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Application of the cellular oxidation biosensor to Toxicity Identification Evaluations for high-throughput toxicity assessment of river water

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HIGHLIGHTS

- A high-throughput cellular oxidation biosensor was applied to the TIE protocol.
- The newly developed TIE method is highly sensitive and rapid.
- The water sample volume was greatly reduced.
- The assay distinguished the toxicity profile in different water samples.
- A TIE protocol compatible with a large sample size is developed.

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ABSTRACT

Toxicity Identification Evaluation (TIE) is a useful method for the classification and identification of toxicants in a composite environment water sample. However, its extension to a larger sample size has been restrained owing to the limited throughput of toxicity bioassays. Here we reported the development of a high-throughput method of TIE Phase I. This newly developed method was assisted by the fluorescence-based cellular oxidation (CO) biosensor fabricated with roGFP2-expressing bacterial cells in 96-well microplate format. The assessment of four river water samples from Langat river basin by this new method demonstrated that the contaminant composition of the four samples can be classified into two distinct groups. The entire toxicity assay consisted of 2338 tests was completed within 12 h with a fluorescence microplate reader. Concurrently, the sample volume for each assay was reduced to 50 μL, which is 600 to 4700 times lesser to compare with conventional bioassays. These imply that the throughput of the CO biosensor-assisted TIE Phase I is now feasible for constructing a large-scale toxicity monitoring system, which would cover a whole watershed scale.

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1. Introduction

More than 30% of the globally accessible renewable freshwater is consumptively used for agricultural, industrial and municipal purposes (Schwarzenbach et al., 2006). Most of these human activities lead to contamination of environmental water with macro- and micro-pollutants that may exert detrimental effects on aquatic ecosystems.

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Whole effluent toxicity (WET) testing is a valuable tool for evaluating aggregated detrimental impacts of effluents (USEPA, 2000). However, WET test is not designed for identification of causative toxicants in the water bodies. Complementary to WET, Toxicity Identification Evaluation (TIE) is a regulatory tool to classify and/or identify the individual chemical substances in water samples, aiming at chemical contamination risk management (USEPA, 1993a, 1993b, 1991). TIE Phase I, which is conducted prior to Phase II and III procedures, consists of several sample manipulations in conjunction with laborious toxicity bioassay practices (USEPA, 1991).

Some studies have attempted to develop high-throughput cytotoxicity bioassays (Arias-Barreiro et al., 2010a; De Zwart and Slooff, 1983; Kim et al., 2003; Ooi et al., 2015; van de Merwe and Leusch, 2015). In the course of such studies, a whole-cell biosensor that detects cellular oxidation (CO) elicited by hazardous chemicals, utilizing genetically encoded fluorescent probe (the reduction-oxidation sensitive green fluorescent protein, roGFP) in a high-throughput fashion was invented (Arias-Barreiro et al., 2010a). It was claimed that this CO biosensor functions as one of the high-throughput cytotoxicity assays, which is assessable for biological responses to wide range of chemicals that evoke CO to the cells, and is rather robust against optical interferences owing to its ratiometric detection (Arias-Barreiro et al., 2010a). The implementation of such high-throughput bioassay would make the application of TIE to a large sample size possible. Consequently, this should promptly provide pertinent stakeholders with the decisive information regarding chemical contaminations occurring at numerous spots through the basin.

To develop a high-throughput TIE procedure, we prospected to confront four technological obstacles: (i) reduction of sample volume; (ii) modification of sample handling procedures for a large-scale application; (iii) streamlining sample manipulations; and (iv) improvement of the throughput of toxicity testing. In this study, we applied the CO biosensor to improve the throughput of TIE and performed TIE Phase I with river water samples collected from a river system running through agricultural and residential areas.

2. Materials and methods

2.1. Study area and sampling

River water was sampled from four sites (S1 − S4) in the Langat river basin as indicated in Fig. 1. The Langat river basin consists of approximately 1815 km^2 of total catchment area and is located in Peninsular Malaysia across the states of Selangor (78%), Negeri Sembilan (19%) and Federal Territories of Putrajaya and Kuala Lumpur (3%) (Farid et al., 2016; Juahir et al., 2011). The population in the basin is rapidly growing and projected to reach 1.68 million by year 2020 (Haque et al., 2014). The Langat river functions as the predominant source of potable water, as well as industrial and agricultural water supply in the region. It also serves as habitats for aquatic organisms. Langat river and its tributaries run through tropical fruits and rubber plantation areas and a densely populated residential district. River water pollution from many sources in this region has been reported (Juahir et al., 2011). Thereafter, contamination of surface water in the basin with heavy metals, radioactive elements, polycyclic aromatic hydrocarbons, endocrine disruptors, cytotoxins, hormones and drugs was reported (Farid et al., 2016; Haque et al., 2014). However, the cytotoxic effect of water samples from this area has not been thoroughly studied. To grasp the effectiveness of the CO assay for a high throughput TIE phase I to characterize the toxicants, the Langat river and its tributaries were chosen as the sampling points in this study.

Five hundred mL of surface water was collected from each site on 20th February 2014 either by using a plastic bucket lowered by rope over the side of the river-wall (S1, S2 and S3) or by direct dipping of the water sampling container into the stream (S4). The sampling was carried out facing upstream in all locations to avoid disturbance to the bottom sediment and the loss of volatile organic compounds. Water sampling containers were rinsed with the same water prior to collection to eliminate potential existing contaminants in the containers. The river water samples were transported immediately to the Chemical Sensor and Biosensor Laboratory (The National University of Malaysia, Bangi, Malaysia) and filtered with a Whatman qualitative filter paper No. 6, a Whatman qualitative filter paper No. 2 and a cellulose acetate filter (pore size = 0.22 μm), successively. This procedure avoids the interference in the following fluorescence assay from the absorption and scattering of light caused by microbial and solid particle contaminations. The filtered water samples were brought to the Laboratory of Environmental Response Systems (Institute of Plant Science and Resources, Okayama University, Kurashiki, Japan) and stored at 4 °C prior to TIE Phase I manipulation and toxicity testing.

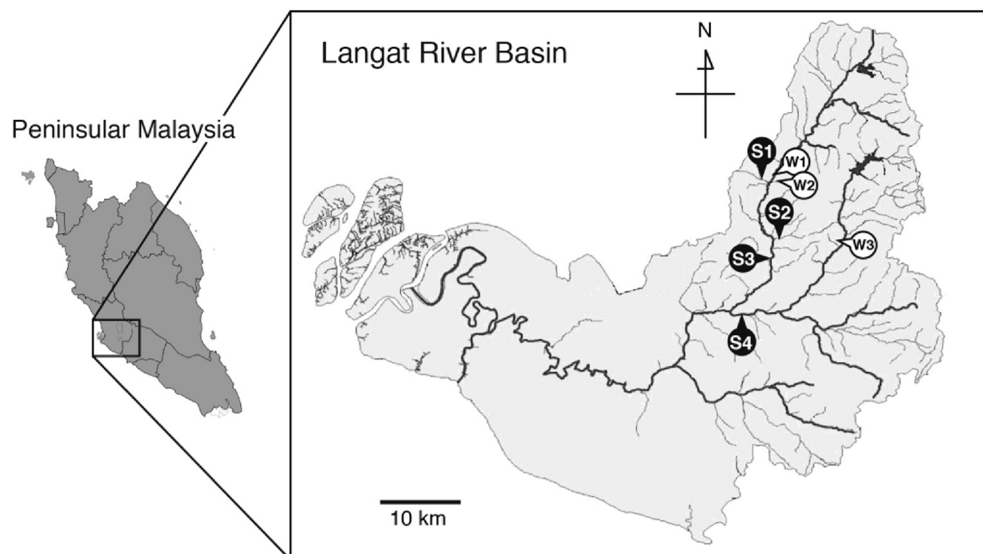
2.2. Physical and chemical characterization

Initial temperature of the water samples was measured with an alcohol thermometer. pH was measured with a glass electrode pH meter (model F-52, Horiba, Kyoto, Japan). Conductivity was measured with a hand-made conductivity meter. The concentration of total dissolved solids was calculated from the thermal conductivity at 20 °C, with a correlation factor of 0.6. Alkalinity was calculated from the carbonate and bicarbonate contents as described earlier (Snoeyink and Jenkins, 1980). Heavy metal concentration in the filtrates was determined by inductively coupled plasma mass spectrometry (ICP-MS, 7500cx, Agilent Technologies) as described previously (Arias-Barreiro et al., 2010b).

2.3. Preparation of *Escherichia coli* roGFP2 cellular oxidation biosensor, measurement of fluorescence and calculation of cellular oxidation index

roGFP2-expressing *E. coli* suspensions were prepared in a 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (pH 7.0) containing 171 mM NaCl according to Arias-Barreiro et al. (2010a). The roGFP2 gene was transcribed under the control of *GAL4* promoter in the strain DH5α (F^- ϕ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17(rk^-, mk+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ^-, Invitrogen Corporation, Carlsbad, CA), which did not require isopropyl β-D-1-thiogalactopyranoside for the *GAL4* induction.

Cellular oxidation (CO) was examined by measuring the ratio of fluorescence intensity of roGFP2 protein expressed in *E. coli* cells with two excitation wavelengths, 400 nm and 485 nm. The formation of disulfide bond between two cysteine residues in the β-sheet promotes the protonation of the tyrosine residue in the chromophore, leading to an increase in excitation spectrum peak near 400 nm at the expense of the peak near 485 nm. This fluorescence shift reflects the reduction-oxidation potentials of living cells (Hanson et al., 2004). Fluorescence of roGFP2 was measured using a fluorescence microplate reader (Powerscan HT, Dainippon Sumitomo Pharma, Osaka, Japan) as described previously (Arias-Barreiro et al., 2010a). In brief, 50 μL of the roGFP2-expressing *E. coli* suspension was dispensed into each well of a black flat-bottom 96-well microplate using an 8-channel pipette. Fluorescence emission at 525 nm with two excitation wavelengths at 400 and 485 nm was measured for 480 s with a 60-sec interval. Exposure was initiated by rapid addition of 50 μL of test solution to the pre-equilibrated cell suspension in wells using a multichannel



	Location	GPS coordinates	Description
S1	Sering River, Batu 9, Cheras	3°04'06"N 101°46'18"E	Sub-urban area connected with forest and agriculture on the upstream and residential area on the downstream
S2	Jelok River, Central Market, Kajang	2°59'27.3"N 101°47'33.3"E	Urban area complexed with commer-cial zone including a big wet market
S3	Langat River, Bandar Baru Bangi	2°57'50.2"N 101°47'01.3"E	Crowded residential area
S4	Langat River, Kampung Bukit Piatu, Dengkil	2°53'31.9"N 101°44'49.4"E	Rural area with agricultural land use
W1	Batu 9, Cheras	3°04'31.7"N 101°47'08"E	Sungai Langat Water Treatment Plant
W2	Kajang	3°02'42.2"N 101°46'46.3"E	Cheras Batu 11 Water Treatment Plant
W3	Taman Desa Mewah, Semenyih	2°57'52.5"N 101°50'44.0"E	Desa Mewah Water Treatment Plant

Fig. 1. River water sampling sites. S1 − S4 indicate sampling sites of river water. W1 − W3 indicate locations of water treatment plants at the upstream of the sampling sites. Grey area represents the catchment area of Langat river basin; thick black lines represent major rivers; thin black lines represent minor tributaries. N in the map indicates the direction of the north pole. GPS indicates the Global Positioning System. The wet market in the central market in Kajang city adjacent to S2 is a semi open-air market selling produce, fresh fish and fresh meat. Its drainage is lead to Jelok river, a tributary of Langat river, where the sample was collected.

pipette. Microplates were shaken for 1 s at the maximum speed of the instrument before each measurement.

The fluorescence ratio ($F_{ex400nm}/F_{ex485nm}$), cellular oxidation index (COI) and the difference in COI from the control, which represents the CO level at the resting condition (ΔCOI) were determined as the biochemical indicator for toxic action of samples on bacterial cells (for details, see Arias-Barreiro et al., 2010a). COI and ΔCOI were calculated as shown in Equation (1) and Equation (2), respectively.

$$COI = \int_{0}^{480} \frac{Fex400_{(t=x)}}{Fex485_{(t=x)}} dt \quad (1)$$

$$\Delta COI = (COI_s \,/\, COI_c - 1) \times 100 \quad (2)$$

where COI_s and COI_c indicate COI values of the sample and the control (ultrapure water), respectively. COI semi-quantitatively represents the cumulative degree of the ratio of oxidized roGFP2 molecule to the reduced molecule during the exposure to the samples. ΔCOI represents a change of COI from the control level in percentage.

2.4. Toxicity identification and evaluation

The TIE Phase I was conducted according to USEPA (1991) with slight modifications (Supplementary Fig. S1). The dilution series of samples, i.e. 6.25, 12.5, 25, 50 and 100%, were prepared by a serial dilution with ultrapure water (Milli-Q, Nihon Millipore KK, Tokyo, Japan). An initial toxicity test was performed prior to other TIE tests without pH adjustment (initial pH, pH_i). Toxicity tests were performed following sample manipulations: pH adjustment at pH 3 and pH 11; pH adjustment and filtration at pH 3, pH_i and pH 11; pH adjustment and aeration at pH 3, pH_i and pH 11; pH adjustment and reverse-phase solid phase extraction (SPE, Sep-Pak C_{18}, 100 mg, Waters Corporation) at pH 3, pH_i and pH 9; sodium thiosulfate ($Na_2S_2O_3$) oxidant reduction at 0.01, 0.1 and 1.0%; graduated pH at pH 6, pH 7 and pH 8; and ethylenediamine tetraacetate (EDTA)

ligand chelation at 1 and 10 mM.

pH adjustment was carried out using 0.1 M HCl and 0.1 M KOH, accordingly, in a 20-mL volume. Where needed, 20 mL of pH-adjusted water samples were filtered with Whatman qualitative filter paper No. 2. Aeration was carried out with 2 mL of water samples in a 13 mm × 100 mm borosilicate test tube by constant shaking at approximately 1250 rpm for 60 min with a test tube mixer (MicroMixer E-36, TAITEC, Koshigaya, Japan). Two mL of pH-adjusted river water samples were subjected to SPE. A Methanol Elution Test was performed according to the method described in USEPA (1991), where indicated. Prior to the toxicity testing of sample water, it was confirmed that the addition of $Na_2S_2O_3$ and EDTA did not affect the basal fluorescence readout of the CO biosensor (Supplementary Fig. S2).

2.5. Data analysis

Hierarchical clustering analysis (HCA) was undertaken using the *hclust* function of *stats* package of R software with complete linkage clustering method (version 3.2.4, R Core Team, 2013). Other statistical analyses are described in figure legends.

2.6. Chemicals

All chemicals were analytical grade and procured from Nacalai Tesque Inc. (Kyoto, Japan), Wako Pure Chemical Co. (Osaka, Japan) or Sigma-Aldrich Inc. (St. Louis, MO, USA).

3. Results

3.1. Initial toxicity test

The CO assay crucially relies on fluorescence measurement. Therefore, an inspection of optical transmittance spectra is compulsory prior to the testing. Transmittance (%T) of the water samples at 400 nm for the excitation of the oxidized form of roGFP2, 485 nm for that of the reduced form of roGFP2 and 525 nm for the emission, were in the ranges of 91.9 − 96.0%, 96.9 − 98.5% and 97.8 − 98.8% in the four samples, respectively (Supplementary Fig. S3). This indicates that COI values measured by CO biosensor may underestimate the actual oxidative stress level by 2.2 − 5.2%. These minor interferences would not cause a practical hindrance to ratiometric analysis of the dynamic changes in roGFP2 fluorescence in this study.

The fluorescence ratios and COIs of the initial toxicity tests of the river water samples are shown in Fig. 2. An increase in fluorescence ratio of the CO biosensor was observed within 10 s after the onset of the exposure, indicating that the *E. coli* cells were oxidized immediately by all four water samples (Fig. 2a). This immediate increase was not of the artifact derived from optical interference of fluorescence measurement as discussed above (Supplementary Fig. S3). The exposure of S1 to the biosensor induced the highest COI level at 100% water sample concentration (WSC) among water samples and reached the maximum at 50% dilution (Fig. 2b). S2 and S3 showed a similar COI level through the dilution series, reaching to the highest COI of approximately 31 at WSC = 100% (Fig. 2c and d). S4 had the apparently least effect on CO among four samples (Fig. 2e). These observations suggest that all 4 water samples from the Langat river basin contained substances that exert oxidative stress on bacterial cells, while the degree of the stress were varied.

3.2. Toxicity identification and evaluation

Since all river water samples exhibited significant CO activities, we then characterized the toxicity in the samples by the TIE phase I procedure (USEPA, 1991). The results of TIE analysis in which toxicity evaluated as ΔCOI are summarized in a heat map (Fig. 3).

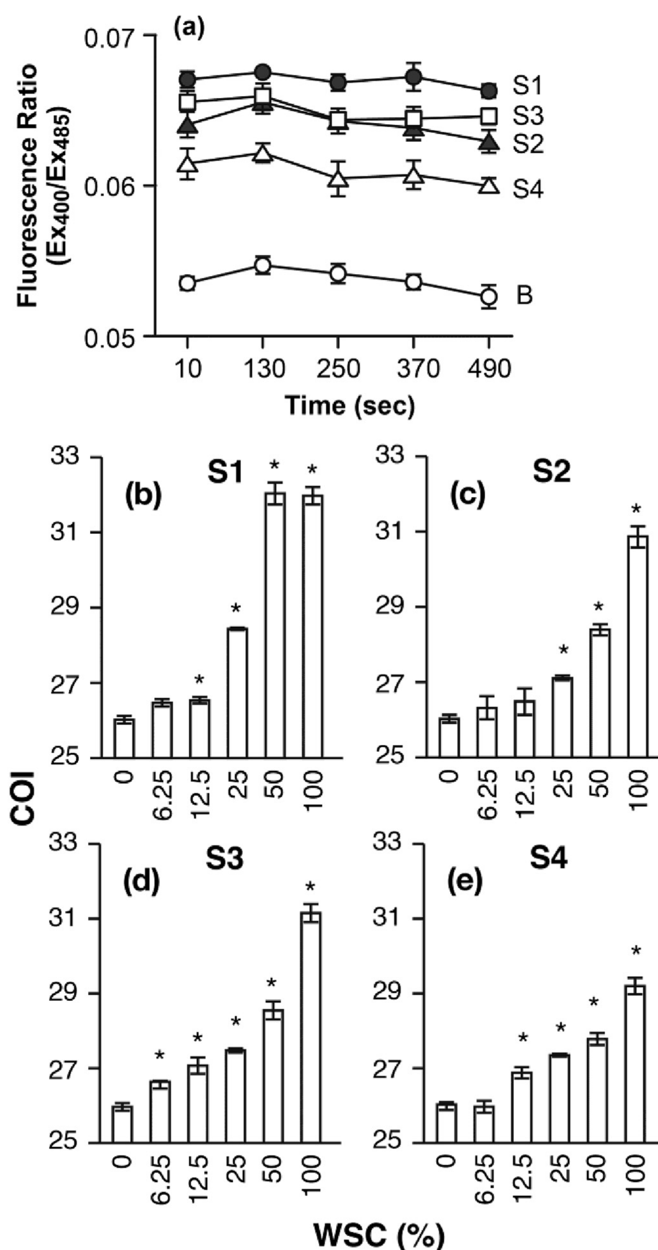


Fig. 2. Initial toxicity assay of river water samples utilizing the *E. coli* roGFP2 CO biosensor. (a) Temporal change in fluorescence ratio of the CO biosensor exposed to 100% river water sample concentration (n = 4). The fluorescence reading started at 10 s after the addition of samples in the microplate wells. B, blank control with pure water. (b)–(e) Effect of river water samples on COI in different concentrations of river water samples (n = 4). COI: cellular oxidation index; WSC: water sample concentration. Error bars indicate standard deviations. Asterisks (*) represent statistical significance from WSC = 0% at $\alpha = 0.05$ by Dunnett's test.

The patterns of enhancement/diminishment in CO activity by sample manipulations were apparently different among samples even though these water samples were collected from the same river system. For example, filtration at pH*i* reduced CO activity in S1 and S4, but not significantly in S2 and S3. SPE increased CO activity in S1 and S4 at any pH, but rather reduced in S2 and S3. A huge reduction of CO activity by aeration was observed in S4. EDTA reduced CO activity in S1, S2 and S4, but not in S3. These observations suggest that each sample is not only different in the degree

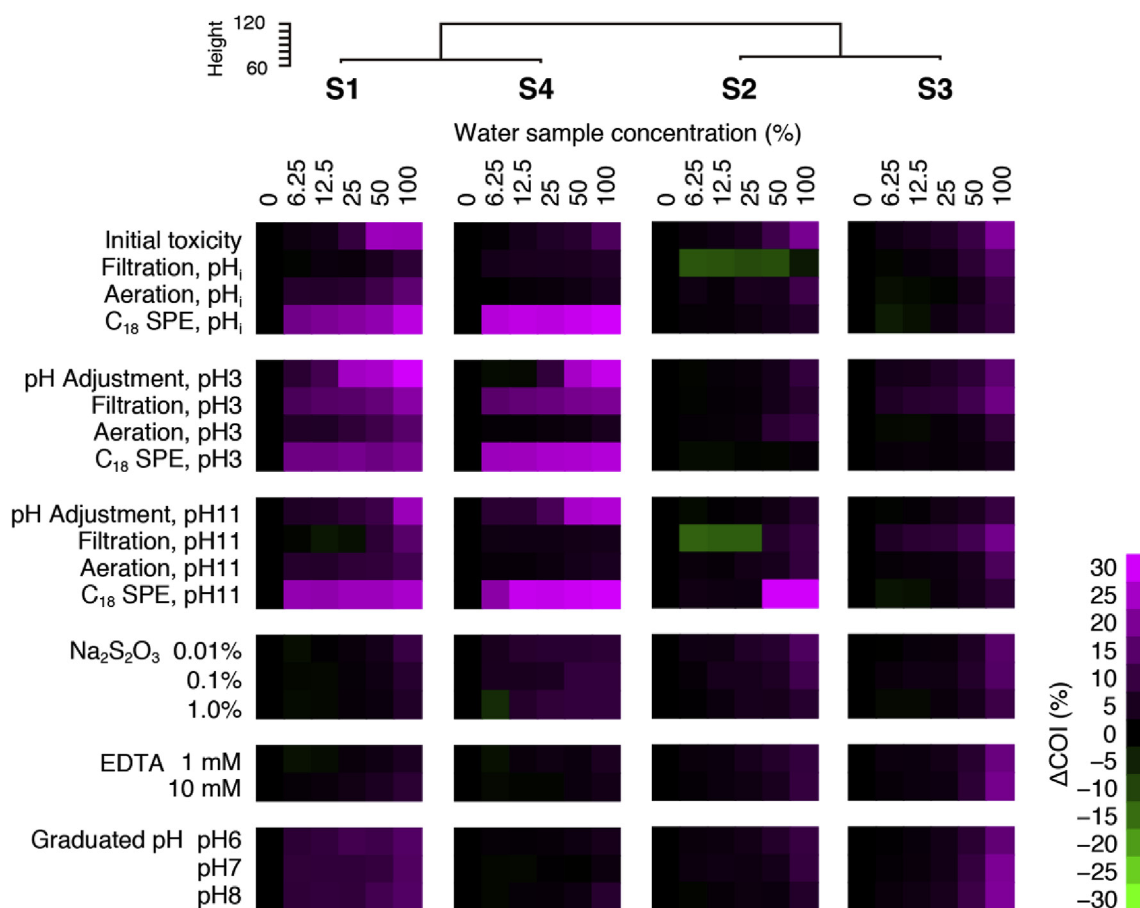


Fig. 3. Heat map presentation of TIE analysis of Langat river basin samples determined as CO activity. Difference in cellular oxidation index (ΔCOI) is displayed in a color gradation scale as indicated on the right. Sample manipulations are indicated on the left. The dendrogram at the top demonstrates similarity in toxicity enhancement/reduction profiles computed by the hierarchical clustering. Each data is represented as graph in Supplementary Figs. S3–S6. The source data for Fig. 3 is provided as an Appendix. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

of CO activity, but also contains different composition of toxicants.

We examined the similarity in toxicity enhancement/diminishment profile among the samples by HCA. The characteristics of toxicity of the four water samples are categorized into two major groups, one comprised of S1 and S4, and another of S2 and S3 (the dendrogram in Fig. 3). In the following, we describe the characteristics of each sample: first, referring to the left branch of the dendrogram comprised of S1 and S4; followed by the right branch comprised of S2 and S3.

3.2.1. *Sample S1*

As mentioned above, sample S1 induced CO in the biosensor (Fig. 3 and Supplementary Fig. S4a). ΔCOI increased along with the increase in water sample concentration (WSC) and reached to 23.5 ± 0.4% at 100% WSC ($\Delta COI_{Initial\ pHi,\ WSC100\%}$). Filtration significantly mitigated CO at pH*i*, $\Delta COI_{Filtration\ pHi,\ WSC100\%} = 8.3 \pm 0.2\%$. Reduction in CO activity of sample S1 by the filtration was also observed at pH 11 ($\Delta COI_{Filtration\ pH11,\ WSC100\%} = 15.0 \pm 0.3\%$) (Supplementary Figs. S4c and g). However, only a weak mitigation was observed at pH 3 compared with pH*i* and pH 11 ($\Delta COI_{Filtration\ pH3,\ WSC100\%} = 21.4 \pm 0.5\%$) (Supplementary Fig. S4b). The reduction was also observed by the addition of $Na_2S_2O_3$ and EDTA, $\Delta COI_{Na2S2O3\ 1\%,\ WSC100\%} = 6.4 \pm 0.2\%$ and $\Delta COI_{EDTA\ 10mM,\ WSC100\%} = 8.1 \pm 0.4\%$, respectively (Supplementary Figs. S4d, e, h and i). Collectively, these results suggest that metal ions were the causative substances for the CO in sample S1. Elemental analysis by ICP-MS revealed that sample S1 contained relatively high concentration of Zn, Mn and Cu (Table 1). Ions derived from these metals have previously been confirmed to induce CO in the biosensor (Arias-Barreiro et al., 2010a).

As $Na_2S_2O_3$ reduced the CO activity of the sample, the involvement of $Na_2S_2O_3$-sensitive oxidants can also be suspected. The

Table 1
Concentrations of selected elements in dissolved fraction of river water samples.

Element	Concentration (μg L^{-1})			
	S1	S2	S3	S4
Mg	318	375	513	241
Al	4.06	4.11	5.66	19.3
Ca	678	949	1639	329
Cr	0.22	0.18	0.20	0.06
Mn	8.37	5.50	1.55	7.48
Fe	240	32.6	31.4	46.5
Co	0.05	0.21	0.17	0.09
Cu	2.30	1.39	3.07	2.42
Ni	2.18	5.61	6.62	2.50
Zn	22.9	3.71	5.51	3.24
As	3.31	9.53	4.55	1.63
Se	0.09	0.11	0.10	0.04
Cd	0.01	0.11	0.06	0.00
Sn	0.08	0.32	0.14	0.05
Pb	0.24	0.18	0.22	0.18

Elements are listed in the order of atomic number.

aeration also caused a reduction in the CO activity of sample S1 at all pH ($\Delta COI_{Aeration\ pHi,\ WSC100\%}$ = 15.7 ± 0.4%, $\Delta COI_{Aeration\ pH3,\ WSC100\%}$ = 14.8 ± 0.4% and $\Delta COI_{Aeration\ pH11,\ WSC100\%}$ = 11.8 ± 0.4%) (Supplementary Figs. S4a, b, c and j). This indicates that volatile constituents and short-chain surfactants may be responsible for the observed CO activity.

The SPE procedure remarkably increased the CO activity in all pH. ΔCOI dramatically increased by the passing of the samples through the SPE cartridge at 6.25% and 12.5% of WSC ($\Delta COI_{Initial\ pHi,\ WSC6.25\%}$ = 2.3 ± 0.2% to $\Delta COI_{SPE\ pHi,\ WSC6.25\%}$ = 18.4 ± 0.2%; $\Delta COI_{Adjustment\ pH3,\ WSC6.25\%}$ = 7.9 ± 0.2% to $\Delta COI_{SPE\ pH3,\ WSC6.25\%}$ = 18.1 ± 0.3% and $\Delta COI_{Adjustment\ pH11,\ WSC6.25\%}$ = 5.8 ± 0.3% to $\Delta COI_{SPE\ pH11,\ WSC6.25\%}$ = 22.7 ± 0.2%) (Supplementary Figs. S4a, b, c and k). This suggests the masking effect of water matrices on the toxicants were removed by the SPE procedure. Presumably, humic substances derived from tropical plantation were the leading cause of the masking effect (Kawahigashi and Sumida, 2010).

The graduated pH manipulations did not show significant difference in ΔCOI (Supplementary Fig. S4f), indicating that the major toxicants were unlikely to be ammonia (NH_3) or hydrogen sulfide (H_2S).

3.2.2. Sample S4

The aeration treatment mitigated the CO activity of sample S4 in all tested pH ($\Delta COI_{Aeration\ pHi,\ WSC100\%}$ = 5.2 ± 0.2%, $\Delta COI_{Aeration\ pH3,\ WSC100\%}$ = 4.8 ± 0.2% and $\Delta COI_{Aeration\ pH11\ WSC100\%}$ = 5.1 ± 0.2%) (Fig. 3; Supplementary Figs. S5a, b, c and g). This indicates that volatile compound(s) and/or non-polar surfactants in the water sample confer oxidative stress to the bacterial cells.

The CO activity of sample S4 was increased by filtration at pH 3 ($\Delta COI_{Filtration\ pH3,\ WSC6.25\%}$ = 15.1 ± 2.4%) comparing to that of pH adjustment at pH 3 alone ($\Delta COI_{Adjustment\ pH3,\ WSC6.25\%}$ = −1.4 ± 0.2%) (Supplementary Fig. S5b). Addition of $Na_2S_2O_3$ and EDTA substantially removed the CO activity from the water sample ($\Delta COI_{Initial\ pHi,\ WSC100\%}$ = 13.3 ± 0.4% to $\Delta COI_{Na2S2O3\ 0.01\%,\ WSC100\%}$ = 8.3 ± 0.2% and $\Delta COI_{EDTA\ 10mM,\ WSC100\%}$ = 3.7 ± 0.7%) (Supplementary Figs. S5d, e, i and j). These indicate that toxicity of sample S4 is partly attributable to cationic metals. ICP-MS analysis showed high concentrations of Al, Cu, Mn and Zn (Table 1). Contamination of these metals can be the reason for the toxicity of sample S4, while we do not deny the involvement of $Na_2S_2O_3$-sensitive oxidants.

The SPE increased CO effect of sample S4 on the biosensor in all tested pH at 6.25% WSC, $\Delta COI_{Initial,\ WSC6.25\%}$ = 0.4 ± 0.3% to $\Delta COI_{SPE\ pHi,\ WSC6.25\%}$ = 27.5 ± 0.3%; $\Delta COI_{Adjustment\ pH3,\ WSC6.25\%}$ = −1.4 ± 0.2% to $\Delta COI_{SPE\ pH3,\ WSC6.25\%}$ = 23.9 ± 0.3% and $\Delta COI_{Adjustment\ pH11,\ WSC6.25\%}$ = 7.9 ± 0.3% to $\Delta COI_{SPE\ pH9,\ WSC6.25\%}$ = 21.7 ± 0.1% (Supplementary Fig. S5h). This enhancement of CO activity may be attributed to removal of the masking effect of organic substance.

The graduated pH test showed no significant effect indicating that NH_3 or H_2S, were not responsible for the toxicity on the bacterial cell (Supplementary Fig. S5f).

3.2.3. Sample S2

The CO activity of S2 was not significantly reduced by filtration except pH 3 and aeration, in contrast to samples S1 and S4 ($\Delta COI_{Initial\ pHi,\ WSC100\%}$ = 19.3 ± 0.5%, $\Delta COI_{Filtration\ pHi,\ WSC100\%}$ = 11.6 ± 0.3%, $\Delta COI_{Aeration\ pHi,\ WSC100\%}$ = 11.0 ± 0.4%; $\Delta COI_{Adjustment\ pH3,\ WSC100\%}$ = 9.4 ± 0.2%, $\Delta COI_{Filtration\ pH3,\ WSC100\%}$ = 7.4 ± 0.3%, $\Delta COI_{Aeration\ pH3,\ WSC100\%}$ = 9.8 ± 0.4%; $\Delta COI_{Adjustment\ pH11,\ WSC100\%}$ = 7.2 ± 0.1%, $\Delta COI_{Filtration\ pH11,\ WSC100\%}$ = 18.1 ± 1.1% and $\Delta COI_{Aeration\ pH11,\ WSC100\%}$ = 9.3 ± 0.4%) (Fig. 3; Supplementary Figs. S6a, b, c and g). A slight decrease in the CO activity was observed after SPE at pH*i* ($\Delta COI_{SPE\ pHi,\ WSC100\%}$ = 6.5 ± 0.5%) and a large decrease was detected at pH 3 ($\Delta COI_{SPE\ pH3,\ WSC100\%}$ = 1.1 ± 0.1%), while a significant increase was observed at pH 11 ($\Delta COI_{SPE\ pH11,\ WSC100\%}$ = 17.3 ± 0.1%) (Supplementary Figs. S6a, b, c and g). Therefore, we deduce that the causative compounds for the CO in sample S2 were organic acid substances and oxidants, and a masking effect was removed at the alkaline pH.

ΔCOI was significantly reduced by the addition of $Na_2S_2O_3$ and EDTA to sample S2 ($\Delta COI_{Initial,\ WSC100\%}$ = 19.3 ± 0.5% to $\Delta COI_{Na2S2O3\ 1.0\%,\ WSC100\%}$ = 7.2 ± 0.7% and $\Delta COI_{EDTA\ 10mM,\ WSC100\%}$ = 9.6 ± 0.2%) (Supplementary Figs. S6d, e, h and i). Cd, Cu and Zn, which were reported to induce CO in the biosensor (Arias-Barreiro et al., 2010a), were detected in sample S2 (Table 1). Consequently, we presumed that cationic metals were the major toxicants in addition to organic acid substances. A high concentration of As was also detected in sample S2 (Table 1). It is noteworthy that arsenite has been reported to induce cellular oxidation in the CO biosensor (Arias-Barreiro et al., 2010a; Ooi et al., 2015) and the application of $Na_2S_2O_3$ reduces arsenite to less toxic arsenate (Anezaki et al., 1999). Taken together, arsenite may be one of the toxicants in sample S2.

The graduated pH manipulation did not significantly affect the CO activity, eliminating the potentiality of NH_3 or H_2S contamination (Supplementary Fig. S6f).

3.2.4. Sample S3

The aeration treatment at pH*i* and pH 3 substantially alleviated the CO activity of sample S3 ($\Delta COI_{Initial\ pHi\ WSC100\%}$ = 20.6 ± 0.4% to $\Delta COI_{Aeration\ pHi\ WSC100\%}$ = 10.7 ± 0.5%; $\Delta COI_{Adjustment\ pH3,\ WSC100\%}$ = 15.7 ± 0.4% to $\Delta COI_{Aeration\ pH3,\ WSC100\%}$ = 8.8 ± 0.3%) (Fig. 3; Supplementary Figs. S7a, b, c and g), indicating the presence of surfactants and volatiles as major toxicants.

The SPE reduced ΔCOI at pH*i* and pH 3 ($\Delta COI_{SPE\ pHi\ WSC100\%}$ = 9.9 ± 0.6% and $\Delta COI_{SPE\ pH3,\ WSC100\%}$ = 4.9 ± 0.2%) (Supplementary Figs. S7a, b, c and h). Besides SPE manipulation, the alkaline treatment of sample S3 per se reduced the CO activity in sample S3 ($\Delta COI_{Adjustment\ pH11,\ WSC100\%}$ = 9.8 ± 0.3%) (Fig. 3; Supplementary Fig. S7c). This suggests that the causative substances are alkaline-labile compounds. To further characterize the causative toxic chemicals associated with the reduction of the

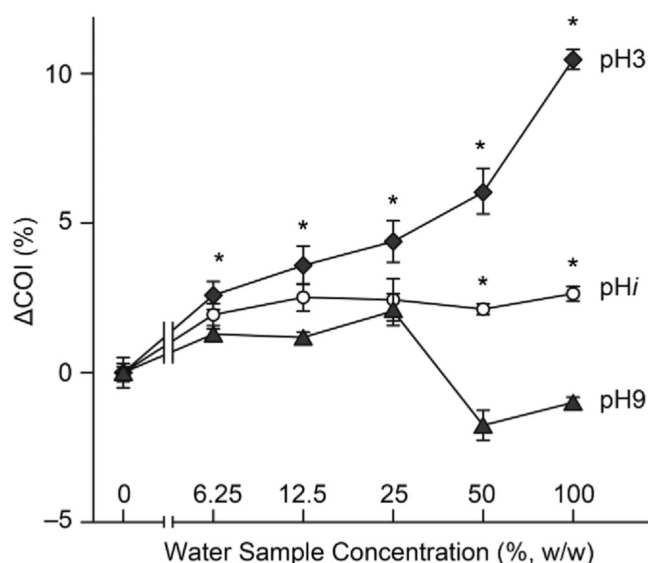


Fig. 4. Cellular oxidation activity of SPE eluate of sample S3 as represented by difference in cellular oxidation index (ΔCOI). pH*i* =7.89. Error bars indicate standard deviations. Some error bars for pH*i* are too small to be seen. Asterisks indicate significance from WSC = 0% control by one-tailed Dunnett's test (n = 3, α = 0.05).

cellular oxidizing activity by SPE, we employed methanol elution. A high ΔCOI value was observed in the eluate at pH 3 (Fig. 4). A weaker CO activity was retrieved at pH*i*. SPE eluates processed at pH 9 did not result in an appearance of CO activity, most likely due to the lability of the toxicant in alkaline pH and/or less affinity of the toxicants to the SPE at alkaline pH. This strongly supports that the toxicity of sample S3 is, at least in part, due to organic substances, most likely the organic acids.

The addition of EDTA showed no apparent effect on ΔCOI of sample S3 ($\Delta COI_{EDTA\ 10\ mM,\ WSC100\%} = 18.8 \pm 0.2\%$) (Supplementary Figs. S7e and i). On the contrary, addition of $Na_2S_2O_3$ to sample S3 reduced ΔCOI significantly ($\Delta COI_{Na2S2O3\ 1.0\%,\ WSC100\%} = 10.6 \pm 0.8\%$) (Supplementary Figs. S7d and j). These results imply the presence of oxidants such as free chlorine or soft Lewis metal ions like Cu^+ (Table 1). The presence of chlorine is compelling, as S3 resides at downstream of congested residential areas in contact with municipal effluents (Fig. 1), while it was not confirmed in this study.

An increase in toxicity was observed when pH is increased from 6 to 7 and 8 (Supplementary Figs. S7f and k). NH_3 demonstrated higher toxicity when pH increases; while H_2S behaves in the opposite due to formation of its HS^- ionic form, which is less toxic (USEPA, 1991). This indicates that NH_3 is another potential toxicant contained in sample S3.

4. Discussion

4.1. Comparison of toxicity composition among water samples

HCA demonstrates that the structure of toxicity composition (organization of factors characterizing the toxicity) in four water samples was categorized into two major branches, one comprised of S1 and S4, and another of S2 and S3 (Fig. 3). It is conceivable that the similarity in toxicity composition structure reflects common features of the contamination sources. Evidently, the former is characterized in part by the enhancement of toxicity by the SPE manipulation (Fig. 3). This might be due to the removal of the masking effect on the toxicity. As S1 and S4 reside around pineapple, rubber tree and other tropical plantations (Fig. 1), it is reasonable to expect a high humic load, which masked the toxicity of heavy metals (Table 1). While the latter comprising of S2 and S3 is most likely to appertain to municipal wastewater as the sampling sites are located in urban residential areas. It is conceivable that S2 and S3 included surfactants, which were originated from the municipal sewage (Field et al., 1992). Collectively, we concluded that this TIE analysis assembled with the CO biosensor has successfully evaluated the structure of toxicity composition of water samples. These results imply that: (1) high-throughput CO biosensors are capable of evaluating toxicity of environmental water samples as an assay incorporated into TIE procedure, and (2) HCA helps to grasp the similarities in the contaminant content among the river water samples in a large sample size.

4.2. Potential sources of contamination and causative agents

Contribution of volatile compounds and/or short-chain anionic surfactants to the toxicity was suggested in S1, S3 and S4 (Fig. 3). These compounds may arise from household and agricultural emissions such as pharmaceutical products, cleaning agents and pesticides as discussed by Soh and Abdullah (2007). The great reduction of the CO activity by SPE in samples S2 and S3 at pH 3 suggests the involvement of organic acid compounds, such as long-chain anionic surfactants, which are also potentially derived from households and/or agricultural activities. We suspect the involvement of such organic acid compounds in samples S1 and S4 as well. The immense CO-enhancing effect by removal of masking substances by SPE might interfere the detection of CO activities associated with toxic organic compounds. In conclusion, it is deducible that significant areas in Langat river basin are contaminated by anionic surfactants from municipal wastewaters and agricultural leachates.

All four water samples showed some sensitivity to $Na_2S_2O_3$ in CO activities (Fig. 3). This may be attributed to the contamination of oxidants in water samples as well as soft Lewis metal ions such as Cd^{2+} and Cu^+ (Table 1). We presumed that the oxidants in the samples were originated from water treatment plants (WTPs) (Sungai Langat WTP [W1], Cheras Batu 11 WTP [W2] and Desa Mewah WTP [W3]) located on the upstream of the sampling sites (Fig. 1). An advanced oxidation process in WTPs uses oxidants such as chlorine, ozone, hydrogen peroxide or photo-catalysts to produce hydroxyl radicals for removing micropollutants in the water (Koepke et al., 2009; Lee and von Gunten, 2010). In addition, S2 is located in the vicinity of a wet market, where chlorine is used for sanitary purposes. Therefore, it was inferred that river water was contaminated with some oxidants from WTPs and the market.

Cationic metals were observed in all four water samples, while the species and quantity were varied among the sites (Table 1). Reportedly, agricultural and industrial activities can be primary sources of heavy mental contamination, although they exist naturally in rocks and the soil crust (Bradl, 2005). Water in the Langat river basin has been reported to contain high concentrations of metals likely from household discharges, economic and industrial activities (Ahmad Zaharin et al., 2015; Lim et al., 2012). It should be mentioned that Fe^{3+}, Co^{2+}, Mn^{2+} and Ni^{2+} were not the metal ions that induced CO in the biosensor in this study, since they did not induce CO in the biosensor (Supplementary Fig. S8).

Graduated pH analysis suggested that S3 contains H_2S and/or NH_3 (Supplementary Fig. S7). This sampling site is located in the middle of a crowded residential area and is highly potential to receive animal and human waste, which can be a likely cause of H_2S and NH_3 contamination (Kafle and Chen, 2014; Sato et al., 2001). Anaerobic respiration activities by sulfate- and sulfur-reducing bacteria is also reported to be a source for H_2S in the water sample (Camacho, 2010). The muddy riverbank bearing tropical shrubs around S3 could be one of the sources of H_2S contamination to the river water.

4.3. Improvement of throughput of TIE

Several advantages of the CO biosensor-assisted TIE Phase I mentioned were noticed over conventional TIE procedures. Table 2 shows comparison of preparation procedure, exposure time and required water sample volume so that the improvement of test throughput can be evaluated.

Conventionally, significant cost and labor are paid for the preparation and the maintenance of the test organisms in acute toxicity assays. As compared to the conventional animal/algae-based bioassays, Microtox®, promoter-reporter gene-based and CO-biosensor bioassays are less laborious in term of preparation of test organisms. This biosensor fabricated using living *E. coli* cells can be stored in a −70 °C deep freezer for decades and routinely cultured from the frozen stock by the common molecular biological procedure (Sambrook, 2001). This preparation procedure of the biosensor is obviously more concise to compare with that of the hatching and the subsequent maintenance of cladocerans, the adaptation of fish to the testing environment and the culture of algae in a culture chamber equipped with an illuminating device. Although bioluminescent-based bacterial respiration inhibition test (Microtox®) only requires minimum effort for organism/reagent preparation, it is needed to maintain the samples at low temperature in the special equipment (3 °C for reconstitution of the

Table 2
Comparison of the conventional methods used in TIE Phase I to our CO biosensor-assisted TIE.

Property	Animal/algae-based Bioassays (USEPA, 1991)	Bioluminescence-based bioassay (Manufacturer's manual, Microtox® model 500 analyzer)	Promoter-reporter gene-based bioassays (Köhler et al., 2000; van der Meer and Belkin, 2010)	CO biosensor-assisted TIE (This study)
Test organisms	Fish, Cladocerans, Crustaceans, algae etc.	*Aliivibrio fishcheri* (marine symbiotic bacterial cells)	Bacterial or cultured cells carrying an inducible reporter genes (Luc, GFP, etc)	Recombinant *Escherichia coli* cells constitutively expressing roGFP2
Preparation of test organisms	2 days to 2 weeks for hatching, preculture and/or adaptation of the organisms in specific testing media	Not required; but preparation of samples is required including salinity adjustment	12–42 h, depending on the species	18–24 h for a preculture in Luria-Bertani broth
Volume for each exposure (mL)	1 to 235	1.0	N/A	0.05
Volume of water sampling (L)	1.8 to 2.7	N/A	N/A	0.25 to 0.3
Exposure/ response time	15 min to 96 h	15 min	2 min to 17 h	6–8 min
Assay temperature	Algae: 21–24 °C Daphnia, fish: 25 °C	15 °C	30 or 37 °C	20–25 °C
Range of detectable toxicants	Any	Very broad	Highly specific	Broad, but not fully comprehensive

reagent and 15 °C for the prior temperature stabilization and the testing).

We also perceived that the working space for the CO assay utilizing the biosensor and promoter-reporter gene assay could be performed in a very compact laboratory space, as the biosensor was ordinarily prepared in a 96-well microplate format (127.7 × 85.4 mm). The sample volume of each assay was reduced to as low as 50 μL. Concurrently, this leads to a great reduction in water sample volume (6 − 10 times lesser). In this study, we sampled 500 mL at each site. This made the sampling and the subsequent sample manipulation procedures faster and less labor-intensive.

As previously reported (Arias-Barreiro et al., 2010a), the response of roGFP2-based CO biosensor to toxic samples is very quick. We detected the CO response within 10 s after the onset of the exposure and the elevated CO level lasted for more than 8 min (Fig. 2). Moreover, this assay can be performed on 96 samples in parallel, suggesting the potential of this assay to allow the evaluation of 96 test samples in as short as 8 min (Table 2). This may enormously improve the throughput of the bioassay inexpensively. Given that 5 dilutions of water samples were analyzed in 4 replicates, five 96-well microplates would be sufficient to complete the whole TIE phase I assay. We estimate that toxicity assay for a whole TIE assay can be completed by one examiner in a few hours. This strongly manifests the high efficiency of the CO biosensor-assisted TIE over the conventional methods in terms of sample processing rate. This great throughput of the CO biosensor shows a significant advantage over the conventional bioassays and the bioluminescence-based bacterial assay.

There are many other GFP-based toxicity assays with different mode of action (for example, see references in Table 2). Majority of GFP-based assays rely on the promoter-reporter gene assay. This assay depends on the specific interaction between stimulant (toxicant) and *cis*-element in the promoter DNA sequences mediated by the associated transcription factor. Therefore, the tests have a tendency toward narrower specificity to detectable toxicants. Furthermore, some promoter response is very quick, but many others take a long time until the reporter gene is induced (up to 17 h) (Table 2). Contrary to the reporter gene assays, the CO assay has a broad specificity to unknown contaminated toxicants in the river water sample. Although, the broad specificity of the CO assay to the toxicants is not fully comprehensive.

4.4. Application of the high-throughput biosensor-assisted TIE to whole watershed scale toxicity evaluation and future prospective

Contamination and overexploitation of the water resource in the upstream of the watershed intercept water use in the downstream. Therefore, one should take account of water usage and water quality management with a comprehension of the entire watershed covering from the springhead regions to the blackish river mouth, as a continuum. Considering conducting ecotoxicological risk assessment in the whole watershed, toxicity assessment at a significant number of sampling sites would be required. While conventional water quality evaluation has been employed to monitor the water quality at a whole-watershed scale (for example, see Juahir et al., 2011), chemical management based on ecotoxicity testing in a watershed scale has not often been exploited. This should be chiefly attributed to the limited throughput of the toxicity bioassays. In this study, we substituted conventional toxicity bioassays with the CO biosensor to increase the assay throughput. Given that 30 sampling stations are needed to reasonably cover a whole basin, the new method developed in this study is capable to evaluate all of the samples by TIE in a few days. We deem that the whole watershed-scale water toxicity management has become progressively more feasible.

In addition to the establishment of high-throughput bioassays, we realized that the improvement of sample manipulation procedure is the next decisive factor to foster a high-throughput TIE in addition to the improvement of bioassays during the pursuance of the experiments. It was not difficult to conduct the filtration, chemicals addition, SPE and aeration procedures even in a large

sample size. On the other hand, pH adjustment procedure was the bottleneck for work efficiency promotion. Development of a high-speed pH adjustment procedure in a small scale or an alternative method that can substitute pH adjustment procedure is desperately needed to further reduce the workload.

We performed classification of the toxicity structure of the water samples by HCA. The development of an efficient data processing method that converts the collected data to interpretable format would be demanded to deal with a huge sample size. Introduction of artificial intelligence would be helpful for the development of a rapid and efficient analytical method to surmise the source of the contamination and the fate of the toxicants from the results of a high-throughput TIE. Studies on technical improvement related to prompt collection and appropriate storage of water sample is also indispensable. It is important to tackle not only the technological advancement but also the social problems. Establishing the social consensus for water samples collection throughout the watershed and a proper procedure of sharing the toxicity data would be necessary in parallel.

This study only aimed at developing a high throughput TIE Phase I. To complete TIE analysis in a high throughput fashion, improvements of Phase II and Phase III are also required. TIE Phase II is a step to identify the causal toxicants by chemical analysis. Recent advance in non-targeted LC-MS analysis can aid in high speed identification of contaminated pollutants in aquatic samples (Bletsou et al., 2015; Herrera-Lopez et al., 2014). Combination of high throughput Phase I and Phase II would accelerate the identification of candidates for causal toxicants in environmental water. On the other hand, acceleration of Phase III may be more difficult. TIE Phase III is a step for validation of the toxicants identified by Phase II with biological assays. One can perform each toxicity assay with a number of candidates promptly taking advantage of a high throughput toxicity assay, such as the CO biosensor or other alternatives. However, the preparation of authentic substances of candidate toxicants may not always be instant. Construction of chemical libraries for potential toxicants in the area which can be shared with pertinent sectors should be undertaken. It should also be noticed that TIE Phase III is highly recommended to be conducted ultimately using conventional test organisms, such as fishes, crustaceans and algae in addition to the high throughput assay to gain insight into the ecotoxicological implications.

We fabricated a high throughput method using the bacterium, *E. coli* as the sensor organism. Bacterial cells are decomposers in the ecosystem. Toxic action on bacterial cells represents only a part in the ecological niche. A battery of tests using several organisms across the ecological niche is required to comprehensively understand the ecotoxicity. We expect that the development of a comprehensive, high-throughput TIE Phase I procedure could contribute to chemical management as a part of the integrated watershed management framework.

We keep in mind that the roGFP2-based CO biosensor is not the only solution for improvement of throughput of toxicity bioassay. The roGFP2-expressing living *E. coli* cell biosensor relies on fluorescent change evoked by cellular oxidation, which is a consequence of disulfide formation between two key cysteine residues in the β-sheet of the roGFP2 protein in the presence of CO-inducing toxicants (Dooley et al., 2004). Other types of high throughput bioassays should be attempted to make a high throughput TIE possible. A biochemically targeted toxic reaction may not predict joint toxic actions in a mixture. Therefore, a combination of several high throughput assays based on different mode of action is desirable. For precise risk management of chemical hazard in environmental water, TIE assisted with high-throughput bioassays and WET with conventional bioassays should be used complementary to each other.

5. Conclusions

This study reports the improvement of the throughput of TIE Phase I. The following improvements have been achieved:

- A 96-well microplate format high-throughput CO biosensor was applied to toxicity assay in TIE phase I, rendering a short assay time and a facile test organism preparation.
- The CO biosensor requires a comparatively small sample volume. This dramatically reduces the amount of water sample and hence transportation, manipulation and sample preparation are simple, facile and inexpensive.
- The CO biosensor-assisted TIE was capable of classifying the river water samples into different clusters based on their toxicity profiles, which were in accordance with the land use of the sampling sites, advocating its suitability in performing toxicity identification and evaluation. It is plausible to be employed for a large-scale watershed cytotoxicity evaluation.

Author contributions

I.C.M, L.Y.H. and L.O. conceived and designed the study; I.C.M., K.O. and C.R.A. developed the analytical method; L.O., K.O. and I.C.M. performed the experiments and analyzed the data. L.O. and I.C.M. wrote the manuscript.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

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