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Revealing interactive toxicity of aromatic amines to azo dye decolorizer *Aeromonas hydrophila*

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ABSTRACT

This study attempted to combine chemostat pulse technique (CPT) and dose-mortality assessment in pursuit of quantitative rankings of toxicity of model aromatic amines (MAAs) in the presence of diazo dye reactive red 141 (or Evercion Red H-E7B; RR141) upon *Aeromonas hydrophila*. As known, bacterial decolorization performance of azo dyes is directly dependent upon both the characteristics of biochemical reactivity and biotoxicity of dyes and related aromatic amines towards color removal. Thus, the findings herein indicated that the relative toxicity series of MAAs were (1) *ortho* > *para* > MAA-free control > *meta* position (for isomeric aminophenols); (2) –OH > $-SO_3H$ > MAA-free control ($-NH_2$ at *ortho* position); (3) –COOH > MAA-free control > –OH ($-NH_2$ at *meta* position) through the CPT at 200 mg/L MAAs. Comparison on results in higher levels of MAAs at 1000 mg/L almost showed parallel relative toxicity rankings at 200 mg/L. Quantification using traditional plate count method also confirmed nearly similar trends for corresponding MAAs except 3-aminophenol, revealing the promising feasibility of CPT for the toxicity assessment in practical applications. In addition, dose-mortality analysis regularly used in toxicology was used to quantitatively determine toxicity rankings of MAAs. In conclusion, this study directly provided a kinetic model to quantify the relative toxicity ranking of MAAs in the presence of RR141. It could provide a feasible guideline for assessment on the toxicity or treatability of azo dyes and MAAs to *A. hydrophila* in wastewater treatment.

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1. Introduction

Since the first synthetic dye, mauevin, was developed in 1856 for practical uses, azo dyes have been the largest chemical class of dyes frequently used for textile dyeing and paper printing. These industrial effluents often contain a significant amount of residual dye due to the inefficiency in dyeing processes. The reactive azo dyes regularly used for textile dyeing and paper printing are characterized by the presence of the azo group, –N = N–, a chromophoric group, that is, a color-producing group. As textile dyes are originally synthesized to be recalcitrant to biodegradation, industrial effluents often contain residual dyes which inevitably affect water quality. Usually 30–70% of reactive azo dyes are hydrolyzed and eliminated into wastewater for dyeing processes [1–4]. In addition, inappropriate disposal of dyes in wastewater apparently causes a threat to public health, as certain azo dyes or their metabolites (e.g., aromatic amines (AAs) or amino-azo compounds) are highly toxic and potentially carcinogenic [5]. Basically, cytotoxicity of typical azo dyes may be relatively low, but the toxicity of related aromatic amine intermediates are very likely still significantly high due to their carcinogenicity or mutagenicity [1]. For example, large "clusters" of bladder cancer cases were reported among dye workers in 1890s due to aromatic amines as the culprits [6]. Azo compounds can be reduced to amines through cometablism and the aid of azoreductase [7,8] for decolorization. With conservation of wild-species in local environment always a concern, indigenous rather than foreign microbiota should be first considered for in situ or ex situ biotreatment. Due to this ecological concern for sustainable development, Chen et al. [9,10] predominantly isolated *Aeromonas hydrophila* with a promising capability for color removal to diazo dye reactive red 141 (ca. 10-fold color-removal efficiency to *Pseudomonas luteola* [11]) from indigenous microbiota for environmental applications (e.g., in situ bioremediation). As aromatic amines are difficult to be removed via traditional wastewater treatment and inevitably tend to be persistent, the toxicity evaluation upon these amines will be apparently crucial to operation success or failure in dye decolorization and biodegradation afterwards. In comparison with *P. luteola* [11], this study was thus intentionally designed to investigate toxic impacts of aromatic amines to decolorizer *A. hydrophila* for optimal operation as well as risk assessment.

As indicated previously, biotoxicity of dyes and their intermediary products to biodecolorizers directly determines the performance of dye decolorization and biodegradation. For example,

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Nomenclature

A	intercept of probit model for dose-response curves
B	slope factor (or steepness measure) of probit model for dose-response curves
C	amine concentration (mg/L)
c_{in}	intracellular amine concentration (mg/L)
c_{ex}	extracellular amine concentration (mg/L)
D	dilution rate (h^{-1})
EC_x	effective concentration of metallic ion to provoke x% response (mg/L)
$erf(x)$	error function of independent variable x
G	Gibbs free energy (J)
Δk	inhibitory (+) or stimulating (−) rate constant to bacterial growth ($L\,mg^{-1}\,h^{-1}$)
m	injected amount of tested amine (mg)
NED	normal equivalent deviation
P	response variable of dose-response curves (1-VCC/VCC_0 for biotoxicity)
S	model aromatic amine concentration (mg/L)
Tox	toxicity
t_0	time delay in response to pulse injection (h)
X	cell concentration (g/L)
Y	probit unit

Greek letters and symbols

$\delta(t)$	Dirac delta function or the unit impulse function (i.e., $\delta(t) = \begin{cases} 0 & t \neq 0 \\ \infty & t = 0 \end{cases}$ and $\int_0^\infty \delta(t)\,dt = 1$)
μ	specific growth rate (h^{-1}; $\mu = d\ln X/dt$)
Σ	sum of all toxicities of MAA and RR141
$\forall$	for all

Mechsner and Wuhrmann [12] pointed out that one of the limiting steps for bacterial degradation of azo dyes is microbial uptake into intracellular compartments. Donlon et al. [13] also reported that mordant orange 1 (MO1; an azo dye) was reductively cleaved to aromatic amines which were relatively less toxic towards methanogens. Since aromatic amines generated by degradation of azo dyes were cytotoxic, respiration-inhibition tests eventually showed increased toxicity in anaerobic treatment [14]. In addition, *Daphnia magna* [15] was used to determine the toxicity of azo dyes in aerobic treatment of textile wastewater. Wang et al. [16] also performed bioluminescence bacteria (Microtox®) to reveal the toxicity of Ramazol Black. Lumistox bacteria were used in evaluation of toxicity of Reactive Black 5 decolorized in a baffled reactor. Moreover, ATA-anaerobic toxicity, respiration/inhibition and *D. magna* tests [17,18] were used to assess the toxicity of Direct Black 38 in an anaerobic/aerobic sequential reactor system. Although aromatic amines may be toxic to biodecolorizers and in feedback repression to decolorization, quantitative evaluation of toxicity of aromatic amines compared to azo dyes still remained open to be discussed due to the specific characteristics of different decolorizers (e.g., *A. hydrophila* and *P. luteola*). Thus, this study tended to combine transient reaction kinetics with biotoxicity assessment in pursuit of explicit toxicity rankings among aromatic amines compared to the model diazo dye (i.e., reactive red 141 or Evercion Red H-E7B; RR141 for short). As intermediate amines of RR141 cannot be easily purified and identified, this study thus intentionally selected certain simple model amines to disclose the technical feasibility of the chemostat pulse technique (CPT) for toxicity assessment. Furthermore, to verify the practicality of the proposed CPT method plate-count method was also provided with aid of dose-response assessment to compare with the results. As known, in the exponential growth phase for batch cultures, cells have adapted to their new environment to multiply in a maximum rate as they can. Hence, the growth rate [11,19–21] was used as an equilibrium outcome of metabolic status in cells in response to a hostile environment (e.g., pollutant-bearing wastewater). Such a core perspective was extended herein to steady-state bacterial cultures in CSTR for toxicity assessment. Kuhn et al. [22] and Goldberg and Er-el [23] mentioned that the CPT via an instantaneous injection could be used to identify growth limiting nutrients for medium optimization [23]. Similarly, this CPT not only was applicable to define the toxic sources, but also quantify toxic level of suspected pollutants present in the culture. After a steady-state growth in LB-bearing continuous culture was achieved, the process of testing chemicals (e.g., aromatic amine and/or RR141) toxic to growth was carried out. First of all, the model aromatic amine (MAA) and/or RR141 were intentionally injected individually into the culture broth as substrate(s) of either growth-stimulation or inhibition. After a single impulse injection, the cell and dye concentration were determined, allowing for a period of ca. 1/3 mean residence time to elapse [23]. So long as the injected substrate stimulated the growth characteristics, an increase in injected substrate concentration should apparently yield a proportional increase in cell concentration. In contrast, if the injected source contained toxic or inhibitory compositions, an increase in injected-source concentration should yield a marked decrease of cell concentration [23]. Note that as CPT was carried out in CSTR, the transient dynamics after impulse injection of the tested pollutant (e.g., MAA) to a steady-state culture would be taking place (i.e., "acute toxicity" response). This is evidently different from the steady-state characteristics in pollutant-bearing CSTR (or "chronic toxicity" response). In addition, we conducted CPT under aerobic conditions to completely exclude confounding factor(s) of decolorization for non-biased toxicity evaluation. Moreover, the benefit of using CPT to replace dose-response assessment [19–21] and Microtox® [16] is to directly disclose toxicity characteristics of toxiants in terms of kinetic parameters of bioreactor operation.

This objective of this study was to provide a first attempt from a toxicological perspective to put forward, in significant terms and quantitative evaluations, biotoxicity of model aromatic amines to the biodecolorizer *A. hydrophila* using the CPT. Using CPT, quantitative rankings of toxicity of model aromatic amines in the presence of diazo dye RR141 upon *A. hydrophila* were obtained. Previous study [24] proposed that bacterial decolorization performance of azo dyes was likely directly dependent upon the characteristics of biochemical reactivity and biotoxicity of dyes and related aromatic amines towards decolorization. However, lack of adequate toxicity assessment upon contaminants makes the biotreatment unreliable for on-site applications. This study clearly revealed a promising viability of CPT as well as dose-response assessment for toxicity evaluation of MAAs to *A. hydrophila* compared to *P. luteola.*

2. Materials and methods

2.1. Microorganism and culture conditions

A. hydrophila [9,10] predominantly isolated from river and mud samples of Lanyang Hsi and Dongshan River in Lanyang River Basin, fountain springs near Chiao-Hsi in the Lanyang Plain was used as a reporter strain of decolorization performance. To obtain all cultures in consistent growth characteristics for study, a loopful of *A. hydrophila* seed taken from an isolated colony on a LB-streak plate was precultured in 50-mL Bacto 1/5X LB broth, Miller (Luria-Bertani) (per liter; 2.0 g Bacto tryptone, 1.0 g Bacto yeast extract, 10 g sodium chloride) for 24 h at 30 °C, pH 7.0, 125 rpm using a water bath shaker (SHINKWANG, SKW-12). Approximately 200 mL precultured broth was then inoculated into 1300 mL fresh

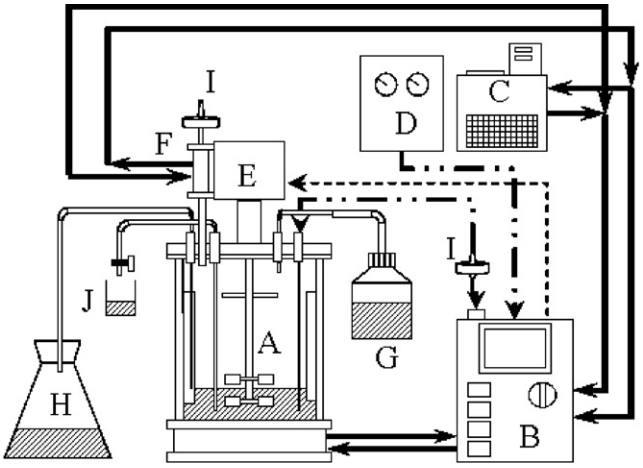


Fig. 1. Experimental setup of fermentor systems for chemostat pulse technique. Lines connected B, C, and F denoted PID control loop for temperature, and lines connected B, D, and E are manual/automatic on-off control loop for aeration. A: fermentor; B: temperature and stirrer controller; C: cooling tank; D: air compressor; E: agitation controller; F: condenser; G: feeding inlet; H: wastewater outlet; I: air filter; J: sampler.

LB broth (ca. 30 μL/L antifoam 204; Sigma) for aerobic continuous culture (1 vvm, 200 rpm, 30 °C) at $t = -48$ h. After 12 h batch cultivation (i.e., $t = -48$ h), the fresh LB broth stream was pumped in 79 mL/h (i.e., $D \approx 0.060 \pm 0.002\ h^{-1}$) to the fermentor to maintain nearly constant working volume at 1300 mL for continuous cultures (refer to Fig. 1 for experimental setup). Once the steady state (i.e., $X_0 = 0.664 \pm 0.067\ g\,L^{-1}$; data not shown) was achieved at 48 h cultivation (i.e., $t = 0$), appropriate amounts of aromatic amine-bearing dye solutions (i.e., tested cases) were injected into the fermentor to reach the levels at ca. 200 or 1000 mg/L AA using sterile syringe. Moreover, the aromatic amine-free control (AFC) was simply the case of injected RR141 alone in the absence of AA. In addition, diazo RR141 was intentionally included in chemostat runs with the aromatic amines to reveal whether the combined toxicity of RR141 and MAA still could be disclosed by the proposed CPT. In these continuous cultures, the pH was not controlled to simulate on-site or in situ practical situations. Note that the concentration of RR141 for all tested cases was set approximately at ca. 3800 mg L^{-1} (or 3200–4000 mg L^{-1}).

For plate count methods, a loopful of *A. hydrophila* seed taken from an isolated colony in LB-streak plate was precultured in 50 mL 1/5X LB broth, Miller for 12 h at 30 °C, pH 7.0, 200 rpm. Approximately 5% (v/v) cultured broth was then inoculated to fresh 1/5X LB medium and a cell culture was harvested in the mid-exponential growth phase (ca. 4 h) for further toxicity assessment. Then, the 1.0 mL cell culture was serially diluted with 9.0 mL sterile saline solution (SSS; NaCl 10.0 g L^{-1}) and only the diluent with appropriate viable cell concentrations (ca. 1500–15,000 cells/mL) was selected as the test seed (TS) for later uses.

2.2. Analytical methods

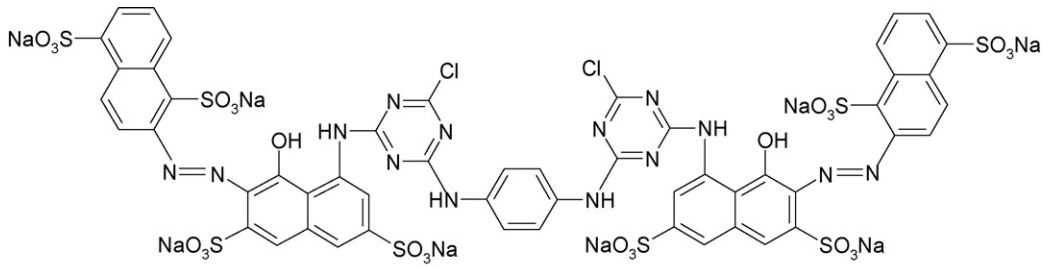
The model diazo dye (Fig. 2) used for study was C.I. Reactive red 141 (RR 141; $\lambda_{max} = 544$ nm), which is often used in dyestuff plants and was obtained from Sumitomo Inc. (Tokyo, Japan). The MAAs (Fig. 2) employed for toxicity assessment are 3-aminobenzoic acid (3ABA; 99 + % Janssen CHIMICA), aniline-2 sulfonic acid (A2SA; 95% Aldrich), 2-aminophenol (2AP; 99% ACROS ORGANICS), 3-aminophenol (3AP; 99% Aldrich), 4-aminophenol (4AP; 97.5% ACROS ORGANICS). Prior to experiments, the dye and amine solutions were sterilized by filtration (Millipore Millex®-GS 0.22 μm filter unit), since RR141 and amines may be unstable in moist-heat sterilization. With appropriate calibrations at specific wavelengths, concentrations of biomass and dyes were determined using an UV-Visible spectrophotometer (HITACHI Spectrophotometer, model UV-2001). The concentration of dye was primarily determined by measuring the optical density (OD) of the supernatant of the sample after centrifugation for 2 min at $700 \times g$ (HSIANGTAI Centrifuge MCD-2000). A sterile cell-free medium was chosen as the control. As all samples contained biomass and dye, concentrations of biomass (i.e., (1)–(2)) and dye (i.e., (3)) were evaluated as follows:

(1) $OD_{600\,nm}$ of sample mixtures without centrifugation: $OD_{600\,nm}^{X+dye} = OD_{600\,nm}^{dye} + OD_{600\,nm}^{X}$,
(2) $OD_{600\,nm}$ of sample supernatant (sup) after centrifugation: $OD_{600\,nm}^{sup} = OD_{600\,nm}^{dye}$, and
(3) $OD_{\lambda_{max}}$ of sample supernatant after centrifugation: $OD_{\lambda_{max}}^{sup} = OD_{\lambda_{max}}^{dye}$.

Samples were diluted to an optical density of less than 0.6 when absorbance was not within the linear range (0.1–0.7). The relationship between the cell concentration and $OD_{600\,nm}$ (ODU) is 1.0 ODU $\cong 0.373\ g\,L^{-1}$ dry cell weight.

2.3. Biotoxicity assessment

Biotoxicity assessment was also specifically conducted through a modification of dose-response analysis [19,20] as follows: aromatic amine solutions were first sterilized by filtration (Millpore Millex®-GS 0.22 μm filter unit) after complete dissolution. Note that to clearly verify the stimulating or inhibitory characteristics of the tested amine, only amine solution was applied as the sole source for plate-count assessment. The concentrations of tested samples were defined here as the concentrations of solutions and their serial diluents which were well mixed with sterile saline solution (SSS; $NaCl_{(aq)}$ 10.0 g L^{-1}). The initial concentration C_0 for toxicity tests of all aromatic amine solutions was chosen at ca. 3000 mg/L (all below the solubility products). Serial-half dilutions of initial concentration C_0 (i.e., $1/2^n\ C_0$; $n \in N$) were carried out by using 5.0 mL derived diluents mixed with 5.0 mL SSS. The 9.0 mL resulted serial diluents (RSDs) were all placed in sterile test tubes for use in quantification of viable cells afterwards. The 1.0 mL freshly harvested TS was then well mixed with RSD via vortex to form serial plate count diluents (SPCDs). Meanwhile, 1.0 mL fresh TS mixed with 9.0 mL pure SSS was used as the amine-free control. It was postulated that AFC denoted a toxicant-free blank and the viable cell numbers present in AFC was thus the maximal number of viable cells in a non-toxic environment. The numbers of survived *A. hydrophila* in SPCD or the control were estimated by the standard plate count method [25]. Standard plate count in 1/5X LB medium was carried out in duplicate as follows: SPCD were serially diluted with SSS immediately after sampling, and then appropriate volumes (e.g., 0.10 mL) of serially diluted SPCD were spread onto agar *Petri* plates. It was assumed that all cells in SPCD would be viable and culturable on



Diazo Dye C.I. Reactive Red 141 (Evercion Red H-E7B)

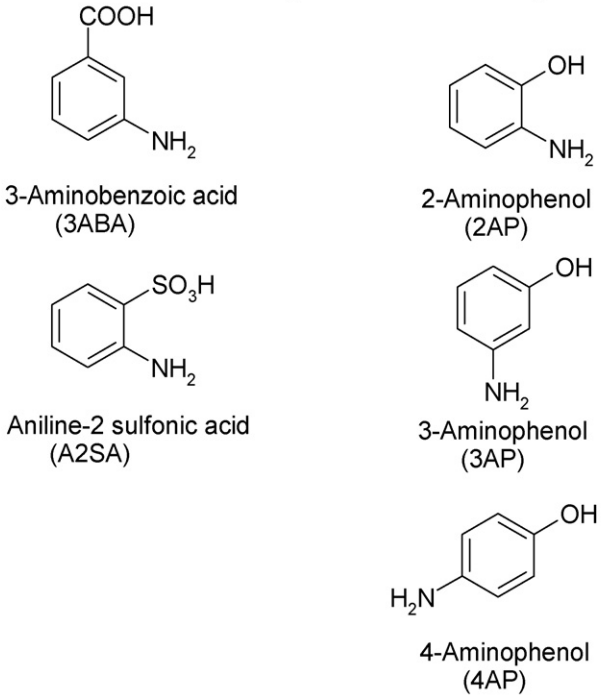


Fig. 2. Chemical structures of C.I. Reactive Red 141 and aromatic amines 3ABA, A2SA, 2AP, 3AP, and 4AP used in the study.

1/5X LB-medium plates due to fresh preparation for exponentially growing cells in all steps [26]. The 1/5X LB-medium plates were then incubated at 30 °C for ca. 16–24 h to form visible colonies for enumeration. After incubation, only plates with between 30 and 300 colonies were considered suitable for counting. The microbial population in the original RSD could then be calculated using the following equation (VCC: viable cell count):

$$\text{cells per liter of broth (VCC)} = \frac{\text{number of colonies}}{\text{amount plated} \times \text{dilution factor}}$$

To have quantitative toxicity for comparison, VCC_0 was chosen as the VCC of AFC. The ratio VCC/VCC_0 of 0 and 1 simply indicated complete inhibition and no inhibitory toxicity to *P. luteola*, respectively. The unity of this ratio simply suggests that the toxicity of this diluent at this concentration is nearly equal to the toxicity of SSS (i.e., zero toxicity). Note that if amine A is more toxic than amine B, a higher dilution factor must be carried out for amine A in order to have "zero" toxicity (i.e., SSS).

2.4. Dose-mortality assessment

Probit analysis [19,20,27,28] was adopted to reveal dose response curves of various amine solutions. Semilogarithmic plot of amine concentration versus the provoked response is assumed to reveal a linear relation. Probit model converts sigmoid-shaped dose-response curve into a linear normal equivalent deviation (NED) scale. Probit unit in the model equals NED scale plus 5. The conversion formulae are shown as follows:

$$Y = A + B \ \log C, \tag{1}$$

$$P = \frac{1}{2}\left\{1 + erf\left(\frac{Y-5}{\sqrt{2}}\right)\right\}, \tag{2}$$

$$erf(x) \equiv \frac{2}{\sqrt{\pi}} \int_0^x e^{-\xi^2} d\xi, \tag{3}$$

where A and B denote the intercept and slope of dose-response relation, C and Y are amine concentration (mg/L) and probit unit, respectively; P is the response (%) corresponding to administered amine, $erf(x)$ is an error function. Note that the response variable is normalized to be located between 0 and 1. The conversion relation between the probit unit and provoked response is listed in Table 1 in Chen et al. [19]. For example, 55% and 85% of response correspond to probit unit of 5.13 and 6.04, respectively. Note that the response variable P of biotoxicity was chosen by $1 - VCN/VCN_0$ with the basic properties of $0 = P\big|_{C \geq MEC} \leq P \leq P\big|_{C \leq TH} = 1$, where *MEC* and *TH* denoted the smallest concentration to have maximal effect (i.e., 100% response) and the largest concentration (i.e., threshold) to have no effect of toxicity (i.e., 0% response), respectively.

3. Results and discussion

3.1. Effects of various amines

3.1.1. Low-level effect of isomeric amines

Time courses of growth characteristics (Figs. 3 and 4) indicated that there were time delays (ca. 5–6 h) to respond changes in cell concentrations apparently longer than ca. 2–3 h for *P. luetola* [11]. It might suggest a slower response for toxic amine than the response for nutrient substrate(s) in cultures. The time course of growth characteristics in the culture without RR141 or MAA (i.e., control w/o RR141 in Figs. 3 or 4) was stably maintained at $X \approx X_0$, indicating that the steady state was actually achieved. There are three possible isomers of disubstituted benzenes, 1,2-substitution (ortho or o-), 1,3-substitution (meta or m-) and 1,4-substitution (para or p-). For 200 mg/L of three isomeric amines (i.e., 4AP(para), 3AP(meta) and 2AP(ortho)) in the presence of ca. 3800 mg/L RR141 (Fig. 3), the simulated kinetic parameters $\Delta k (= k - k_0$; refer to Appendix A for parameter estimation) for 3AP, amine-free control (i.e., RR141 alone), 2AP and 4AP were obtained to be -1.70×10^{-6}, 0.0, 9.90×10^{-6} and 2.40×10^{-6} L/mg-h, respectively. Note that the kinetic parameter k of RR141 alone (i.e., amine-free control) was 1.10×10^{-6} L/mg-h, clearly indicating that RR141 was slightly inhibitory to *A. hydrophila* and indirectly explaining why *A. hydrophila* could play a role to be an azo decolorizer [9,10]. In addition, these results simply suggested that amino group at meta-position of 3AP ($\Delta k < 0$) might play a role of stimulant to bacterial growth. In contrast, 2AP and 4AP were inhibitory

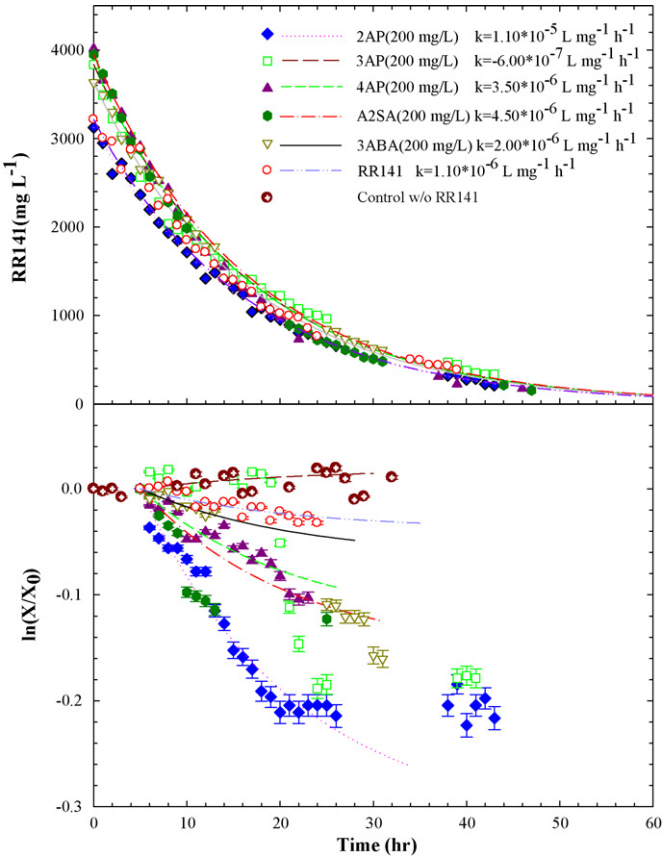


Fig. 3. Time courses of cell and dye concentrations in continuous cultures containing different MAAs at 200 mg/L via CPT. Amine-free control is the case of RR141 alone. Control w/o RR141 denoted the control without dye or amines. Kinetic parameters k were determined by the plots of $\ln(X/X_0)$ versus time t (i.e., Eq. (A4)), where X and X_0 denoted cell concentrations at $t = t$ and $t = t_0$, respectively.

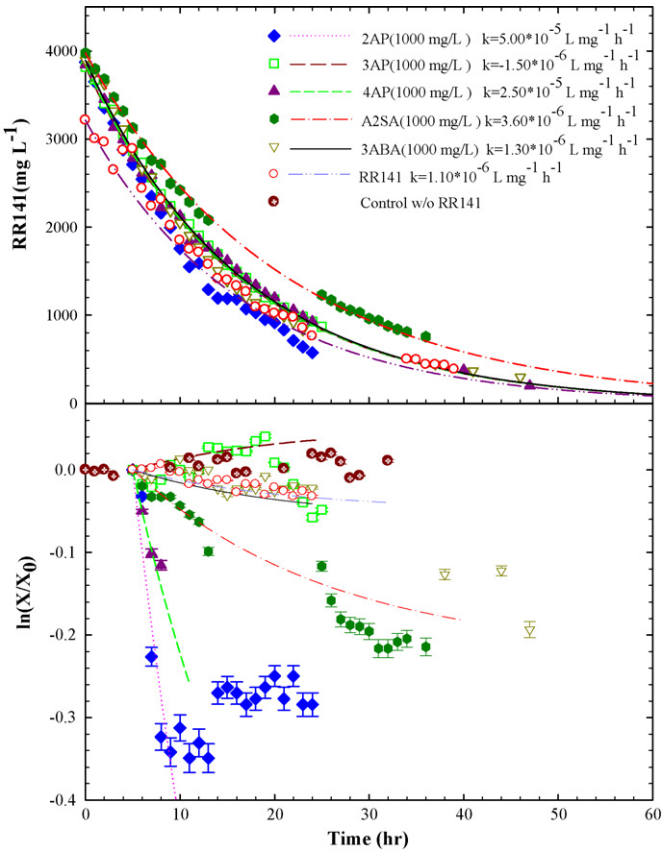


Fig. 4. Time courses of cell and dye concentrations in continuous cultures containing different MAAs at 1000 mg/L via CPT. Amine-free control is the case of RR141 alone. Control w/o RR141 denoted the control without dye or amines. Kinetic parameters k were determined by the plots of $\ln(X/X_0)$ versus time t (i.e., Eq. (A4)), where X and X_0 denoted cell concentrations at $t = t$ and $t = t_0$, respectively.

to bacterial growth. This clearly disclosed that the relative ranking of biotoxicity of isomeric aminophenols was 2AP > 4AP > (RR141 alone) > 3AP. This ranking was slightly different from the ranking of *P. luteola* [11] due to diverse metabolic capability of bacterial strains. Although 3AP (Fig. 3) seemed to be slightly stimulating to cell growth of *A. hydrophila*, all aminophenol isomers (2AP, 3AP, 4AP) might be considered to be inhibitory (i.e., $\Delta k < 0$). Ottoboni [28] mentioned that with chronic exposure of toxicants, cells inevitably require more energy to maintain the defense mechanism and thus provide less positive change in free energy handling toxicant in a manner to prevent a toxic effect from occurring. Due to this possible inhibitory characteristic and extra energy cost to the growth of *A. hydrophila*, color removal efficiency of *A. hydrophila* might gradually decrease once similar functional group(s)-containing intermediates after decolorization were gradually accumulated. That is, although *A. hydrophila* expressed an excellent decolorization performance [9,10], the gradual decreases in azo dye decolorization due to the formation of related intermediates might still take place. Acclimation in intermediate-containing cultures would be needed to improve and maintain color removal as indicated in Chen et al. [9,10].

3.1.2. Low-level effect of functional group

As shown in Fig. 3 for comparison, in the presence of RR141 at 3800 mg/L the toxicity ranking of aniline-based disubstituted benzenes at 200 mg/L could be determined by simulated kinetic parameters for A2SA ($\Delta k = 3.40 \times 10^{-6}$ L/mg-h) < 2AP($\Delta k = 9.90 \times 10^{-6}$ L/mg-h) and 3AP ($\Delta k = -1.70 \times 10^{-6}$ L/mg-

h) < 3ABA ($\Delta k = 0.90 \times 10^{-6}$ L/mg-h), indicating that the relative toxicity series of functional groups are –OH > –SO_3H > (amine-free control) and –COOH > (amine-free control) > –OH (Fig. 3).

3.1.3. High-level effect of isomeric amines

According to Fig. 4, in the presence of RR141 at 3800 mg/L 2AP, 3AP and 4AP at 1000 mg/L all expressed inhibitory characteristics of biotoxicity for $\Delta k = 4.89 \times 10^{-5}$, -2.60×10^{-6} and 2.39×10^{-5} L/mg-h, respectively. This revealed that the relative inhibitory ranking to *P. luteola* was 2AP > 4AP > (amine-free control or RR141 alone) > 3AP. Compared to low-dose amines (200 mg/L; refer to Section 3.1.1), relatively higher concentrations of toxic amines (1000 mg/L) might be easier moved across the membrane from the region of high concentration to low concentration via passive diffusion [29] due to relatively higher concentration gradient as a driving force. Chen [11] mentioned that amines at 1000 mg/L are more thermodynamically favored to move across the membrane than at 200 mg/L. Although 1000 mg L^{-1} of 3AP might still appreciably pentrate into the plasma membrane to establish significant toxicity to cells, 3AP still expressed slightly stimulating effects on *A. hydrophila*.

3.1.4. High-level effect of functional group

As shown in Fig. 4, in the presence of RR141 at 3800 mg/L the toxicity ranking of aniline-based disubstituted benzenes at 1000 mg/L could be determined by simulated kinetic parameters for A2SA ($\Delta k = 2.50 \times 10^{-6}$ L/mg-h) < 2AP ($\Delta k = 4.89 \times 10^{-5}$ L/mg-h) and 3AP ($\Delta k = -2.60 \times 10^{-6}$ L/mg-h) < 3ABA ($\Delta k = 0.20 \times 10^{-6}$ L/mg-h), indicating that the relative toxicity series of functional groups in aniline-based disubstituted benzenes are –OH > –SO_3H > (amine-free control) and –COOH > (amine-free control) > –OH (Fig. 4).

3.2. Serial plate count

To verify whether CPT is scientifically sound and technically feasible for biotoxicity assessment, traditional plate count method coupled with dose-response analysis was also carried out to confirm its practicability from a perspective in toxicology. As indicated in Fig. 5, comparison upon colony forming phenomena in various amine-bearing plate cultures revealed that the toxicity of 3ABA can be negligible (i.e., almost all identical to amine-free control) at least up to 3000 mg/L (ca. maximal solubilities). Although the effects due to amines should be thought of as being unfavorable, this is not always to be factual, in particular for azo decolorizer *A. hydrophila*. Fig. 5 clearly represented how the concept of "sufficient challenge" (i.e., reverse effect) changes the shape of "dose-response curve" [28]. Note that the phenomenon of beneficial effects from exposures to trace amounts of "toxic chemicals" (e.g., amines) are termed "sufficient challenge" (ca. <100 mg L^{-1} for 3ABA and 4AP). This "sufficient challenge" or reverse effect might provide some indirect explanations to the occurrence of slightly stimulating or inhibitory characteristics as shown in Figs. 3 and 4 (Section 3.1).

Table 1
Critical effective concentrations of toxicity in A2SA, 2AP and 4AP predicted from the probit model. The response variable P is defined as 1-VCC/VCC_0 and C denotes the amine concentration (mg/L).

	EC_0	EC_{50}[a]	EC_{100}[a]	$Y = A + B \log C$
2AP	0.570	73.3	3.92×10^4	$Y = 2.88 + 1.14 \log C$
3AP	0.040	8790	6.97×10^6	$Y = 3.22 + 0.45 \log C$
4AP	1.62	1420	8.99×10^6	$Y = 2.43 + 0.828 \log C$
A2SA	0.041	1.40×10^7	1.46×10^{18}	$Y = 2.99 + 0.28 \log C$
A2SA[b]	57.3	220.3	1253	$Y = -4.61 + 4.10 \log C$
2AP[b]	550	1203	3296	$Y = -16.8 + 7.08 \log C$
4AP[b]	457	1438	6165	$Y = -10.3 + 4.84 \log C$

[a] Certain predicted values might exceed the solubility of chemicals and thus be used only for comparison.
[b] *P. luteola* data adopted from Chen [11].

3.3. Dose-mortality analysis

To provide more clear figures of these toxic responses among various amines, dose-response analysis was conducted by excluding the regions of "sufficient challenge" in curves (Fig. 5). Note that if the EC_x for chemical M is greater than that of chemical N, chemical M may be said to be less toxic (inhibitory) than chemical N. Thus, compared to *P. luteola* (superscript *) the ranking of biotoxicity, in increasing order, based upon viable cell count using various amines is presented as follows (Table 1 and Fig. 6):

EC_0: 3AP (0.040 mg/L) ≈ A2SA (0.041 mg/L) > 2AP (0.570 mg/L) > 4AP (1.62 mg L^{-1}) > A2SA* (57.3 mg/L) > 4AP* (457 mg/L) > 2AP* (550 mg/L) ≫ (3ABA);

EC_{50}: 4AP (73.3 mg/L) > A2SA* (220 mg/L) > 2AP* (1210 mg/L) > 2AP (1420 mg/L) > 4AP* (1440 mg/L) > 3AP (8790 mg/L) > A2SA (1.40 × 10^7 mg L^{-1}) ≫ (3ABA);

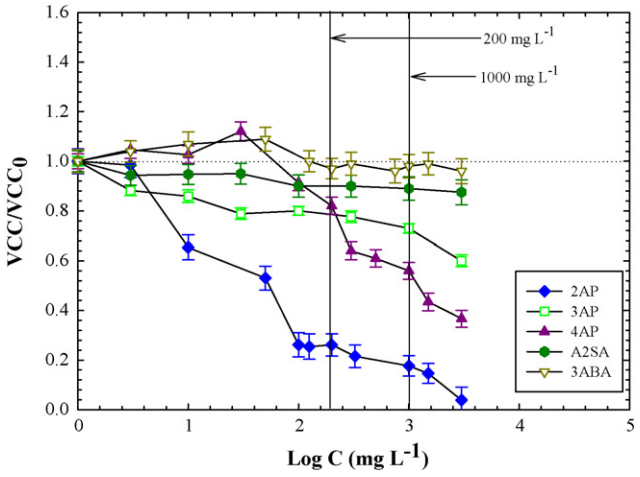


Fig. 5. Comparison on cell growth in various concentrations of amines (C: mg/L). At points above the line VCC/VCC_0 = 1.0, the curves passed from "sufficient challenge" regions back to normal growth regions. Lines at 200 and 1000 mg/L (i.e., arrows) indicated the concentration levels to compare with CPT results.

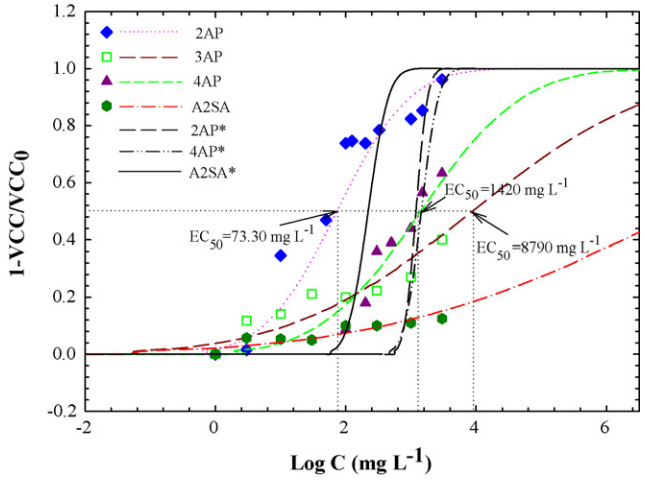


Fig. 6. Dose-mortality curves of "chronic toxicity" of tested amines predicted from the probit model. The response variables (1-VCC/VCC_0) were estimated from data of the viable cell counts (VCCs) with respect to viable cell counts of amine-free cultures (VCC_0). Superscript (*) denoted data adopted from *P. luteola* [11].

EC_{100}: A2SA* (1250 mg/L) > 2AP* (3920 mg/L) > 4AP* (6170 mg/L) > 2AP (3.92×10^4 mg/L) > 3AP (6.97×10^6 mg/L) > 4AP (8.99×10^6 mg/L) > A2SA (1.46×10^{18} mg L^{-1}) ≫ (3ABA);
Slope B: 2AP* (7.08) > 4AP* (4.84) > A2SA* (4.10) > 2AP (1.14) > 1.0 > 4AP (0.82) > 3AP (0.45) > A2SA (0.28).

The largest value (1.14) of slope B for 2AP (Table 1) suggested the smallest tolerance range from EC_0 to EC_{100} and the steepest dose-response curve for the toxicity of 2AP. In contrast, the smallest value (0.28) of slope B for A2SA directly implies the widest range of tolerance to amine toxicity from the threshold dose (EC_0) to a maximum effect dose (EC_{100}). Compared to *P. luteola*, *A. hydrophila* seemed to express a higher tolerance (slope B ≤ 1.0 and higher EC_{100} values; Fig. 6) to aromatic amines. This might cause a relatively higher color removal efficiency (ca. 10-fold to *P. luteola*; [9,10]). The reasons why the slopes of dose-response curves differ for different amines involve the particular cellular and/or enzymic mechanisms and metabolic functions which they affect [30]. In addition, for 2AP with the steepest dose-response curve it is required to be treated with caution as there may be only a small difference between a dose producing no effect and a dose producing serious outcome. Note that extraploted EC_{50} values of certain MAAs (e.g., 3AP, 4AP, A2SA) went far beyond their solubility limits (Table 1). This implied that these MAAs should express insignificant toxicities to *A. hydrophila*. That is, these MAAs could be very likely non-harmful to *A. hydrophila*. In addition, almost all EC_x values (e.g., EC_{50}, EC_{100}) were in similar ranking, indicating that biotoxicity based upon the viable cell count is an appropriate indicator for quantitative comparison on combined toxicity responses of amine solutions to *A. hydrophila*.

4. Conclusions

This assessment clearly indicated the toxicity series of model aromatic amines to *P. luteola* in terms of kinetic models from a toxicological perspective. It clearly indicated inhibitory characteristics of MAAs in RR141-bearing cultures, suggesting technical feasibility of using CPT for toxicity assessment upon decolorizer *A. hydrophila*. Almost all aromatic amines were inhibitory to *A. hydrophila* (except 3AP), the occurrence of some beneficial effects still existed for exposures of trace levels of 4AP, 3ABA (i.e., sufficient challenge; Fig. 5). As indicated in Table 3, the toxicity figure of 3AP determined by CPT was apparently underestimated (i.e., stimulating). This exception of 3AP might be explained as follows: when cells were spread onto 3AP-laden agar-plates for assay, each cell could be fixed at specific point on plates. However, cells in liquid cultures might "swim" away to prevent such a toxic pressure for survival (i.e., toxicity reduction). Moreover, CPT results (i.e., this study and Chen [11]; Table 2) indicated that A2SA and 3ABA expressed parallel toxic characteristics to *P. luteola* and *A. hydrophila*. However, as different isomeric aminophenols showed varied degrees to penetrate into the plasma membrane of cells, diverse toxic/stimulating responses on both decolorizers (Table 2 and [11]) were established. As shown in the comparison between CPT and plate-count results (Table 3), it was apparent to perceive almost parallel trends of CPT (quantitative) and plate-count (qualitative) results (except 3AP). In addition, Table 2 also revealed that 3AP might have synergistic interaction(s) with diazo RR141 [28] in chemostat cultures, resulting in different predicted parameter Δk values between the CPT and plate-count method. Dose-mortality analysis also revealed that a negligible toxicity of 3ABA to cells was observed at least up to 3000 mg/L. Regardless of diverse biochemical characteristics of decolorizers, this study apparently revealed a promising feasibility of CPT as well as dose-mortality assessment for toxicity assessment of MAAs to *A. hydrophila*. However, similar to a possible human pathogen, *Pseudomonas aeruginosa* (that is popularly used for bioremediation of petroleum-contaminated soil and groundwater), *Aeromonas* species is probably more appropriate to be cultivated in closed-vessel bioreactor due to its likely pathogenicity.

Table 2
Comparison between CPT results of decolorizers (i.e., *P. luteola* and *A. hydrophila*) for toxicity assessment.

MAAs	Δk or $\Delta k\|_{MAA}$ ($\times 10^{-6}$ L/mg-h)			
	200 mg/L		1000 mg/L	
	A. hydrophila	*P. luteola*[a]	*A. hydrophila*	*P. luteola*[a]
2AP	9.90	6.72	48.9	25.7
3AP	−1.70	4.66	−2.60	2.10
4AP	2.40	−37.9	23.9	18.8
A2SA	3.40	5.36	2.50	3.55
3ABA	0.90	12.0	0.20	2.50

Note: The difference of kinetic parameter k of studied MAA and kinetic parameter of amine-free control k_0 (i.e., $\Delta k = k - k_0 = \Delta k|_{MAA}$).
[a] Data adopted from Chen [11].

Table 3
Comparison between CPT (quantitative) and plate-count (qualitative) results for toxicity assessment of *A. hydrophila*.

MAAs	Δk or $\Delta k\|_{MAA}$ ($\times 10^{-6}$ L/mg-h)			
	200 mg/L		1000 mg/L	
	CPT	Plate count	CPT	Plate count
2AP	9.90	>0	48.9	>0
3AP	−1.70	>0	−2.60	>0
4AP	2.40	>0	23.9	>0
A2SA	3.40	>0	2.50	>0
3ABA	0.90	>0	0.20	>0

Note: The difference of kinetic parameter k of studied MAA and kinetic parameter of amine-free control k_0 (i.e., $\Delta k = k - k_0 = \Delta k|_{MAA}$).

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Appendix A. Mathematical model

In continuous operation, the transient dynamics of cell (X), dye (RR141) and aromatic amine (S) concentrations could be represented as follows:

$$\frac{dX}{dt} = (\mu - D)X; \qquad X(0) = X_0 \tag{A1}$$

$$\frac{dS}{dt} = -D \cdot S + \frac{m}{V}\delta(t); \qquad S(0) = S_0 \tag{A2}$$

Chemostat pulse injection was carried out at the steady state was achieved at $\forall t > 0^+$.

$$m \int_0^{+\infty} \delta(t)\, dt = m, \tag{A3}$$

where $\delta(t)$ is Dirac delta function or the unit impulse function (i.e., $\delta(t) = \begin{cases} 0 & t \neq 0 \\ \infty & t = 0 \end{cases}$ and $\int_0^\infty \delta(t)\,dt = 1\ \forall t \in R^+$). Eq. (A2) can be reformulated via Laplace transform to obtain $S(t) = (m/V)e^{-Dt} = S_0\,e^{-Dt}$, where m and D denoted amount of injected amine (mg) and dilution rate (h^{-1}). Suppose that the initial specific growth rate can be approximated by a linear approximation of $\mu \cong \mu^*(S_0) + (d\mu/dS)|_{S=S_0}(S - S_0) = \mu_0^* - k(S - S_0) \cong \mu_0 - k \cdot S \cong D - k \cdot S$, since a steady state was reached (i.e., $\mu_0 = D$). Since introduced amine and RR141-bearing solutions are assumed to be toxic, positive and negative value of k directly suggested the inhibitory and stimulating characteristics to overall bacterial growth, respectively. In the initial short period of time, Eq. (A1) can thus be written as

$$\frac{d\ln X}{dt} = -kS_0\,e^{-Dt}. \tag{A4}$$

After integration, one may obtain

$$\ln\left(\frac{X}{X_0}\right) = \frac{kS_0}{D}\left(e^{-Dt} - e^{-Dt_0}\right), \tag{A5}$$

where t_0 indicated the time delay in response to injection as suggested by Goldberg and Er-el [23]; and X and X_0 denoted cell concentrations at $t = t$ and $t = t_0$, respectively. Using Eq. (A4), one may obtain the kinetic parameter k to quantify whether the introduced chemical(s) are either a toxicant or stimulant to overall bacterial growth of *A. hydrophila*. It was also assumed that the difference of kinetic parameter k of studied case and kinetic parameter of amine-free control k_0 (i.e., $\Delta k = k - k_0$ or $\Delta k = \Delta k\big|_{\mathrm{MAA}} = k\big|_{\mathrm{MAA+RR141}} - k_0\big|_{\mathrm{RR141}} = k - k_0$) quantitatively indicated the stimulating (−) and inhibitory (+) effect of amine to *A. hydrophila*. When the toxicity of RR141 was subtracted from the combined toxicity of MAA and RR141-containing cultures, the sum of the toxicity of MAA and interactive toxicity between RR141 and MAA was apparently obtained (i.e., $\mathrm{Tox}_\Sigma - \mathrm{Tox}_{\mathrm{RR141}} \cong \mathrm{Tox}_{\mathrm{MAA}} + \mathrm{Tox}_{\mathrm{MAA\&RR141}}$). Thus, the postulate behind this study was that the interactive toxicity between RR141 and MAA was far smaller than the toxicity of MAA (i.e., $\mathrm{Tox}_\Sigma - \mathrm{Tox}_{\mathrm{RR141}} \cong \mathrm{Tox}_{\mathrm{MAA}}$).

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