 mg L^{-1}, respectively. However, the results indicate that heavy metal ions, such as Cu^{2+}, Pb^{2+}, and Ni^{2+}, did not exhibit any notable toxicity towards *E. coli.* [34]. Yu et al. developed a rapid and highly sensitive toxicity bioassay utilizing *E. coli* as the test organism and *p*-benzoquinone as the artificial electron mediator [39]. Four heavy metal ions were tested as model toxicants, and the corresponding IC_{50} were determined to be 0.95, 8.14, 11.69 and 42.76 mg L^{-1}, respectively.

The aim of this study is to develop a new electrochemical bioassay based on rotating droplet electrochemistry using an electron-transfer mediator. Ferricyanide was used as the electron-transfer mediator due to its fast electron transfer and reversible redox reaction, high membrane permeability, and low toxicity. It is approximately 50,000 times more water soluble than oxygen. This allows for concentrated microbial cell suspensions to be used without ferricyanide restricting respiration. The mediator participates in the respiration chain of *Escherichia coli* by accepting the electron in periplasmic space and oxidizing at the electrode surface as shown in Figure 1. Thus, the oxidation current of the mediator can reflect the microbial activity. In the presence of pollutants, the capacity of the central function of cellular metabolism is disturbed, causing a change in current flow compared to the healthy cells. With respect to the transduction mechanism, electrochemical methods such as amperometry and bulk electrolysis have been in use for many years in microbial toxicity testing because of their high sensitivity and wide detection range. In amperometric detection for the oxidation of ferrocyanide on the electrode surface, a diffusion-limited current is obtained that is quantitatively related to its concentration. However, amperometric detection does not provide a direct real-time view of ferricyanide reduction kinetics because it relies on interfacial mass transport processes. The technique proposed in this study which enables the hydrodynamic amperometric analysis in a microliter volume of sample solution, the rotating disk electrode (RDE), exhibits dual functions, such as being used to efficiently blend the sample solution and concurrently acting as an electrochemical detection device. The RDE promotes the convective mass transport to the surface of the electrode because of the fast rotation speed. The result causes effective mixing that reduces the incubation time for the biochemical reaction. The advantages acquired from RDE detection in a microdroplet (40 µL) reduced the diffusional distance, which results in shorter assay times, rapid detection, and, therefore, faster assay times. In addition, another benefit of using microdroplets is the decrease in dilution of the sample, which causes lower detection limits [40,41]. Thus, using RDEs for detecting the electrochemical reaction in the microliter sample solution is of great interest. To the best of our knowledge, this is the first report on the development of a toxicity assessment method based on a hydrodynamic electrochemical measurement using RDE for microdroplet sample containing microorganisms suspended.

One of the advantages of this method is that no additional incubation time is required to detect toxicity. In this study, we optimized and investigated the catalytic response of *E. coli* in the presence of a mediator by using hydro-dynamic chronoamperometry. In addition, the toxicity of various antibiotics and their recovery from different matrixes were tested using this test method. Based on these results, we aimed to show that this electrochemical assay can contribute to the rapid and simple toxicity evaluation of antibiotics present in drinking and environmental water containing colored and suspended components.

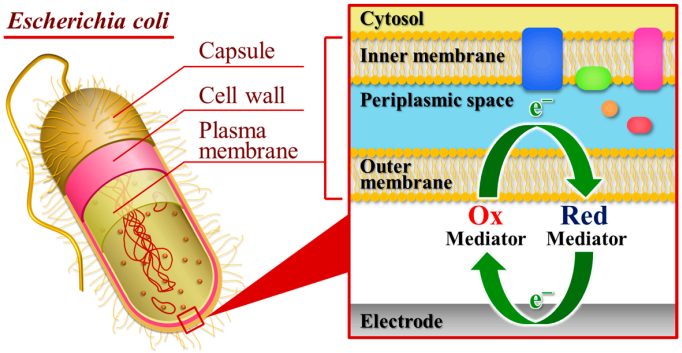

Figure 1. Principle of electrochemical bioassay using mediator.

2. Materials and Methods

2.1. Reagent and Solution Preparation

Potassium hexacyanoferrate (III) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in a phosphate buffer solution. Ampicillin, tetracycline, sulfamonomethoxine, chloroteracycline, and oxytetracycline were obtained from Sigma-Aldrich (St. Louis, MO, USA). The phosphate buffer solution (PBS, pH 7.4) contained 0.044 M NaH_2PO_4 and 0.056 M NaH_2PO_4. All reagents used were of analytical grade, and the water was sterile deionized water. Bacto tryptone was purchased from Difco Company (Detroit, MI, USA). The Luria–Bertani (LB: 10.0 g/L bacto tryptone, 5.0 g/L yeast extract, and 10.0 g/L NaCl) broth was sterilized in high-pressure steam at 120 °C for 20 min.

2.2. Culture, Maintenance, and Harvesting the E. coli

Escherichia coli K-12 strain was used as a bacterial stain. *E. coli* K-12 was cultured on nutrient agar plates at 4 °C and replicated regularly to confirm its viability. A 200 mL solution of autoclaved broth was inoculated with a colony of *E. coli* and then incubated anaerobically at 37 °C for 5 h to reach the stationary phase. Later on, the microorganisms were harvested by centrifuging at 3000 rpm for 30 min and re-suspended in PBS. The solution was diluted 10-fold with PBS buffer and the absorbance value was checked at 600 nm (OD_{600}) by using a UV-2450 UV/Vis detector (Japan Spectroscopic Company Co., Tokyo, Japan). The bacterial suspension was used for the experiments on the day of harvesting.

2.3. Instrumentation and Selection of Electrodes

Hydrodynamic amperometry with a rotating disk electrode (RDE) was carried out using an electrochemical analyzer (ALS-1200, Bioanalytical Systems Inc. (BAS) (West Lafayette, IN, USA)). The thick-film screen-printed electrochemical micro-device was printed onto a PVC pad with a size of 3.4 × 1.0 × 0.05 cm (DRP-110, Metrohm DropSens, Oviedo, Spain) and embedded into a solvent-resistant three-electrode system, like reference, working, and counter electrodes. The glassy carbon, carbon, and Ag/AgCl of screen-printed electrodes were used as working, counter, and reference electrodes, respectively. The box connector (DSC, Metrohm DropSens) was used to obtain the electrochemical interface between the RDE, SPCE, and the potentiostat. Before starting the measurement, the surface area of the glassy carbon disk electrode was polished, by consecutively adding 0.3 and 0.05 µm alumina paste and later washed well with water.

2.4. Procedures of Experimental Equipment Settings and Electrochemical Detection

The RDE was placed perpendicular to the screen-printed electrode and contacted at a distance of 2.5 mm. The *E. coli* solution (40 µL) was kept in the center position between the SPCE and the RDE. The optimum potential for the ferricyanide (300 mV) for electrochemical oxidation to ferrocyanide was applied to the electrode, and the rotation rate was fixed at 3000 rpm. The assay for antibiotic toxicity on *E. coli* was demonstrated by a pre-incubation test method. The measurement was carried out with 40 µL of *E. coli* and a toxicant mixture, and 30 s later, 10 µL of mediator solution was added. In this test method, the antibiotic was first added during the incubation of *E. coli*. The hydrodynamic chronoamperograms were recorded for 300 s (Figure 2).

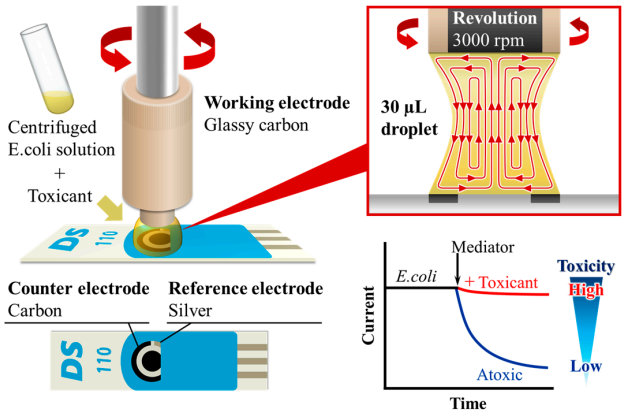

Figure 2. The experimental set-up for the hydrodynamic electrochemistry for water toxicity bioassay using an electron-transfer mediator, and the hydrodynamic chronoamperograms is expected to be obtained.

3. Results and Discussion

3.1. Investigation of Catalytic Response of E. coli

Figure 3 demonstrates the catalytic response of *E. coli* in the presence of a ferricyanide mediator by using hydrodynamic chronoamperometry. In comparison with the baseline current, a sharp increase in response current after adding the ferricyanide (1 mM) with the *E. coli* was observed at 300 s. The obtained current response for the oxidation of ferrocyanide to ferricyanide was increased to 20 µA. This result indicated that ferricyanides were mediated by the catalytic response of *E. coli*. The current response was measured at higher µA levels and even at higher concentrations of ferricyanide (45 mM; nA current) reported in another study [42]. This might be due to the high rotation rate of RDEs which allows for the rapid mass transfer [43] to the electrode surface.

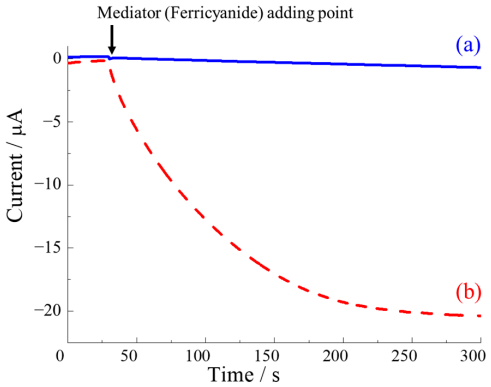

Figure 3. The catalytic response of *E. coli* in the presence of a ferricyanide mediator by using hydrodynamic chronoamperometry. The hydrodynamic chronoamperograms for 1 mM ferricyanide with (a) PBS buffer and (b) *E. coli*.

3.2. Effect of E. coli on Electrochemical Detection

The effect of *E. coli* on the electrochemical detection was evaluated by the hydrodynamic linear sweep voltammetry (Figure 4). A total of 40 µL of droplets including 10 µL of ferrocyanide with *E. coli* or PBS buffer were measured with different rotation rates of 500, 1000, 1500, 2000, 2500, and 3000 rpm at a scan rate of 100 $mV \cdot s^{-1}$. Levich's equation [40] was used to find the relationship between mass-transfer limited current for the oxidation of ferrocyanide and the rotation of angular velocity. The equation is as follows:

$$i_l = 0.620nFAC_0D^{2/3}\nu^{-1/6}\omega^{1/2} \quad (1)$$

where i_l is the mass-transfer limited current, n is the number of electrons involved in the reaction, C_0 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 limiting currents showed a positive linear relationship with the square root of ω in the presence of *E. coli*. This indicates that the electrochemical response of *E. coli* depends on the convective mass transport of the mediator resulting from the hydrodynamic flow caused by RDEs. The limiting currents also showed a similar positive linear relationship with the square root of ω in the absence of *E. coli*. This indicates that the electrochemical reaction of the mediator is not influenced by the high concentration of *E. coli*. Therefore, *E. coli* did not affect the electrochemical reaction of the mediator.

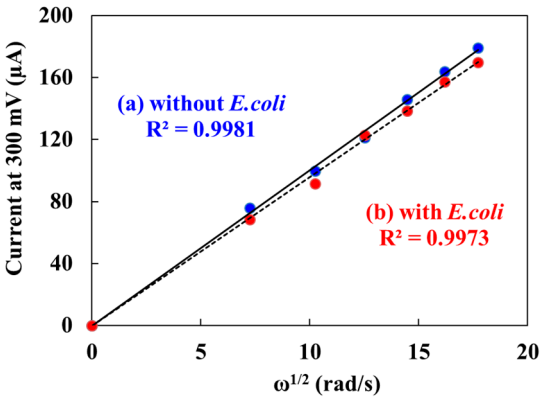

Figure 4. Correlation between the mass-transfer limited current for the oxidation of ferrocyanide and the rotation of angular velocity ($\omega^{1/2}$). The hydrodynamic linear sweep voltammograms were obtained in a microdroplet solution comprising 10 µL ferrocyanide with 40 µL buffer solution (a) or buffer solution containing *E. coli* (b).

3.3. Effects of Mediator Concentration on E. coli Catalytic Response

Figure 5 shows the results for demonstration of the hydrodynamic chronoamperograms obtained from different concentrations of ferricyanide. Each concentration of ferricyanide (10 µL) was injected into 40 µL of *E. coli* solution. There was an increment of anodic current with increasing the concentration of ferricyanide added into the droplet solution containing *E. coli*. This result was found within only 300 s. The increase in the catalytic response of *E. coli* was observed with the increment of concentration of ferricyanide. When the concentration of ferricyanide was increased to 20 mM, there was more ferricyanide transforming into ferrocyanide and oxidized at the electrode surface. As a result, the greatest response current was observed at high concentrations of ferricyanide. On the other hand, at a minimum concentration of 0.25 mM of ferricyanide, the smallest response current was detected, indicating that the catalytic response of *E. coli* depends on the concentration of ferricyanide which was consistent with a previous report [42].

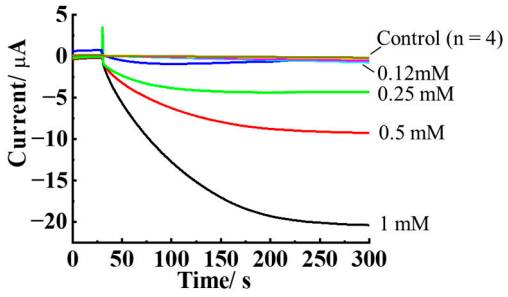

Figure 5. The hydrodynamic chronoamperograms obtained from different concentrations of ferricyanide at 0.12, 0.25, 0.5, and 1.0 mM. Each concentration of ferricyanide (10 μL) was injected into 40 μL of *E. coli* solution (20×10^9 cells/mL). The measurements were performed by the RDE system at 300 mV applied potential with a rotation rate of 3000 rpm.

The hydrodynamic amperometric responses of *E. coli* with different concentrations of ferricyanide were superbly fitted by using the Michaelis−Menten equation:

$$v = v_{max}\,[S]/(K_M + [S]) \quad (2)$$

The Michaelis–Menten constant (K_M) and the maximum velocity (v_{max}) acquired from the Hanes–Woolf plots as shown in Figure 6 was 0.27 mM at a rotation rate of 3000 rpm. This clearly indicated that the hydrodynamic amperometry with the RDE system is applicable in investigating the catalytic activity of *E. coli* for performing the toxicity test. In addition, the catalytic response of *E. coli* was even observed at a low concentration of ferricyanide.

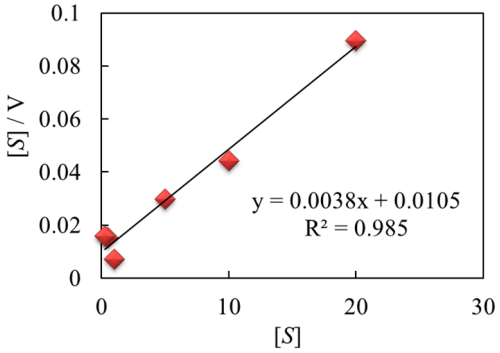

Figure 6. Hanes–Woolf plots of ferricyanide in *E. coli* solution obtained by the RDE system at 300 mV applied potential with a rotation rate of 3000 rpm.

3.4. Effects of E. coli Concentration on Catalytic Response

The relationship between catalytic response and the concentrations of *E. coli* was investigated as shown in Figure 7. The increase in current mediated by ferricyanide was observed with the increment in the cell densities of *E. coli*. When the cell density of *E. coli* increased to 20×10^9 cells/mL, there was more *E. coli* to show the catalytic response. As a result, the greatest response current was observed at high call densities of *E. coli*. On the other hand, at a minimum cell density of 1.25×10^9 cells/mL of *E. coli*, the smallest response current was detected, indicating that the catalytic current depends on the *E. coli* cell densities. In addition, the proposed method can detect the catalytic response at a low concentration of *E. coli* solution.

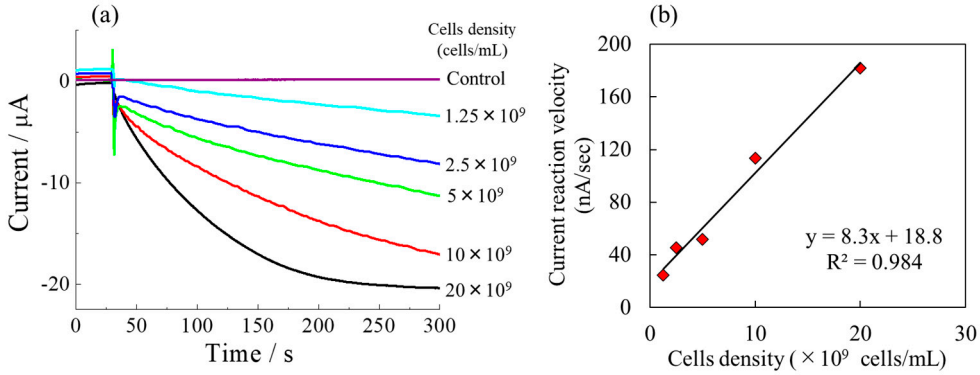

Figure 7. (**a**) The hydrodynamic chronoamperograms for ferricyanide exposed to 40 μL of *E. coli* with different cell densities of 1.25 × 10^9, 2.5 × 10^9, 5 × 10^9, 10 × 10^9, and 20 × 10^9 cells/mL. The measurements were carried out by the RDE at an applied potential of 300 mV. (**b**) Relationship between the current reaction velocity and the cell density of *E. coli*.

3.5. Evaluation of Toxicities of Antibiotics on E. coli

Five common antibiotics—ampicillin, tetracycline, sulphamonomethoxin, chlorotetracycline, and oxytetracycline—were selected for toxicity testing using the methods proposed in this study. The dose–response curves of antibiotics obtained by using the proposed pre-incubation method are shown in Figure 8. The electrochemical response of *E. coli* is decreased resulting in a deviation of current flow compared to the unaffected cells. This change in current is considered as the bacterial inhibition of those pollutants. The EC_{50} value for ampicillin, sulfamonomethoxine, chlorotetracycline, oxytetracycline, and tetracycline was calculated as 0.26, 0.77, 5.25, 19.0, and 18.5 μM, and the toxicity order was ampicillin > sulfamonomethoxine > chlorotetracycline > tetracycline > oxytetracycline.

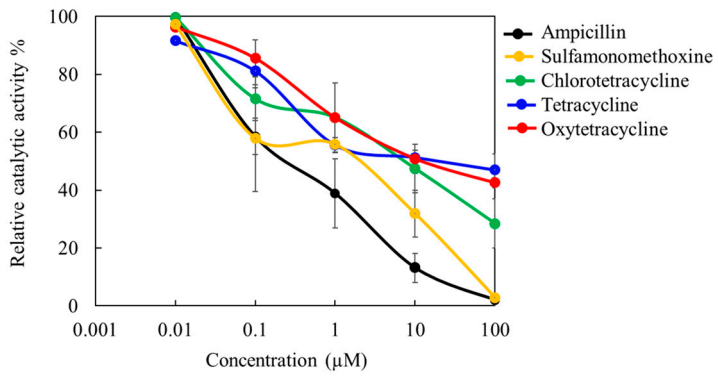

Figure 8. The dose–response curves for 5 antibiotics (ampicillin, chlorotetracycline, tetracycline, oxytetracycline, and sulfamonomethoxine). The measurements were performed by the RDE system at 300 mV applied potential with a rotation rate of 3000 rpm.

3.6. Mean Recovery Test

Antibiotics have been detected in milk and environmental water. In order to evaluate the possible analytical applications of the proposed toxicity test, 1 or 10 μM of tetracycline was evaluated in different matrixes, such as powder milk, pond water, and raw milk. The powdered milk and formula were purchased from a local supermarket. The pond water was taken from a pond on the university campus. The obtained results are shown in Table 1. The concentrations of tetracycline detected in milk powder, pond water, and raw milk were consistent with the concentrations added. In addition, a recovery test was performed to check the accuracy of the proposed method. As shown in Table 1, the average recovery rate was 90–99% in all cases. Relative standard deviation (RSD) values were calculated as (S × 100)/x, where S is the standard deviation and x is the mean of the data (n = 3). These

observations indicate that the proposed method can accurately and reproducibly detect antibiotics in milk and surface water.

Table 1. Recovery study of tetracycline in different matrixes.

Matrix	Additional Concentration of Tetracycline (μM)	Found Concentration (μM)	Recovery (%)	RSD (%)
Pond water	1	0.97	97	2.1
	10	9.61	96	3.8
Milk	1	0.90	90	10.0
	10	9.02	90	10.0
Powder milk	1	0.99	99	0.2
	10	9.27	93	7.7

4. Conclusions

The results presented in this study confirm that the electrochemical bioassay based on rotating droplet electrochemistry successfully evaluates the catalytic response of *E. coli* in the presence of ferricyanide. The pre-incubation test method can evaluate the toxicities of antibiotics in a wide variety of real samples with good accuracy. Because the proposed method did not require additional incubation time, it can evaluate the water toxicity within a short period of time.

Author Contributions: Conceptualization, H.K.; methodology, K.S. (Kazuto Sazawa) and H.K. (Hideki Kuramitz); investigation, K.S. (Kazuto Sazawa), Y.S. and H.K.; resources, K.S. (Kazuto Sazawa), K.S. (Kazuharu Sugawara), and H.K.; writing—original draft preparation, Y.S.; writing—review and editing, K.S. (Kazuto Sazawa), K.S. (Kazuharu Sugawara), and H.K.; visualization, K.S. (Kazuto Sazawa), Y.S. and H.K.; supervision, K.S. (Kazuto Sazawa) and H.K.; project administration, H.K.; funding acquisition, H.K. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the Japan Society for the Promotion of Science (JSPS) via a Grant-in-Aid for Scientific Research (Grant JP16H02976 and 23K22894).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data is contained within the article.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Sarmah, A.K.; Meyer, M.T.; Boxall, A.B.A. A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. *Chemosphere* **2006**, *65*, 725–759. [CrossRef] [PubMed]
2. Schlüsener, M.P.; Bester, K. Persistence of antibiotics such as macrolides, tiamulin and salinomycin in soil. *Environ. Pollut.* **2006**, *143*, 565–571. [CrossRef] [PubMed]
3. Elmund, G.K.; Morrison, S.M.; Grant, D.W.; Nevins, M.P. Role of excreted chlortetracycline in modifying the decomposition process in feedlot waste. *Bull. Environ. Contam. Toxicol.* **1971**, *6*, 129–132. [CrossRef] [PubMed]
4. Lindsey, M.E.; Meyer, M.; Thurman, E.M. Analysis of trace levels of sulfonamide and tetracycline antimicrobials in groundwater and surface water using solid-phase extraction and liquid chromatography/mass spectrometry. *Anal. Chem.* **2001**, *73*, 4640–4646. [CrossRef]
5. Kolpin, D.W.; Furlong, E.T.; Meyer, M.T.; Thurman, E.M.; Zaugg, S.D.; Barber, L.B.; Buxton, H.T. Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999−2000: A national reconnaissance. *Environ. Sci. Technol.* **2002**, *36*, 1202–1211. [CrossRef] [PubMed]
6. Ye, Z.; Weinberg, H.S.; Meyer, M.T. Trace analysis of trimethoprim and sulfonamide, macrolide, quinolone, and tetracycline antibiotics in chlorinated drinking water using liquid chromatography electrospray tandem mass spectrometry. *Anal. Chem.* **2007**, *79*, 1135–1144. [CrossRef]
7. Lin, A.Y.-C.; Tsai, Y.-T. Occurrence of pharmaceuticals in Taiwan's surface waters: Impact of waste streams from hospitals and pharmaceutical production facilities. *Sci. Total Environ.* **2009**, *407*, 3793–3802. [CrossRef]

8. Dolliver, H.; Kumar, K.; Gupta, S. Sulfamethazine uptake by plants from manure-amended soil. *J. Environ. Qual.* **2007**, *36*, 1224–1230. [CrossRef]
9. Kong, W.D.; Zhu, Y.G.; Liang, Y.C.; Zhang, J.; Smith, F.A.; Yang, M. Uptake of oxytetracycline and its phytotoxicity to alfalfa (*Medicago sativa* L.). *Environ. Pollut.* **2007**, *147*, 187–193. [CrossRef]
10. Kim, S.; Aga, D.S. Potential ecological and human health impacts of antibiotics and antibiotic-resistant bacteria from wastewater treatment plants. *J. Toxicol. Environ. Health Part B* **2007**, *10*, 559–573. [CrossRef]
11. Flaherty, C.M.; Dodson, S.I. Effects of pharmaceuticals on *Daphnia* survival, growth, and reproduction. *Chemosphere* **2005**, *61*, 200–207. [CrossRef]
12. Van der Schalie, W.H.; Shedd, T.R.; Knechtges, P.L.; Widder, M.W. Using higher organisms in biological early warning systems for real-time toxicity detection. *Biosens. Bioelectron.* **2001**, *16*, 457–465. [CrossRef] [PubMed]
13. Thomulka, K.; McGee, D.; Lange, J. Use of the bioluminescent bacterium *Photobacterium phosphoreum* to detect potentially biohazardous materials in water. *Bull. Environ. Contam. Toxicol.* **1993**, *51*, 538–544. [CrossRef] [PubMed]
14. Ma, J.; Xu, L.; Wang, S.; Zheng, R.; Jin, S.; Huang, S.; Huang, Y. Toxicity of 40 herbicides to the green alga *Chlorella vulgaris*. *Ecotoxicol. Environ. Saf.* **2002**, *51*, 128–132. [CrossRef] [PubMed]
15. Jiang, H.; Islam, M.S.; Sazawa, K.; Hata, N.; Taguchi, S.; Nakamura, S.; Sugawara, K.; Kuramitz, H. Development of an electrochemical bioassay based on the alkaline phosphatase activity of to assess the toxicity of heavy metals. *Int. J. Electrochem. Sci.* **2016**, *11*, 5090–5102. [CrossRef]
16. Zhu, B.; Wu, Z.-F.; Li, J.; Wang, G.-X. Single and joint action toxicity of heavy metals on early developmental stages of chinese rare minnow (*Gobiocypris rarus*). *Ecotoxicol. Environ. Saf.* **2011**, *74*, 2193–2202. [CrossRef]
17. Nguyen-Ngoc, H.; Durrieu, C.; Tran-Minh, C. Synchronous-scan fluorescence of algal cells for toxicity assessment of heavy metals and herbicides. *Ecotoxicol. Environ. Saf.* **2009**, *72*, 316–320. [CrossRef]
18. Xiong, J.; Fu, G.; Tao, L.; Zhu, C. Roles of nitric oxide in alleviating heavy metal toxicity in plants. *Arch. Biochem. Biophys.* **2010**, *497*, 13–20. [CrossRef]
19. Liu, Q.; Cai, H.; Xu, Y.; Xiao, L.; Yang, M.; Wang, P. Detection of heavy metal toxicity using cardiac cell-based biosensor. *Biosens. Bioelectron.* **2007**, *22*, 3224–3229. [CrossRef]
20. Wang, F.; Yao, J.; Si, Y.; Chen, H.; Russel, M.; Chen, K.; Qian, Y.; Zaray, G.; Bramanti, E. Short-time effect of heavy metals upon microbial community activity. *J. Hazard. Mater.* **2010**, *173*, 510–516. [CrossRef]
21. Hassan, S.H.A.; Van Ginkel, S.W.; Kim, S.-M.; Yoon, S.-H.; Joo, J.-H.; Shin, B.-S.; Jeon, B.-H.; Bae, W.; Oh, S.-E. Isolation and characterization of acidithiobacillus caldus from a sulfur-oxidizing bacterial biosensor and its role in detection of toxic chemicals. *J. Microbiol. Methods* **2010**, *82*, 151–155. [CrossRef] [PubMed]
22. Tizzard, A.; Webber, J.; Gooneratne, R.; John, R.; Hay, J.; Pasco, N. MICREDOX: Application for rapid biotoxicity assessment. *Anal. Chim. Acta* **2004**, *522*, 197–205. [CrossRef]
23. Fulladosa, E.; Debord, J.; Villaescusa, I.; Bollinger, J.C.; Murat, J.C. Effect of arsenic compounds on *Vibrio fischeri* light emission and butyrylcholinesterase activity. *Environ. Chem. Lett.* **2007**, *5*, 115–119. [CrossRef]
24. Hernando, M.; Malato, O.; Farré, M.; Fernandez-Alba, A.; Barceló, D. Application of ring study: Water toxicity determinations by bioluminescence assay with *Vibrio fischeri*. *Talanta* **2006**, *69*, 370–376. [CrossRef]
25. Li, A.; Lee, P.Y.; Ho, B.; Ding, J.L.; Lim, C.T. Atomic force microscopy study of the antimicrobial action of Sushi peptides on Gram negative bacteria. *Biochim. Biophys. Acta-Biomembr.* **2007**, *1768*, 411–418. [CrossRef]
26. Pasco, N.; Joanne, H.; Webber, J. Biosensors: MICREDOX—A new biosensor technique for rapid measurement of BOD and toxicity. *Biomarkers* **2001**, *6*, 83–89. [CrossRef]
27. Evans, M.R.; Jordinson, G.M.; Rawson, D.M.; Rogerson, J.G. Biosensors for the measurement of toxicity of wastewaters to activated sludge. *Pestic. Sci.* **1998**, *54*, 447–452. [CrossRef]
28. Farré, M.; Pasini, O.; Carmen Alonso, M.; Castillo, M.; Barceló, D. Toxicity assessment of organic pollution in wastewaters using a bacterial biosensor. *Anal. Chim. Acta* **2001**, *426*, 155–165. [CrossRef]
29. Hsieh, C.-Y.; Tsai, M.-H.; Ryan, D.K.; Pancorbo, O.C. Toxicity of the 13 priority pollutant metals to *Vibrio fisheri* in the Microtox® chronic toxicity test. *Sci. Total Environ.* **2004**, *320*, 37–50. [CrossRef]
30. Wang, X.; Liu, M.; Wang, X.; Wu, Z.; Yang, L.; Xia, S.; Chen, L.; Zhao, J. *P*-benzoquinone-mediated amperometric biosensor developed with *Psychrobacter* sp. for toxicity testing of heavy metals. *Biosens. Bioelectron.* **2013**, *41*, 557–562. [CrossRef]
31. Stauber, J.L.; Davies, C.M. Use and limitations of microbial bioassays for assessing copper bioavailability in the aquatic environment. *Environ. Rev.* **2000**, *8*, 255–301. [CrossRef]
32. Walsh, G.E.; Merrill, R.G. Algal bioassays of industrial and energy process effluents. In *Algae as Ecological Indicators*; Schubert, L.E., Ed.; Academic Press: London, UK, 1984; pp. 329–360.
33. Liu, C.; Yong, D.; Yu, D.; Dong, S. Cell-based biosensor for measurement of phenol and nitrophenols toxicity. *Talanta* **2011**, *84*, 766–770. [CrossRef] [PubMed]
34. Liu, C.; Sun, T.; Xu, X.; Dong, S. Direct toxicity assessment of toxic chemicals with electrochemical method. *Anal. Chim. Acta* **2009**, *641*, 59–63. [CrossRef] [PubMed]
35. Yong, D.; Liu, C.; Yu, D.; Dong, S. A sensitive, rapid and inexpensive way to assay pesticide toxicity based on electrochemical biosensor. *Talanta* **2011**, *84*, 7–12. [CrossRef] [PubMed]

36. Yong, D.; Liu, L.; Yu, D.; Dong, S. Development of a simple method for biotoxicity measurement using ultramicroelectrode array under non-deaerated condition. *Anal. Chim. Acta* **2011**, *701*, 164–168. [CrossRef] [PubMed]
37. Catterall, K.; Robertson, D.; Hudson, S.; Teasdale, P.R.; Welsh, D.T.; John, R. A sensitive, rapid ferricyanide-mediated toxicity bioassay developed using *Escherichia coli*. *Talanta* **2010**, *82*, 751–757. [CrossRef]
38. Ramsay, G.; Turner, A.P.F. Development of an electrochemical method for the rapid determination of microbial concentration and evidence for the reaction mechanism. *Anal. Chim. Acta* **1988**, *215*, 61–69. [CrossRef]
39. Yu, D.; Zhai, J.; Yong, D.; Dong, S. A rapid and sensitive *p*-benzoquinone-mediated bioassay for determination of heavy metal toxicity in water. *Analyst* **2013**, *138*, 3297. [CrossRef]
40. Kuramitz, H.; Dziewatkoski, M.; Barnett, B.; Halsall, H.B.; Heineman, W.R. Application of an automated fluidic system using electrochemical bead-based immunoassay to detect the bacteriophage MS2 and ovalbumin. *Anal. Chim. Acta* **2006**, *561*, 69–77. [CrossRef]
41. Kuramitz, H.; Halsall, H.B.; Heineman, W.R. Magnetic microbead-based enzyme immunoassay for ovalbumin using hydrodynamic voltammetry and fluorometric detection. *Anal. Methods* **2012**, *4*, 1783. [CrossRef]
42. Yong, D.; Liu, C.; Zhu, C.; Yu, D.; Liu, L.; Zhai, J.; Dong, S. Detecting total toxicity in water using a mediated biosensor system with flow injection. *Chemosphere* **2015**, *139*, 109–116. [CrossRef] [PubMed]
43. Kuramitz, H.; Sazawa, K.; Nanayama, Y.; Hata, N.; Taguchi, S.; Sugawara, K.; Fukushima, M. Electrochemical genotoxicity assay based on a SOS/*umu* test using hydrodynamic voltammetry in a droplet. *Sensors* **2012**, *12*, 17414–17432. [CrossRef] [PubMed]