*Desalination and Water Treatment*
www.deswater.com

doi: 10.1080/19443994.2015.1040853

57 (2016) 10934–10940
May

Taylor & Francis
Taylor & Francis Group

# Simplification and sensitivity study of Alamar Blue bioassay for toxicity assessment in liquid media

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Received 15 December 2014; Accepted 8 April 2015

**ABSTRACT**

Bacteria, as an essential component of most wastewater treatment plants (WWTPs), play a critical role in the biological stage of WWTPs due to their role in cycling mineral compounds and in the decomposition of organic material. In this study, a modified Alamar Blue-based bioassay was used to evaluate the toxicity of metal plating wastewaters on sequence batch reactor (SBR) bacteria. For confirming the robustness, reproducibility, and efficiency of the optimized method, it was compared with the standard colony count protocol and oxygen consumption method. The influence of pH on the Alamar Blue reduction was examined over a pH range of 1.0–13.0. The optimum pH for Alamar Blue bioassay using SBR bacteria was 6.5–7. The correlation between the reduction of Alamar Blue by SBR bacteria and their oxygen consumption coefficient was 0.945. According to our optimized bioassay, Alamar Blue was rapidly reduced by active SBR bacteria. This protocol successfully assessed the toxicity of metal plating wastewaters. As most of the WWTPs are inefficient for elimination of toxic effect of metals, it is desirable to use bioassays that can determine the toxicity of such pollutants. We simplified and optimized such an assay that can be manipulated for real metal plating wastewater samples.

*Keywords:* Bioassay; Alamar Blue; Dehydrogenase enzyme activity; Heavy metals; Toxicity

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## 1. Introduction

Most of the cost-effective and standard methods for toxicity evaluation on wastewater micro-organisms use bioassays that determine the activity of some bacterial enzymes [1]. It has been shown that electron transport system (ETS) activity in wastewater sludge correlates significantly with oxygen uptake rate [2]. ETS activity in sludge samples can be evaluated by measuring the reduction of redox dyes which can efficiently compete with oxygen atoms as electron acceptors. Different standard compounds with such characteristics have been studied; the most common compounds in such fields are colorless tetrazolium salts which are soluble and when reduced by bacteria, convert to insoluble colored formazan products [3].

But tetrazolium salts are generally cytotoxic to many of micro-organisms because their products (formazan crystals) that are originated from reduction of the salts must be solubilized with dimethyl sulfoxide or hydrogen chloride/isopropanol which destroys the cells, so time-dependent experiments cannot be performed [3]. 5-diphenyltetrazolium bromide as one of the tetrazolium salts is not soluble in conventional culture medium and is best suited for manipulate with adherent cell lines [4]; however, some other tetrazolium salts (sulfonic acid hydrate) are soluble in culture medium, but these compounds are not efficiently reduced without phenazine methosulfate [4]. Alamar Blue in comparison with tetrazolium salts circumvents many of the incompatibility problems described and provided many advantages over tetrazolium salts [4].

Alamar Blue is an oxidized and non-fluorescent dye that can be reduced to the highly fluorescent and pink-colored resorufin. Based on Kreft's dichromaticity index, its solution is highly dichromatic [5]. Alamar Blue acts as an intermediate electron acceptor without interference of the normal transfer of electrons [6]. As the dye accepts electrons, its color changes from blue state to the fluorescent pink, reduced, state [6]. In addition to mitochondrial reductases, other enzymes such as the diaphorases, NAD(P)H:quinine oxidoreductase, and flavin reductase located in the mitochondria and the cytoplasm may be able to reduce Alamar Blue [4]. Hence, Alamar Blue bioassay may signify an impairment of bacterial metabolism and is not necessarily specific to interruption of mitochondrial dysfunction and electron transport [7]. For such reasons, Alamar Blue has been broadly used over the past 50 years in investigations on cytotoxicity and cell viability in a range of environmental and biological systems. It is one of the most highly referenced compounds used for viability and cytotoxicity assays. Generally, its use has been carried out to various aspects of monitoring cellular health [8,9], cytotoxicity [10], test compound toxicology in environmental risk assessments and in medicine [6], and antimicrobial susceptibility testing [11].

Since unidentified components may be present in the field samples, appropriate referencing of the sample toxicity via physical and chemical methods may not be practical. As a solution, whole sample analysis using bioindicators was proposed by the US Environment Protection Agency [12].

But to our knowledge, an Alamar Blue-based bioassay has not been simplified, so that it can be used in the small and decentralized wastewater treatment plants (WWTPs) for measure the impact of toxicants. Also to date, no study has addressed the effect of industrial effluents on the sequence batch reactor (SBR) bacterial community.

The aim of this study was to simplify and sensitivity study of Alamar Blue bioassay for toxicity assessment of metal plating industry on the SBR bacterial community.

## 2. Materials and methods

### 2.1. Preparation of bacterial culture

Freshly harvested SBR bacteria were obtained by cultivation of 250 µl of SBR aeration basin samples in nutrient broth. The dominant bacterial species in the SBR are suspected to be related to *Pseudomonas*, *Aeromonas*, *Bacillus*, *Micrococcus*, and *Nitrobacter* [13]. For keeping the sludge age in logarithmic growth phase during the period of the experiments, the harvested SBR bacteria were transferred every day to a fresh nutrient broth.

A pure sample of harvested bacteria using an aseptic preparation method and the aid of a centrifuge (6,000 rpm for 10 min) was prepared for bioassays. The bacterial pellets were then resuspended in 20 ml of sterile phosphate buffer (pH 7). The optical density of isolated bacteria in suspension was recorded at 540 nm. Serial dilutions were performed until the optical density was 1.0 ± 0.05.

### 2.2. Optimized Alamar Blue assay

Alamar Blue bioassay method of Liu [14] was simplified and used to calculate the $EC_{50}$ of the heavy metals and metal plating wastewater using SBR bacteria.

The assay mixture contained 50 µl of Alamar Blue solution at 1 g/l concentration, 500 µl phthalate–HCl buffer (pH 7), 500 µl of nutrient broth (×10), and

distilled water (pH 7) to a volume of 4 ml. Finally, this volume was increased to 5 ml by adding 1 ml of SBR harvested bacteria. As needed, pH values were adjusted to 7 by adding 1 N NaOH or 1 N HCl.

The reaction was started by adding 1 ml of freshly harvested SBR bacteria. For each separate bioassay, bacteria-free experiments were set up as controls. The final mixtures were incubated on a shaker at 120 rpm and 21˚C. After 0 and 30 min, a 1 ml sample was removed from the experiments. The bacteria were removed from the samples by vortexing and centrifugation (4 min at 10,000 rpm), and Alamar Blue reduction was measured spectrophotometrically at 600 nm. All assays were performed in duplicate and the means were determined.

In the presence of active SBR bacteria, dehydrogenase enzyme activity changes Alamar Blue to pink-colored resorufin. Dead or inactive bacteria cause no change in Alamar Blue color and it stays blue. Therefore, the Alamar Blue color is used as an indicator of bacterial activity. The experiment data were analyzed as the quantity of toxicants in mg/l required for reducing the growth of micro-organism to two standard deviations (no observed effect concentration (NOEC)), to 100% (100% mortality concentration), and to 50% (50% effective concentration ($EC_{50}$)), of the mean growth level of control cultures.

For confirming the robustness, reproducibility, and efficiency of the optimized method, it was compared with the previous described method [14], standard colony count method [15], and oxygen consumption method [16]. Correlation between these methods was determined using Pearson's correlation test ($p < 0.05$).

### 2.3. Wastewater samples

Metal plating wastewater samples were collected from plating facilities, located in Isfahan, Iran (Jun 2013). The full-scale SBR was operated in an 8 h cycle with 6 h of reaction and feeding phase, 1.5 h of settling phase, and 0.5 h of decanting. The SBR reactor was run at 15–28˚C and dissolved oxygen level was maintained at 1.0–3 mg/l. Other operating parameters of the SBR were as follows: food to micro-organism ratio: 0.15–0.35 g COD/g MLVSS.d; flow rate: 900 $m^3$/d; solids retention time: 8 d; and hydraulic retention time : 1.5–2.5 d.

The samples were transported in glass containers on ice and stored at 4˚C. Afterward, initial toxicity experiments were established and wastewater characteristics were determined.

Since suspensions may be toxic on micro-organism at conductivities over 10 mS/cm [17], the $EC_{50}$ for this parameter was determined as previously described [17]. For this aim, toxicity experiments were manipulated using a solution of 10–70 g/l of sodium chloride which induced a conductivity of about 15–100 mS/cm.

### 2.4. Analytical methods

In total, three heavy metals were used in assessment. All heavy metals were obtained from Sigma Chemical Co., Poole, UK. The water-soluble salts for the three metals were $Pb(NO_3)_2$, $ZnCl_2$, and $K_2Cr_2O_7$. Assays were performed in disposable borosilicate tubes with constant shaking.

To remove the suspended solids, wastewater samples were allowed to settle at room temperature for 2 h and then clear supernatants were analyzed for total organic carbon (TOC), pH, color, alkalinity, total suspended solids (TSS), volatile suspended solids (VSS), electrical conductivity (EC), and chemical oxygen demands (COD) contents according to the standard methods [15].

The concentration of heavy metals was measured using an ICP-OES (Thermo-Electron Corporation, Dreieich, Germany). A clear description on the reagents and method used is given by Alonso et al. [18].

Absorption spectra of Alamar Blue and resorufin (10 mg/l) and also raw metal plating wastewater were recorded using a spectrophotometer (Hach's DR 5000).

## 3. Results and discussion

### 3.1. Optimization of Alamar Blue reduction assay

In the previous final protocol [14], the bioassay mixture (5 ml) included 1 ml of Alamar Blue solution (100 mg/l), 1 ml of a microbial cell suspension, 0.25 ml of dimethyl sulfoxide, and 2.750 ml of growth medium. To each bioassay tube, 1 ml of 0.05 M phthalate–HCl buffer (pH 3.5) and 10 ml of n-amyl alcohol were added at time zero and after 30 min. After centrifugation for 5 min (at 1,000 g), the upper layer was transferred to sodium bicarbonate-contained tube and then the optical density of the suspension was determined at 610 nm [14]. Since the present study aimed to develop a simple and rapid bioassay for measuring bacterial viability level in WWTP biomass, major considerations were simplicity of the method for the small and decentralized plant employees, and minimum usage of reagents and equipment. Therefore, we adapted the Alamar Blue method of Liu [14] to achieve these criteria. In the first part of Materials and Methods section, our optimized method using SBR bacteria has been described. In our optimized method, (a) the phosphate buffer has been used instead of

phthalate–HCl buffer; (b) sodium bicarbonate and toxic amyl alcohol usage has been eliminated; and (c) nutrient broth (a common growth medium) has been replaced with the defined growth medium. A comparison of Liu Alamar Blue method [14] with our optimized bioassay using SBR bacteria showed no statistically significant difference in the rates of Alamar Blue reduction (Fig. 1(a)).

This study is the first one that uses a dehydrogenase enzyme assay for the evaluation of viability of SBR bacteria [2,3,8,16,19]. In the case of other bacteria, to our knowledge, previous investigations have not used a modified dehydrogenase enzyme assay, or if they have used this type of assay, the efficiency, robustness, and reproducibility of the method have not been considered [2,3,8,16,19]. As any reliable protocol for measuring bacterial viability as a monitoring tool of WWTP should present a significant correlation between its results and the biomass oxygen uptake, the relationship between the reduction of Alamar Blue by SBR bacteria and their oxygen consumption was determined (Fig. 1(b)). According to the Fig. 1(b), the correlation coefficient was 0.945. Therefore, Alamar Blue reduction would be a robust and reliable indicator of microbial viability. McNicholl et al. [20] also found a significant correlation between oxygen uptake by cultured Polytox cells and Alamar Blue consumption with an $r^2$ value of 0.991, although other studies have questioned the consistency of this strong correlation [21]. In this regard, in the present study, for confirming the reproducibility, robustness, and efficiency of the method, it also was compared with the plate count method that is already standardized and accepted [15]. Pearson's correlation coefficients revealed that Alamar Blue reduction assay for toxicity assessment of metals had a good and positive linear correlation with the colony count assay. The correlation coefficients for Pb, Zn, and Cr were 0.894, 0.940, and 0.977, respectively (Fig. 1(c)).

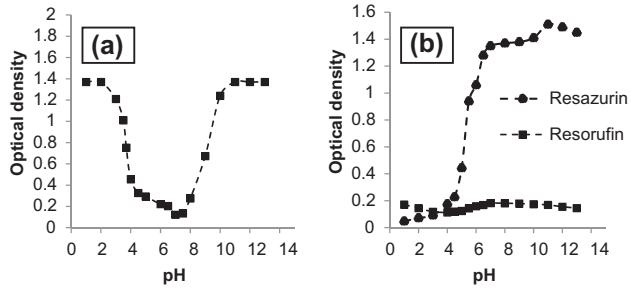


Fig. 2. The effect of pH on the (a) reduction of Alamar Blue using SBR bacteria; and (b) optical density (at 600 nm) of Alamar Blue (resazurin) and resorufin; inoculum cell density = 0.300 ± 0.01 at 540 nm, Alamar Blue 10 mg/l, and incubation time 30 min.

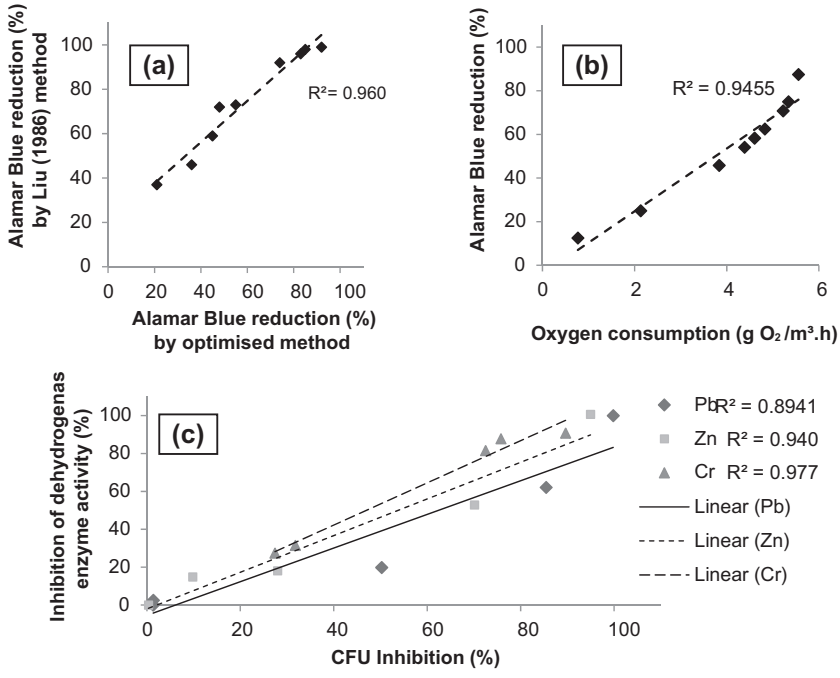


Fig. 1. Relationship between (a) our optimized bioassay and Liu method [14]; (b) oxygen consumption and Alamar Blue reduction; and (c) Alamar Blue reduction assay and CFU inhibition assay using SBR biomass, incubation time: 30 min.

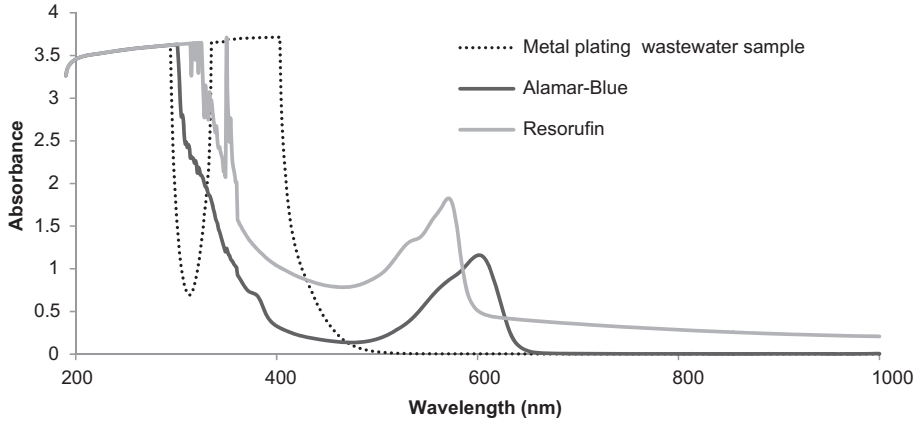


Fig. 3. Absorption spectra of metal plating wastewater sample (raw wastewater), Alamar Blue (Resazurin), and resorufin (10 mg/l).

The influence of pH on the Alamar Blue reduction was examined over a pH range of 1.0–13.0, and it was proved that the optimum pH for SBR bacteria activity is 6.5–7 (Fig. 2(a)). It could be said, at this pH level, all of the Alamar Blue was reduced by SBR bacteria following 30 min incubation. In the lower or higher pH values, a part of the Alamar Blue remained without any change.

Fig. 2(b) shows the pH dependence of the optical density of resorufin and Alamar Blue at $\lambda$ 600 nm. In this regard, the results proved the sensitivity of Alamar Blue spectrum in the pH range of 4–6.5. At such pH levels (4–6.5), the optical density sharply increases.

The absorption of Alamar Blue in the UV–visible spectrum in liquid media includes a weak absorption at 380 nm and an intense absorption at 605 nm (Fig. 3). These values also proved by previous studies [22]. This result proved that no interference in Alamar Blue measurement was occurred by resorufin at 605 nm. In fact, it can be concluded that metal plating wastewater or resorufin may not have any interfering effect in the measurement of Alamar Blue optical density at 605 nm wavelength.

### 3.2. The effect of metal plating wastewater on the SBR bacterial activity

In order to more fully setup the applicability of our modified bioassay, we used it to analyze the effect of metal plating wastewaters on the enzyme activity of SBR bacteria.

Metals in real environmental samples do not exist as pure solutions. In real wastewaters, obtained samples are complex suspensions and may have unrecognized synergistic effects on micro-organisms [23]. So, it would be advantageous to have a simple and rapid bioassay to determine the potential influence of whole of the industrial waste on the WWTP processes.

The characteristics of the metal plating wastewater sample are given in Table 1. The dose–response graphs for metal plating wastewater sample and EC are shown in Fig. 4. Since wastewater samples may be toxic at EC over 5–10 mS/cm [15], the EC level of the sample was high enough to be toxic for some micro-organisms (8.8 mS/cm). For that reason, the $EC_{50}$ for EC was determined. But results showed that EC

Table 1
Raw wastewater characteristics of the metal plating industries

| Parameters* | Values |
|---|---|
| Chemical oxygen demand (mg/l) | 543 |
| Total organic carbon (mg/l) | 9.1 |
| Electrical conductivity (mS/cm) | 8.8 |
| Total suspended solids (mg/l) | 5.4 |
| Volatile suspended solids (mg/l) | 2.2 |
| Alkalinity (mg/l) | 0.0 |
| Color (Pt-Co) | 145 |
| pH | 1.93 |
| Na (mg/l) | 26 |
| Ca (mg/l) | <0.1 |
| Cr (mg/l) | 92.4 |
| Cd (mg/l) | 37.1 |
| Rb (mg/l) | 6.5 |
| S (mg/l) | <0.05 |
| Bi (mg/l) | <0.05 |
| Si (mg/l) | 0.9 |
| Sb (mg/l) | 0.5 |

*Concentrations of other metals were below 0.1 mg/l. These metals were including Ba, Be, Ce, Ag, Al, As, Co, K, La, Li, Mg, Cu, Fe, Mn, Sc, Sn, Sr, Mo, Ni, V, W, Pb, Th, Ti, Y, and Zn.

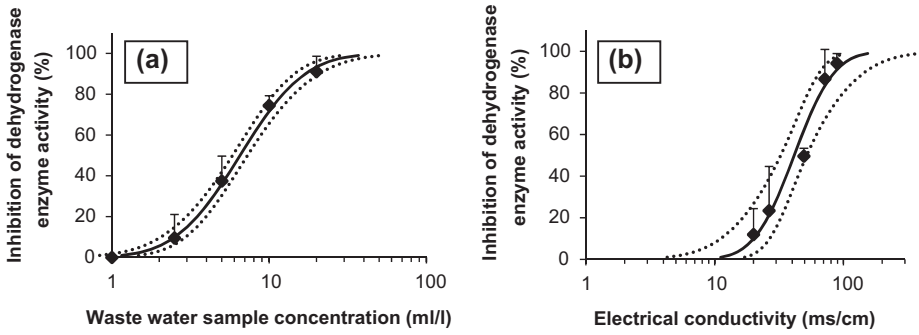


Fig. 4. Representative metal plating wastewater sample and EC dose–response graphs from experiments for SBR bacteria; wastewater sample (a) and EC (b); each value is the mean of duplicates with vertical bars representing the standard error.

Table 2
Results of 30 min NOEC, $EC_{50}$, and 100% mortality tests (with 95% confidence interval) for SBR bacteria exposed to different values of EC and metal plating wastewaters

| Toxic substances | $EC_{50}$* | NOEC** | 100% mortality |
|---|---|---|---|
| Wastewater sample (ml/l) | 6.43 (5.76–7.19) | 1.35 (1.14–1.78) | 37.93 (27.74–52.18) |
| Conductivity (mS/cm) | 41.43 (32.59–51.83) | 11.41 (4.27–17.11) | 154 (102−381) |

*NOEC = no observed effect concentration.
**$EC_{50}$ = 50% effective concentration; values are means of duplicates measurements. The $EC_{50}$, NOEC, and 100% mortality values were calculated by probit analysis using the SPSS ver. 16.0 software.

cannot be toxic to SBR bacteria in such values. In addition, 6.5 mg/l Rb in the wastewater sample was not toxic for the SBR biomass and in general, this element serves as a satisfactory substitute for potassium [7]. The toxicity of the metal plating sample can be mostly attributed to Cr and Cd with a concentration of 143.3 and 37.1 mg/l, respectively [12].

Considering the results of $EC_{50}$, NOEC, and 100% mortality tests for wastewater sample (Table 2), it is revealed that the concentration of 6.43 and 37.93 ml/l of the metal plating wastewater can induce 50 and 100% inactivity in the SBR bacterial community, respectively. Therefore, where the wastewater contains heavy metals, WWTPs which are dependent on bacterial activity should employ bioassay for early detection of the toxic effect of heavy metals. In fact, a rapid bioassay with bacteria in line with the other experiments can more efficiently reveal the toxicity effects of heavy metals.

Suspended or soluble forms of metals entered the biological basin of WWTPs may be adsorbed to the activated bacteria and lead to damage the treatment process [24]. As most of the WWTPs are inefficient for elimination of toxic effect of metals, it is desirable to use bioassays that can determine the toxicity of such pollutants. We simplified and optimized such an assay that can be manipulated for real metal plating wastewater samples.

## 4. Conclusion

We optimized a rapid and simple Alamar Blue-based method, in which the level of reduction of the Alamar Blue is correlated with respiration rate and bacterial biomass.

The modified Alamar Blue reduction method successfully assessed the toxicity of metal plating wastewaters on the activities of SBR bacteria. According to the results, where the wastewaters contain metals, wastewater treatment facilities especially small and decentralized facilities can use this bioassay for primary detection of toxicants.

## Acknowledgments

The authors would like to thank the Environment Research Center at the Isfahan University of Medical Sciences, Isfahan, Iran, for funding support (No. 2741-2) during the preparation of this manuscript. This work was also supported by Department of Environmental Health Engineering, School of Health, Isfahan University of Medical Sciences, Isfahan, Iran.

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