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Chemical pollutants are entering aquatic system globally at increasing rates, highlighting the need for fast, economical and effective assays for measuring their toxicity. This paper presents the Bacterial Luminescence Toxicity Screen (the BLT-Screen): an assay measuring inhibition of bacterial bioluminescence in response to chemical exposure that is rapid, high throughput and highly sensitive to a wide range of contaminants (organic and inorganic). The BLT-Screen is a novel and cost effective analytical tool with a wide range of research, monitoring and operational applications, particularly in the areas of contaminant exposure and impacts in aquatic environments.

# A sensitive and high throughput bacterial luminescence assay for assessing aquatic toxicity – the BLT-Screen

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**Abstract**

Bioassays using naturally luminescent bacteria are commonly used to assess the toxicity of environmental contaminants, detected by a decrease in luminescence. Typically, this has involved the use of commercial test kits such as Microtox and ToxScreen. These commercial assays, however, have limitations for routine environmental monitoring, including the need for specialized equipment, a low throughput and high on-going costs. There is therefore a need to develop a bacteria bioassay that is sensitive, high-throughput and cost effective. This study presents the development and application of the BLT-Screen (Bacterial Luminescence Toxicity Screen), a 96-well plate bioassay using *Photobacterium leiognathi*. During development of the method, the concentration of the phosphate buffer in the experimental medium was adjusted to maximize the sensitivity of the assay, and protocols for analyzing both solid-phase extracts and raw water samples were established. A range of organic compounds and metals were analyzed in the assay, as well as extracts of various water samples, including drinking water, wastewater effluent and river water. The $IC_{50}$ values of the organic compounds and metals tested in the BLT-Screen were comparable to previously published ToxScreen and Microtox data. In addition, the assay was sensitive enough to detect toxicity in all water types tested, and performed equally well for both solid-phase extracts and raw water samples. The BLT-Screen therefore presents a cost-effective, sensitive and high throughput method for testing the toxicity of environmental contaminants in a range of water types that has widespread applications for research, as well as for routine monitoring and operation of wastewater and drinking water plants.

Key words: BLT-Screen, Microtox, *Photobacterium leiognathi*, ToxScreen, *Aliivibrio fischeri*, water quality

## 1. Introduction

Increasing production, use and disposal of chemical pollutants worldwide has driven a need for improved methods for detecting their occurrence and effects, particularly in aquatic environments where a large proportion of pollutants accumulate [1]. Chemical analyses [2, 3] and passive sampling devices [4, 5] are commonly used to collect and screen for the presence of a wide range of contaminants in aquatic systems, but need to be targeted to specific individual or groups of compounds, and generally do not provide an indication on the biological availability or toxicity of pollutants, or potential mixture interactions. Direct toxicity testing provides a clearer understanding of the impacts of contaminants in aquatic systems, and traditionally involves acute and chronic assays on fish, crustaceans and algae [6-8]. However, these assays are often expensive and time consuming and carry a heavy ethical cost, limiting their application in routine environmental monitoring. As a result, sensitive, low-cost and high throughput bacterial bioassays are gaining popularity as rapid initial screening tools for toxicity and exposure to contaminants at biologically relevant concentrations, particularly in the assessment of water quality [9-11].

There are various bacterial bioassays with a number of different endpoints, including substrate consumption [12], respiration [13], ATP luminescence [14] and bioluminescence [15, 16]. Bacterial bioassays measuring inhibition of bioluminescence are particularly used to assess the toxicity of micropollutants in water samples [7, 17], because these assays are rapid and easy to use, and highly sensitive to a wide range of micropollutants. As the toxicity of water samples to bacteria is often strongly correlated with toxicity to other aquatic organisms (*e.g.*, $LD_{50}$ values) including, algae, invertebrates and fish [18, 19], bacterial assays can provide an ethical indicator of the ecotoxicity potential of a particular water sample. Generally, bacterial luminescent bioassays utilize naturally bioluminescent bacteria such as *Aliivibrio fischeri*

(previously *Vibrio fischeri* and *Photobacterium phosphoreum*), *Vibrio harveyi,* and *Photobacterium leigonathi* [15, 16]. Light production of these bacteria is directly proportional to their metabolic activity, so any toxicant that inhibits enzymatic activity results in a decrease in luminescence that can be easily measured in a luminometer [20].

There are a number of commercially available assays using naturally luminescent bacteria that are commonly used to assess the toxicity of water samples, including Microtox [15] and ToxScreen [16]. Microtox, in particular, has been published by the International Organisation for Standardization (ISO) as a standard method for measuring toxicity of water samples - ISO 11348-3 [21]. However, these commercial tests are not without their limitations. Both Microtox and ToxScreen utilize freeze dried cultures of bacteria, which must be continually purchased from commercial suppliers. Microtox requires purchase of a specialized luminometer that keeps the temperature sensitive *Aliivibrio fisheri* within a narrow temperature range throughout the assay, and can only house up to 30 microtubes (low-medium throughput). These limitations of Microtox have, in part, been recently overcome by miniaturization into 96-well plate format [7], although the on-going cost of purchasing the bacteria and the temperature sensitivity issues remain. The more recently developed ToxScreen uses the naturally luminescent bacterium *Photobacterium leiognathi*, which can be used at a room temperature and has been shown to be more sensitive than *Aliivibrio fisheri* to a range of organic and inorganic contaminants [16]. However, ToxScreen also requires a specialized luminometer capable of processing only one sample at a time (very low throughput), and has in recent years become increasingly difficult to obtain commercially.

In the absence of completely suitable bacteria luminescence toxicity assays to provide cost-effective and high throughput toxicity assessments there is a need to further develop this type

of assay. Due to its superior sensitivity to micropollutants and minimal sensitivity to temperature changes, *Photobacterium leiognathi* was a preferred bacterium for further development of toxicity assays. This study presents a bacterial luminescence toxicity assay, the "BLT-Screen" (Bacterial Luminescence Toxicity Screen), using a commercially available strain of *Photobacterium leiognathi* that can be continually cultured and cryo-preserved, reducing the need to continually purchase freeze-dried cultures. The BLT-Screen has also been designed to be high throughput (96-well plate format), sensitive to a range of organic and inorganic compounds commonly occurring in aquatic systems, and compatible with water quality testing of a wide range of both raw and solid-phase extracted water samples.

## 2. Methods

### 2.1. Culture and cryopreservation of *Photobacterium leiognathi*

A freeze-dried aliquot of commercially available *Photobacterium leiognathi* (ATCC® 33469™) was reconstituted in sterile growth medium (pH = 7.4) containing bacto-peptone (5 g/L), yeast extract (3 g/L), NaCl (450 mM), $MgSO_4.7H_2O$ (50 mM), $MgCl_2.6H_2O$ (30 mM), $CaCl_2.2H_2O$ (10 mM) and KCl (10 mM) in ultra-pure laboratory grade water. The culture was incubated at 23 °C on an orbital shaker (200 rpm) and harvested in late exponential phase for cryopreservation: the culture was centrifuged at 9,500 g for 10 min, the supernatant removed, and the pellet reconstituted in growth medium containing 30% glycerol at a concentration yielding ~2 x $10^7$ relative light units (RLU). Aliquots (1 mL each) were kept at -80°C until use in the assay.

### 2.2. Assay procedure – the BLT-Screen

The BLT-Screen was performed in flat bottom white 96-well microplates (Greiner Bio-One, Austria), configured to include an eight point serial dilution standard curve for the reference

compound (pentachlorophenol, PCP), and eight samples, a solvent control and an inter-assay sample, each with a four point serial dilution in duplicate (Figure 1).

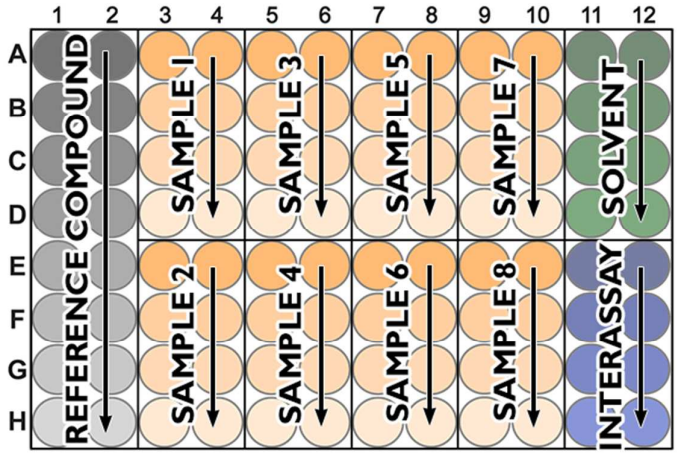


Figure 1. Layout of a 96-well plate for the BLT-Screen, including 8 samples, reference compound (pentachlorophenol), solvent control and inter-assay sample.

The BLT-Screen was validated to analyze both solid-phase extracts (in methanol) and raw water samples, using methanol or ultra-pure laboratory grade water, as solvent controls, respectively. The solvent, or negative, controls were included to ensure no toxicity of the carrier solvents, and were also used as the controls in the calculation of inhibition of luminescence (see Equation 1). Inter-assay samples (a methanolic extract of drinking water or a treated sewage effluent water sample, respectively) were also included in each plate to monitor the consistency of the assay in assessing the toxicity of the same sample between plates.

For methanolic extracts, the standard procedure for sample processing involved extraction of a 1 L filtered (GF-C; Whatman, U.K.) water sample (pH 2, following addition of ~1 mL of 12 M HCl) in an Oasis HLB solid-phase extraction (SPE) cartridge (6cc 500mg; Waters,

U.S.A.) preconditioned and eluted with equal volumes (10 mL) of methanol and hexane/acetone (1:1), and reconstituted in 500 µL methanol [22]. This process resulted in an enrichment factor (EF) of 2000 for each sample.

Sterile experimental medium (pH = 4.0) containing $KH_2PO_4$ buffer (100 mM), NaCl (450 mM), $MgSO_4.7H_2O$ (50 mM), and KCl (10 mM) was added to each well. For methanolic extracts, 249 µL of experimental medium was added to each well of the top row (A) and wells 3-12 of row E, and 200 µL was added to all other wells. For raw water samples, 125 µL of experimental medium was added to each well of the top row (A) and wells 3-12 of row E, and 200 µL of a 1:1 diluted (in ultra-pure water) test medium was added to all other wells. Either 1 µL (methanolic extracts) or 125 µL (raw water samples) of reference compound (PCP), sample, solvent control (methanol or water) or inter-assay sample was then added in duplicate to each well of the top row (A) and wells 3-12 of row E (Figure 1). An eight point (PCP reference compound) or a 4-point (samples, solvent control, inter-assay sample) 5-fold (50 µL to 200 µL) serial dilution was then performed for each sample/standard using a multichannel pipette. The highest relative enrichment factors (REFs) in the final assay volumes were therefore 8 and 0.5 for the methanolic extracts (REF = 2000× SPE enrichment / 250× assay dilution) and raw water samples (REF = 1 / 2× assay dilution), respectively.

Following serial dilutions, a cryopreserved aliquot of *Photobacterium leiognathi* was removed from the -80ºC freezer, thawed on ice and diluted 1:10 in sterile growth medium. The RLU of the diluted stock was measured in a Fluostar plate reader (BMG Labtech, Germany) to ensure that no more than 10% decrease in luminescence had occurred during cryopreservation. When the decrease in luminescence of the diluted cryopreserved bacteria exceeded 10%, a new batch of cryopreserved bacteria was prepared for future analyses. Five

microliters of diluted *Photobacterium leiognathi* was then added to each well of the assay plate using a multi-channel pipette. Exactly 30 min after the addition of the *Photobacterium leiognathi* the luminescence of each well was measured in a Fluostar plate reader (BMG Labtech, Germany).

The percent inhibition of luminescence was calculated for each well using Equation 1:

$$\% \text{ Inhibition} = \left[1 - \left(\frac{\text{lum}_{\text{sample}}}{\text{lum}_{\text{control}}}\right)\right] \times 100 \qquad \text{……Equation 1}$$

The $IC_{50}$ for the PCP standard curve was calculated from the log-logistic dose-response curve ($y = 100/(1 + 10^{(\log IC50\text{-}x)*slope})$) of % inhibition plotted against log concentration (M) using Prism (GraphPad Software, U.S.A.). The limit of detection of the BLT-Screen was calculated for each plate as three times the standard deviation of the % inhibition values of the solvent controls, and was generally <10%. A 20% inhibition of luminescence was therefore considered to be a conservative value of the minimum response that could be quantified in the assay. Results of all samples and the inter-assay controls were expressed as toxic unit (TU), the reciprocal of the $IC(REF)_{20}$, as determined by a straight line regression of % inhibition against log REF, for all results <40% inhibition (Figure 2). A $TU_{IC(REF)20} = 1$ equates to an undiluted sample causing a 20% inhibition of bacterial luminescence.

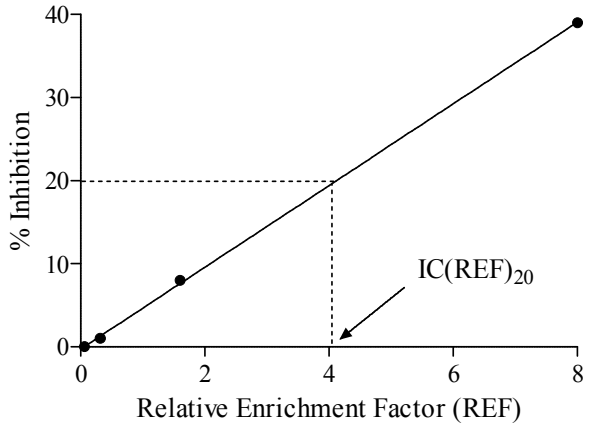


Figure 2. For each sample, the $IC(REF)_{20}$ (relative enrichment factor that results in 20% inhibition of luminescence) was calculated from the straight line regression between luminescence inhibition (%) and the REF of the sample dilutions. The $TU_{IC(REF)20}$ value is then calculated as the reciprocal of $IC(REF)_{20}$.

### 2.3. **Optimization of the test buffer**

To optimize the concentration of the buffer in the experimental medium to yield most sensitive results, the $IC_{50}$ for PCP was analyzed in a series of experimental media with different concentrations of monobasic potassium phosphate ($KH_2PO_4$, CASRN: 7778-77-0; 99+% purity; Acros Organics, Belgium): *i.e.*, all containing NaCl (450 mM), $MgSO_4.7H_2O$ (50mM) and KCl (10 mM), but varying concentrations of $KH_2PO_4$ of 0, 3.1, 6.2, 12.5, 25, 50 and 100 mM. The layout of the 96-well microplate for this experiment was modified so that each column contained a 7-point serial dilution (1:5) of PCP, leaving the final well of each column (H) as the solvent control. The $IC_{50}$ values for PCP were determined from log-logistic fit of the concentration-effect curves of duplicate analyses for each of the different media and calculated as described in Equation 1.

### 2.4. Water samples *vs.* solid-phase extracts

To test the performance of the BLT-Screen in assessing the toxicity of raw water samples (not extracted), the $IC_{50}$ for PCP (in methanol) was analyzed in a series of experimental media diluted with ultrapure water - 10, 20, 30, 40 and 50 % water - alongside a control of undiluted experimental medium. Again, the layout of the plate was modified to allow a 7-point serial dilution for each sample, with the final well (H) of each column used as the solvent (methanol) blank. In addition, two samples of treated wastewater were analyzed as both raw water and solid-phase extract (in methanol), and the $TU_{IC(REF)20}$ values of these samples were compared to ensure repeatability of the method irrespective of the sample processing.

### 2.5. Toxicity of organic contaminants and metals

To assess the sensitivity of the BLT-Screen to a range of contaminants, 16 organic compounds (in methanol) and seven metals (in ultra-pure laboratory grade water) commonly found in wastewater, surface water and drinking water were tested in the assay (Tables 1 and 2). The layout of the 96-well microplate was modified so that each column contained a 7-point serial dilution (1:5) of each compound, leaving the final well of each column (H) as the solvent control. All compounds were run in duplicate and the PCP standard was included in the first two columns of each plate for quality control. The $IC_{50}$ values for each compound were determined from log-logistic fit of the concentration-effect curve of duplicate analyses and calculated as described in Equation 1. Each compound was analysed twice (on separate days) and the mean ± SD $IC_{50}$ for each compound was calculated.

The $IC_{50}$ values for the compounds analyzed in the BLT-Screen were compared to Microtox and ToxScreen literature values. ScienceDirect, Scopus, ProQuest, Web of Science and Google Scholar databases were searched using the terms '*name of compound*' and 'Microtox' or 'ToxScreen'. Due to the large number of studies using Microtox to assess toxicity of organic compounds and metals, only the highest and lowest literature $IC_{50}$ values were included in these comparisons.

### 2.6. Toxicity of water extracts from different sources

The application of the BLT-Screen for testing the toxicity of a range of different water types was validated for water extracts from four different water sources within Southeast Queensland, Australia: 1) treated effluent from a conventional sewage treatment plant (STP), 2) water from within the tidal range of the Logan River, 3) chlorinated drinking water from a local council supply, and 4) ultra-pure laboratory grade water. To maximize the likelihood of detecting a response in these samples, the enrichment factor after solid phase extraction was increased to 3333 by reconstituting the extracted 1 L sample in a smaller volume of methanol (300 μL). All samples were analyzed twice (on separate days) in 4-point serial dilutions (Figure 1). The mean ± SD % inhibition (Equation 1) was plotted against log REF, and the $IC_{50}$ was calculated for each sample from the log-logistic fit of the concentration-effect curve.

### 2.7. Quality assurance and quality control

Pentachlorophenol (PCP) was established as the reference compound for the BLT-Screen, by analyzing the PCP standard on eight separate occasions, using different frozen bacterial aliquots, and calculating the mean and standard deviation of the $IC_{50}$ values.

The precision of the BLT-Screen was established by calculating the repeatability (within-run precision) and reproducibility (between-run precision) of the assay, based on the analysis of

eight samples (wastewater purification pond solid-phase extracts) in duplicate on two separate occasions [9]. The standard deviation ($S_r$) and coefficient of repeatability ($VC_r$) were calculated using equations 2 and 3, where $X_i$ are the $TU_{IC(REF)20}$ values of the two replicates in run 1:

$$S_r = \sqrt{\frac{\sum_{i=1}^{n}(X_{i1}-X_{i2})^2}{2n}} \quad \text{.....Equation 2}$$

$$VC_r = \sqrt{\frac{\sum_{i=1}^{n}\left(\frac{X_{i1}-X_{i2}}{0.5(X_{i1}+X_{i2})}\right)^2}{2n}} \quad \text{.....Equation 3}$$

The standard deviation ($S_R$) and coefficient of reproducibility ($VC_R$) were calculated using equations 4 and 5, where $X_i$ and $Y_i$ are the mean $TU_{IC(REF)20}$ values of run 1 and run 2, respectively:

$$S_R = \sqrt{\frac{\sum_{i=1}^{n}(X_i-Y_i)^2}{2n}} \quad \text{....Equation 4}$$

$$VC_R = \sqrt{\frac{\sum_{i=1}^{n}\left(\frac{X_i-Y_i}{0.5(X_i+Y_i)}\right)^2}{2n}} \quad \text{....Equation 5}$$

The robustness of the BLT-Screen, a measure of the sensitivity of the assay to operational variations, was calculated as $VC_R/VC_r$ [9].

## 3. Results

### 3.1. Optimization of the test buffer

The concentration of monobasic potassium phosphate ($KH_2PO_4$) buffer in the experimental medium influenced the sensitivity of the BLT-Screen (Figure 3). The assay was least sensitive ($IC_{50}$ = 4.5 µM) in the unbuffered saline medium, and the sensitivity generally

increased as the concentration of buffer increased, reaching a maximum sensitivity at 50 mM phosphate ($IC_{50}$ = 0.077 μM). Interestingly, there was minimal difference in sensitivity between 50 mM and 100 mM ($IC_{50}$ = 0.091 μM), suggesting that the assay would be equally sensitive for both water extracts (100 mM phosphate buffer) and raw water samples up to half the assay volume (50 mM phosphate buffer). This was further investigated in section 3.2.

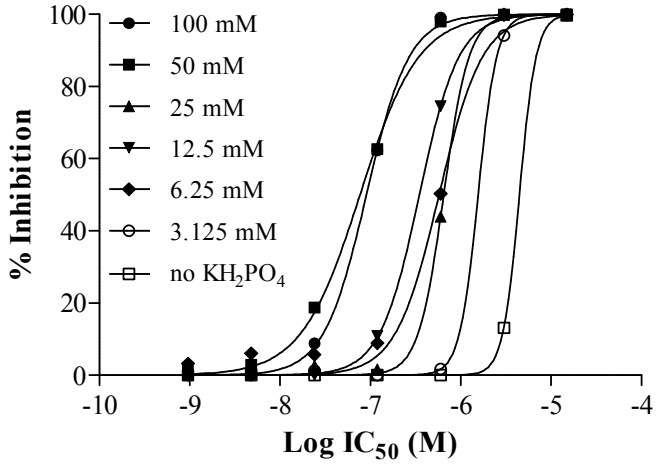


Figure 3. Mean ± SD luminescence inhibition (%) vs log concentration (M) of pentachlorophenol analyzed in the BLT-Screen using experimental media containing monobasic potassium phosphate ($KH_2PO_4$) concentrations ranging from 0 to 100 mM.

### 3.2. **Water samples *vs.* solid-phase extracts**

Dilution of the experimental medium in ultra-pure water affected the $IC_{50}$ for PCP slightly, ranging from 0.12 ± 0.01 μM in the undiluted medium to 0.58 ± 0.01 μM in the 40% diluted medium (Figure 4). However, all $IC_{50}$ values were within half a log unit of the mean $IC_{50}$ of PCP in the undiluted medium, 0.17 ± 0.08 μM (Figure 7).

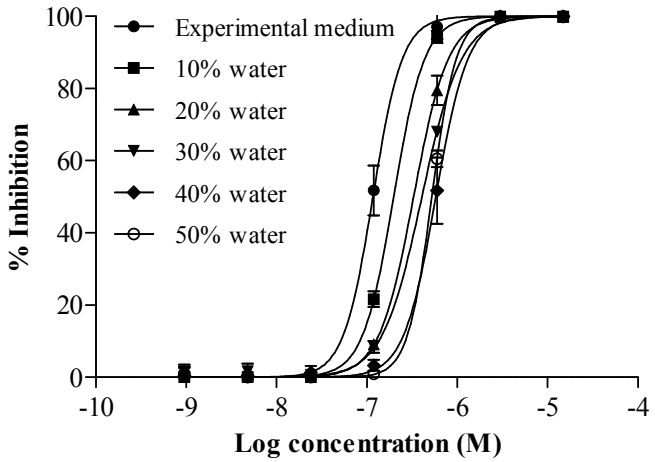


Figure 4. Mean ± SD luminescence inhibition (%) vs log concentration (M) of pentachlorophenol analyzed in the BLT-Screen using experimental medium and media diluted with ultra-pure laboratory grade water from 10 to 50%.

The two treated wastewater effluent samples that were analyzed as both raw water and solid-phase extracts showed similar dose-response curves over the range in REFs for which there was overlap, although the raw water samples had slightly higher % inhibition values (Figure 5). This is reflected by the slightly higher $TU_{IC(REF)20}$ values in the raw water extracts (1.1 ± 0.4 and 1.4 ± 0.6), compared to the solid-phase extracts (0.54 ± 0.05 and 0.86 ± 0.15).

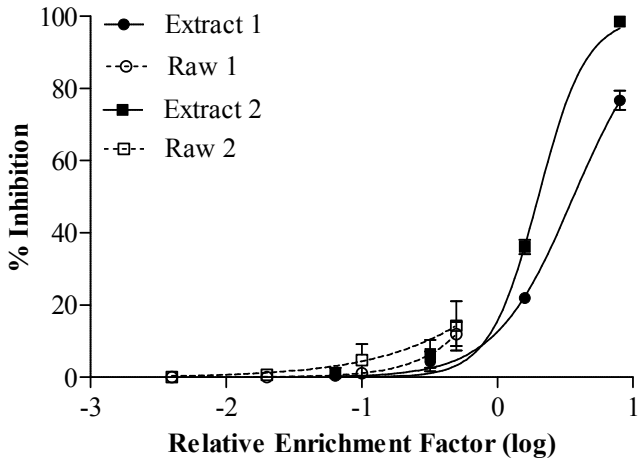


Figure 5. Mean ± SD luminescence inhibition (%) *vs.* log relative enrichment factor (REF) of two samples that were analyzed in the BLT-Screen as both raw water and solid-phase extracts.

### 3.3. **Toxicity of organic contaminants and metals**

The BLT-Screen was sensitive to a wide range of organic compounds and metals commonly present in drinking water, wastewater effluent, and surface water. Most $IC_{50}$ values were in the same order of magnitude as literature Microtox and ToxScreen values, with some compounds (e.g. naproxen, pentachlorophenol, and iodo-, bromo- and chloro-acetic acids) several orders of magnitude lower than Microtox (Table 1). The $IC_{50}$ values for metals analysed in the BLT-Screen were also generally in the same order of magnitude as literature Microtox values, although generally (with the exception of mercury) higher than ToxScreen values (Table 2).

Table 1. $IC_{50}$ values (μM) for organic compounds tested in the BLT-screen (this study), and compared to ToxScreen and Microtox literature values.

| Compound | Class of compound | BLT-Screen | ToxScreen | Microtox |
|---|---|---|---|---|
| Atrazine | Herbicide | ~41 * | NF | 187 [k] - 2500 [c] |
| Bisphenol A | Industrial chemical | 130 ± 66 | NF | 27 [g] |
| Bromoacetic acid | Disinfection by-product | 43 ± 11 | NF | 100 [f] - 430 [p] |
| Bromochloroacetic acid | Disinfection by-product | 43 ± 6 | NF | NF |
| Carbamazepine | Anti-convulsant drug | 800 ± 100 | NF | 221 [j] - 332 [i] |
| Chloroacetic acid | Disinfection by-product | 620 ± 190 | NF | 9150 [p] |
| Chlorpyrifos | Insecticide | 20 ± 5 | 2.9 [a] | 29 [c] - 130 [b] |
| 2,6-dibromo-1,4-benzoquinone | Disinfection by-product | 0.24 ± 0.04 | NF | NF |
| 2,6-dichloro-1,4-benzoquinone | Disinfection by-product | 0.07 ± 0.01 | NF | NF |
| 3,5-Dichlorophenol | Industrial chemical | 24 ± 3 | NF | 32 [e] |
| Gemfibrozil | Lipid reduction drug | 360 ± 130 | NF | 116 [h] - 1000 [c] |
| Iodoacetic acid | Disinfection by-product | 2.1 ± 1.2 | NF | 84 [p] |
| Naproxen | Anti-inflammatory drug | 0.5 ± 0.1 | NF | 135 [p] - 1600 [c] |
| 4-*tert*-Octylphenol | Industrial chemical | 19 ± 6 | NF | NF |
| Pentachlorophenol | Pesticide | 0.17 ± 0.08† | 0.06 [a] | 1.9 [d] - 3.8 [b] |
| Triclosan | Anti-bacterial/fungal agent | 2.3 ± 1.4 | NF | 0.2 [m] - 2.5 [n] |

* extrapolated $IC_{50}$ when > 20% but <50% inhibition reached in assay; † calculated from 8 replicates; NF, not found

References: [a] Ulitzur, et al. [16]; [b] Kaiser and Palabrica [23]; [c] Tang, et al. [24]; [d] Blondin, et al. [25]; [e] Ricco, et al. [26]; [f] Domart-Coulon, et al. [27]; [g] Debenest, et al. [28]; [h] Rosal et al. [29]; [i] Jos et al. [30]; [j] Kim et al. [31]; [k] Tchounwou et al. [32]; [m] DeLorenzo et al. [33]; [n] Villa et al. [34]; [p] Halmi et al. [35]

Table 2. Mean (SD) $IC_{50}$ values (µM) for metals (expressed as cationic concentrations) tested in the BLT-screen (each on two separate occasions), and compared to ToxScreen and Microtox (15-min) literature values.

| Metal | Chemical used* | BLT-Screen | ToxScreen | Microtox |
|---|---|---|---|---|
| Cadmium ($Cd^{2+}$) | $Cd(NO_3)_2.4H_2O$ | >1400 | 89 [a] | 2.7 [j] - 2230 [e] |
| Copper ($Cu^{2+}$) | $CuSO_4.5H_2O$ | 840 ± 190 | 0.31 [a] - 3150 [d] | 1.6 [f] - 130 [c] |
| Lead ($Pb^{2+}$) | standard solution | 13 ± 1.7 | 3.9 [a] | 1.1 [f] - 170 [g] |
| Mercury ($Hg^{2+}$) | $HgCl_2$ | 0.02 ± 0.004 | 0.015 [a] - 0.2 [d] | 0.23 [c] - 1.9 [j] |
| Nickel ($Ni^{2+}$) | $NiCl_2$ | >1200 | 850 [a] | 57 [i] - 3560 [e] |
| Silver ($Ag^{+}$) | $AgNO_3$ | 11 ± 2.3 | 7 [a] | 0.9 [f] - 5.5 [e] |
| Zinc ($Zn^{2+}$) | $ZnSO_4.7H_2O$ | >2800 | >380 [a] | 3.9 [j] - 260 [b] |

* purity ≥ 98%

[a] 25-30 min values when run in the Pro-Organic buffer [16]; [b] Kahru [36]; [c] Dutka and Kwan [37]; [d] van der Schalie, et al. [38]; [e] Sankaramanachi and Qasim [39]; [f] McClosky et al. [40]; [g] Rosen et al. [41]; [h] McFetters et al. [42]; [i] Codina et al. [43]; [j] Sillanpää and Oikari [44]

### 3.4. Toxicity of water extracts from different sources

Ultra-pure laboratory grade water did not produce any response in the BLT-Screen even at the highest relative enrichment factor (13×). River, STP effluent and drinking water extracts, however, caused inhibition of bacterial luminescence (Figure 6), with STP effluent most toxic ($IC_{50}$ = 1.6 REF), followed by drinking water ($IC_{50}$ = 5.8 REF) and river water ($IC_{50}$ = 11.8 REF).

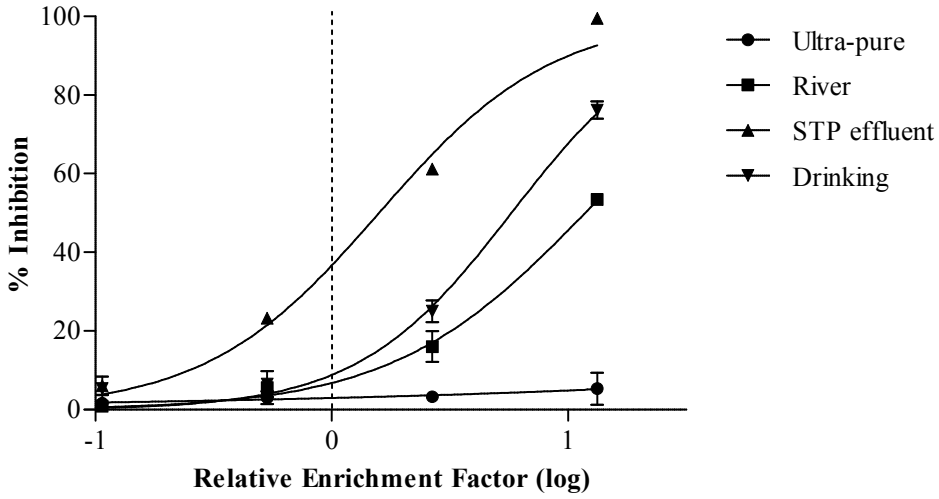


Figure 6. Mean (± SD) luminescence inhibition (%) *vs.* log relative enrichment factor (REF) of ultra-pure, river, drinking and STP effluent water extracts analyzed in the BLT-Screen. The vertical dotted line at log REF = 0 indicates the point where samples have essentially not been enriched. Only the STP effluent produces an inhibition of luminescence >10% at this point.

### 3.5. Quality assurance and quality control

The mean ± SD $IC_{50}$ for eight replicates of pentachlorophenol analyzed by the BLT-Screen on separate days (using different aliquots of cryopreserved bacteria) was 0.17 ± 0.08 μM, and ranged from 0.089 to 0.31 μM (Figure 7). For quality control, assay runs where the $IC_{50}$ for

PCP is more than half a log unit either side of 0.17 μM (*i.e.*, below 0.048 or above 0.48 μM) should be discarded and repeated.

The standard deviation ($S_r$) and coefficient ($VC_r$) of repeatability were 0.10 and 0.08, respectively. The standard deviation ($S_R$) and coefficient ($VC_R$) of reproducibility were 0.15 and 0.10, respectively. These values were well within the limit of 0.2 for acceptable repeatability and reproducibility [9]. The robustness index of the BLT-Screen was 1.3 indicating that the assay yields similarly consistent results both within and between runs [9].

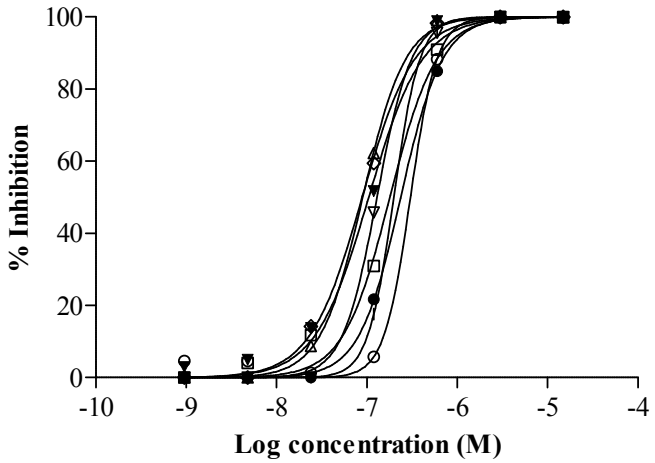


Figure 7. The % inhibition *vs.* log concentration (M) for the pentachlorophenol standard analyzed in the assay on eight separate occasions.

## 4. Discussion

The BLT-Screen assay presented here is a sensitive, rapid and high throughput method for analyzing the toxicity of water samples from a wide range of sources. The BLT-Screen was sensitive to a wide range of organic and inorganic contaminants, and comparable to previously published Microtox and ToxScreen data. In addition, the BLT-Screen could detect inhibition of luminescence in water samples from a wide range of sources, including

wastewater effluent, drinking water and surface water samples. The assay performed equally well for analyzing concentrated solid-phase extracts and raw water samples, and satisfied critical benchmarks of repeatability, reproducibility and robustness. Importantly, the BLT-Screen uses cryo-preserved cultures of *Photobacterium leiognathi* that can be continually sub-cultured, reducing the need to repeatedly purchase freeze-dried aliquots for use in other commercial bioassays. The BLT-Screen is therefore a cost-effective and powerful tool for use in a range of research (*e.g.*, exposure assessments, relative toxicity), routine monitoring (*e.g.*, receiving environments), and operational (*e.g.*, drinking water and wastewater plants) capacities.

### 4.1. Sensitivity of the BLT-Screen

Sensitivity is a critical factor in selecting a suitable method for water quality assessment. The $IC_{50}$ values for most organic compounds analysed by the BLT-Screen in this study were in the same order of magnitude as previously published Microtox data. The BLT-Screen was particularly sensitive for some pesticides (*e.g.*, pentachlorophenol), pharmaceuticals (*e.g.*, naproxen), and drinking water disinfection by-products, producing $IC_{50}$ values orders of magnitude lower than published Microtox values (Table 1). The ToxScreen assay (which uses the same bacterium, *Photobacterium leiognathi*) was slightly more sensitive than the BLT-Screen for pentachlorophenol and chlorpyrifos [16], although the BLT-Screen $IC_{50}$ values of both compounds were within an order of magnitude of the ToxScreen $IC_{50}$ values. For metals, the BLT-Screen showed similar sentitivity to Toxscreen and Microtox for mercury silver and lead, but was less sensitive for nickel, zinc, copper and cadmium (Table 2). However, it is important to note here that there were large variations in the reported ToxScreen and Microtox $IC_{50}$ values for metals analyzed in more than one study, with the most extreme variation observed for copper in the ToxScreen, where $IC_{50}$ values ranged from

0.31 [16] to 3100 μM [38]. This suggests that the assay may be quite variable for analysing metals, and that direct comparisons in bioassay sensitivity to metals, in particular, should therefore be treated with caution.

The relative toxicity of metals in different bacterial luminescence assays should also be discussed in terms of the chemistry of the assay media. The pH, anions and chelating agents in the assay media can change the speciation of metals and hence affect their toxicity in the assay [45, 46]. Indeed, the differences observed in metal toxicity between the BLT-Screen and Microtox may be due to differences in pH and chelating agents. The ToxScreen assay developed by Ulitzur, et al. [16] used different buffers for analysis of metals and organics (with different pH), which have been manipulated to yield maximum sensitivity for both contaminant classes. This requires the user to make a choice on which buffer to use (based on the types of contaminants expected in the sample), and is not particularly applicable to samples that may have mixtures of organic and metals (*e.g.*, water samples). The BLT-Screen was developed with a single buffer optimized for both organics and metals. Overall, the BLT-Screen is very sensitive to both groups of compounds, although it is more applicable to samples containing organic contaminants (*e.g.*, water extracts, from which metals and other matrix interferences are removed).

### 4.2. **Application of the BLT-Screen**

In water quality assessments using bacterial luminescence assays, the bacteria are exposed to water extracts that contain a wide range of compounds. Therefore, while comparing sensitivity of the different assays using individual compounds is valid, it is arguably more important to compare the assays when analyzing similar water extracts. The utility of the BLT-Screen for assessing water quality is exemplified in the responses observed in analysis

of solid-phase extracts of STP effluent, drinking water and river water (Figure 6). All samples produced some inhibition of luminescence above the ultra-pure laboratory water control, with toxicity ranging from STP effluent > drinking water > river water (Figure 6). However, drinking water and river water needed to be concentrated (log REF > 0) to produce an inhibition of luminescence > 10%. This indicated that as raw water samples, river and drinking water are not particularly toxic to bacteria. In addition, the $IC_{50}$ values for all water types presented here were similar to those reported in previous studies using Microtox [2, 11, 47], supporting the application of the BLT-Screen for benchmarking water quality over a range of water types.

The BLT-Screen is also useful for detecting changes in toxicity due to water treatment. In this study, drinking water ($IC_{50}$ = 5.8 REF) was about two times more toxic to bacteria than river water ($IC_{50}$ = 11.8 REF). While this does not necessarily indicate that these drinking water samples were particularly toxic (*i.e.*, they needed to be concentrated 6 times to cause 50 % inhibition of luminescence), it does support previous studies using the Microtox assay, which suggested that increased bacterial toxicity in drinking water was due disinfection and the presence of disinfection by-products [17, 48]. Similarly, chlorination of STP effluent prior to release can also increase the toxicity of the water to bioluminescent bacteria, as illustrated by Watson, et al. [49].

The application of the BLT-Screen in analyzing a range of water samples is largely dependent on the enrichment of water samples during the extraction process. Figures 5 and 6 clearly show that at lower REF values, the differences in luminescence inhibition between samples are difficult to detect. In comparison, as the REF increases, the separation in luminescence inhibition is much larger, allowing a much clearer comparison between the

different samples and water types. Generally samples are enriched 2000 times (which once assay dilution is included translates into a maximum REF of 8 in the assay), but enrichment of the sample can be increased, through increasing the volume of water extracted and decreasing the volume of methanol used in reconstitution. For example, a 2 L water sample extracted and reconstituted in 100 µL methanol would have an enrichment factor of 20,000 and a maximum REF of 80 in the assay. This essentially allows responses to be observed in less toxic samples, allowing comparisons between many different water types and quantification of treatment efficacy[50]. In fact, even ultra-pure laboratory grade water can cause a response in bacterial luminescence bioassays if enriched enough, as previously illustrated by Microtox detecting inhibition of luminescence in MilliQ water extracts enriched to a maximum of 38 in the assay [50].

Despite the optimal application of the BLT-Screen in analyzing organic compounds in water extracts, the assay also performed well when analyzing raw (unextracted) water samples. This study clearly showed that diluting the experimental medium by up to half with ultra-pure laboratory grade water had minimal influence on the sensitivity of the assay (Figure 4). This is further supported by the similar relationships between % inhibition and REF and similar $TU_{IC(REF)20}$ values when the same STP effluent samples were analyzed as both raw water and solid-phase extracts (Figure 5). Important to note here, the slightly higher $TU_{IC(REF)20}$ values in the raw water samples compared to the methanolic extracts may be due to the presence of contaminants that are not retained by the SPE sorbent (such as metals). The analysis of raw water samples may therefore be the preferred application for many users, as it accounts for a more complete (organic and metals) assessment of toxicity. However, it is important to keep in mind that when analyzing samples as raw water, the maximum REF that can be measured is 0.5 (a 1:1 dilution of the sample in the assay). Only particularly toxic samples (such as STP

effluents) will cause inhibition of luminescence when analyzed as raw water, and the assay will not be sensitive enough to measure less toxic samples such as drinking water and surface water. When analyzing "clean" water samples, sample enrichment via solid-phase extraction remains necessary.

Another important application of the BLT-Screen is in direct toxicity assessments of the aquatic environment. Bacteria are ubiquitous in rivers, estuaries and oceans, and perform important roles in nutrient recycling [51]. Bioluminescent bacteria, such as *Photobacterium leiognathi*, are particularly prevalent in estuarine and oceanic ecosystems, and can exist as free living cultures that are involved in the decomposition of fish, or as symbionts in certain fish and squid species that have light organs [52]. Understanding the effects of contaminants on aquatic bacteria (using assays such as the BLT-Screen) therefore provides important information on the health and functioning of aquatic ecosystems. In addition, the strong relationships between toxicity of aquatic pollutants to bacteria and other aquatic organisms (algae, invertebrates and fish)[18, 19] mean that low cost, high throughput, sensitive bacterial assays, like the BLT-Screen, are important tools in the initial assessment of aquatic ecotoxicity. Because the BLT-Screen uses aquatic bacteria, it is essentially an *in vivo* assay for the assessment of the aquatic ecotoxicity. In the BLT-Screen, a reduction in bacterial luminescence >20% in an undiluted environmental sample (*e.g.*, estuarine water), expressed here as $TU_{IC(REF)20} > 1$, can therefore indicate an impact of chemical pollutants in that ecosystem.

## 5. Conclusions

This study has established the BLT-Screen (Bacterial Luminescence Toxicity Screen) that is:

- A robust, sensitive and cost-effective alternative to commercial bacterial toxicity assays
- Responsive to a wide range of organic and inorganic toxicants, and a variety of water types, including wastewater, drinking water and surface water.
- Compatible with both raw (unextracted) and solid-phase extracted water samples

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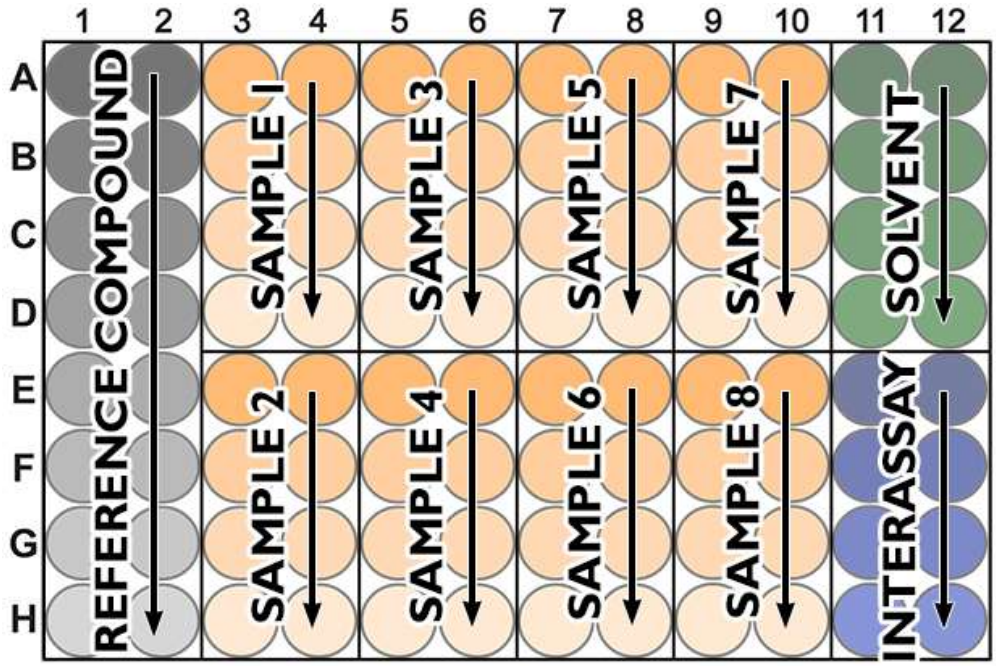
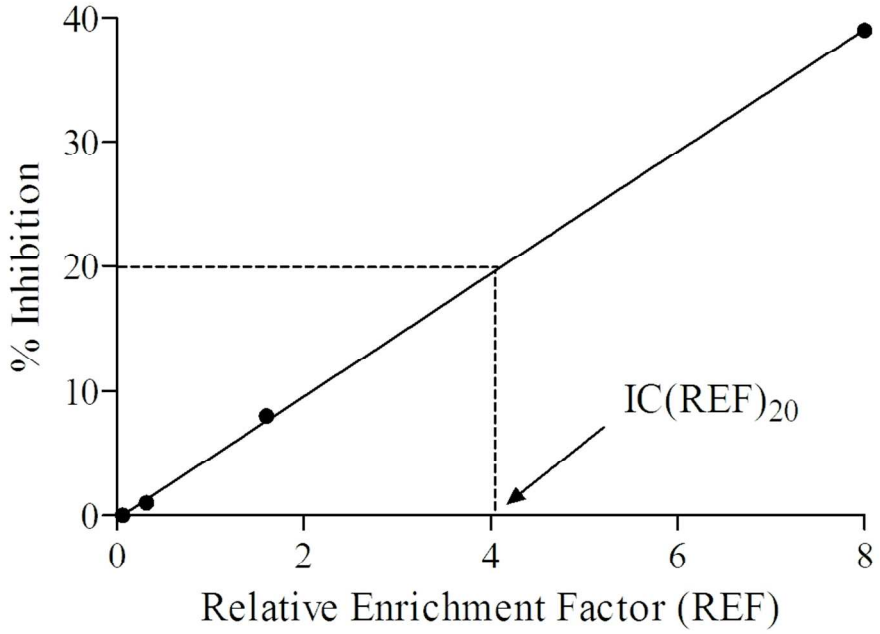



Layout of a 96-well plate for the BLT-Screen, including 8 samples, reference compound (pentachlorophenol), solvent control and inter-assay sample.
52x34mm (300 x 300 DPI)




For each sample, the IC(REF)20 (relative enrichment factor that results in 20% inhibition of luminescence) was calculated from the straight line regression between luminescence inhibition (%) and the REF of the sample dilutions. The TUIC(REF)20 value is then calculated as the reciprocal of IC(REF)20.
102x73mm (300 x 300 DPI)

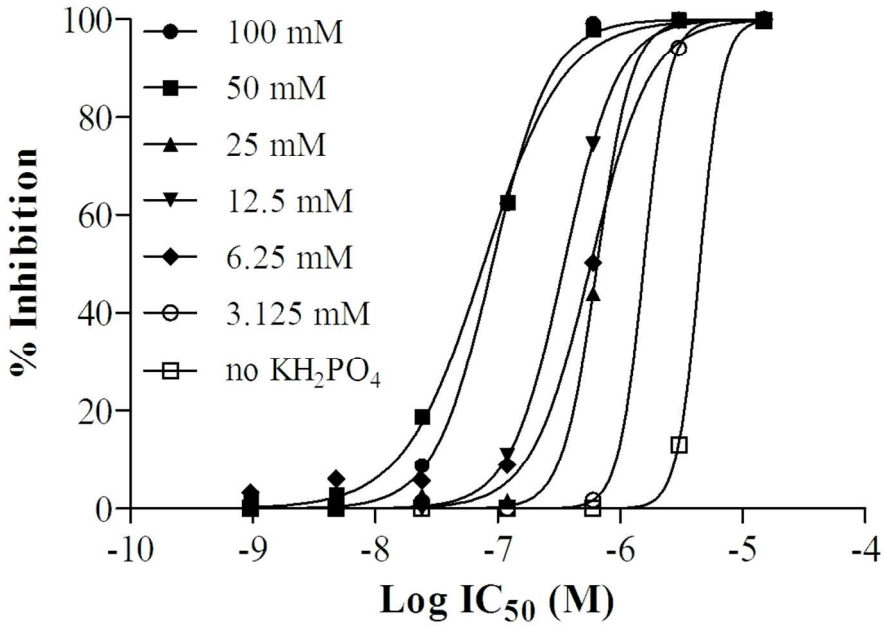
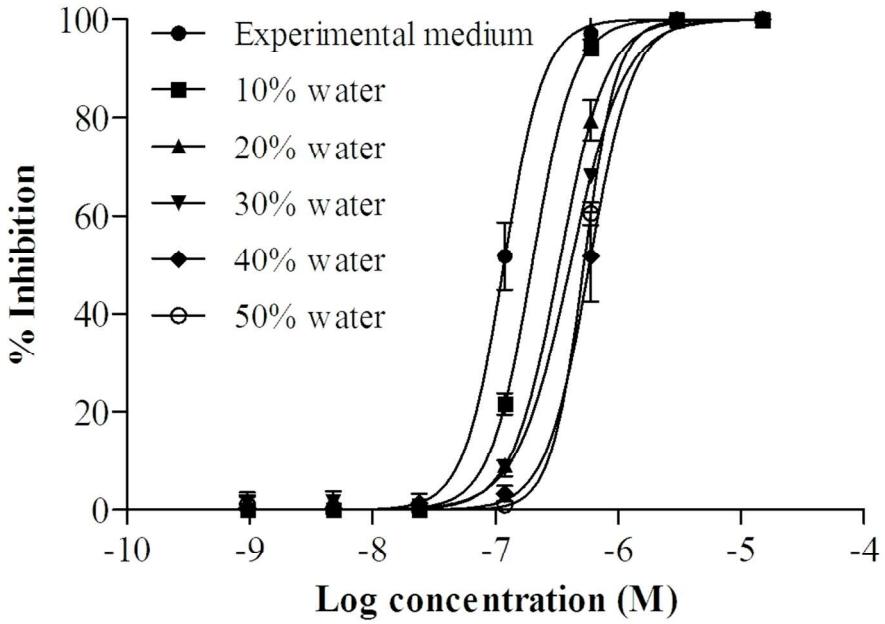
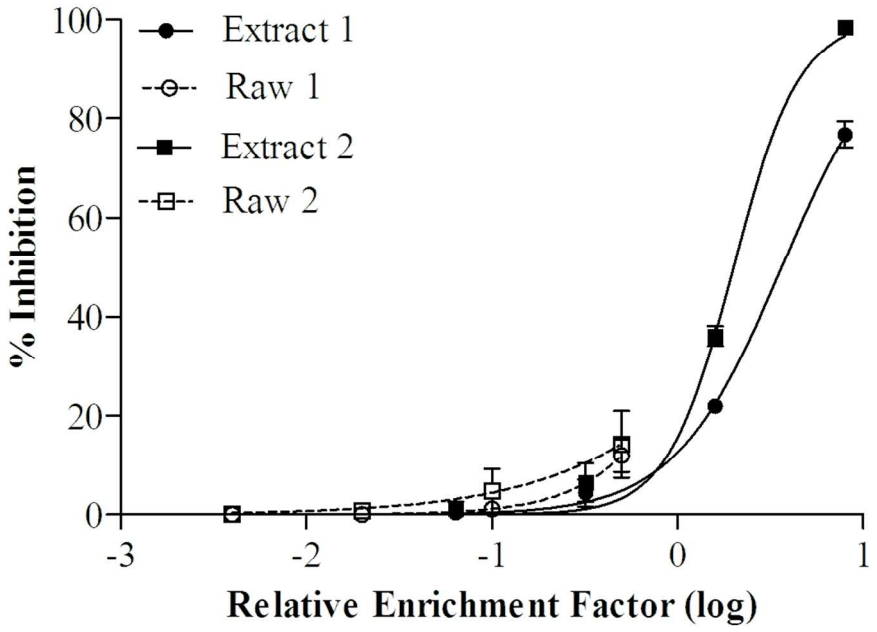


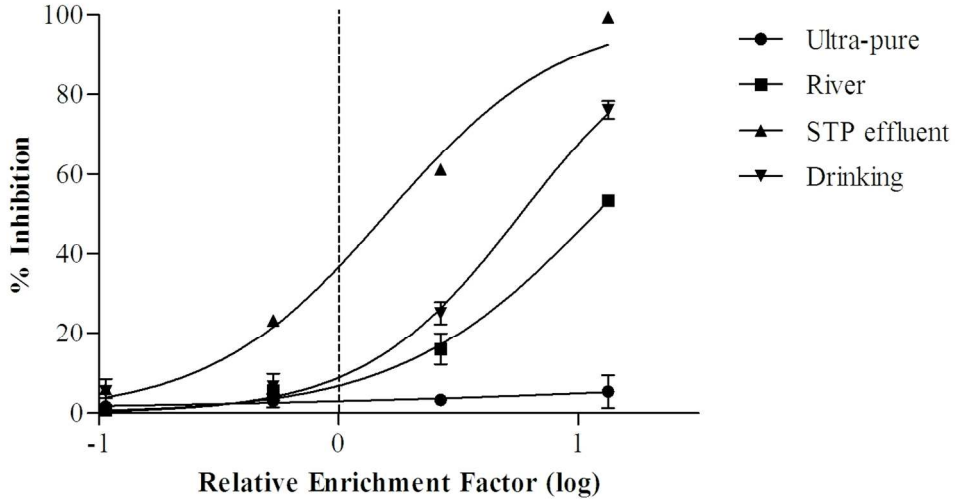

Figure 3. Mean ± SD luminescence inhibition (%) vs log concentration (M) of pentachlorophenol analyzed in the BLT-Screen using experimental media containing monobasic potassium phosphate (KH2PO4) concentrations ranging from 0 to 100 mM.
104x73mm (300 x 300 DPI)



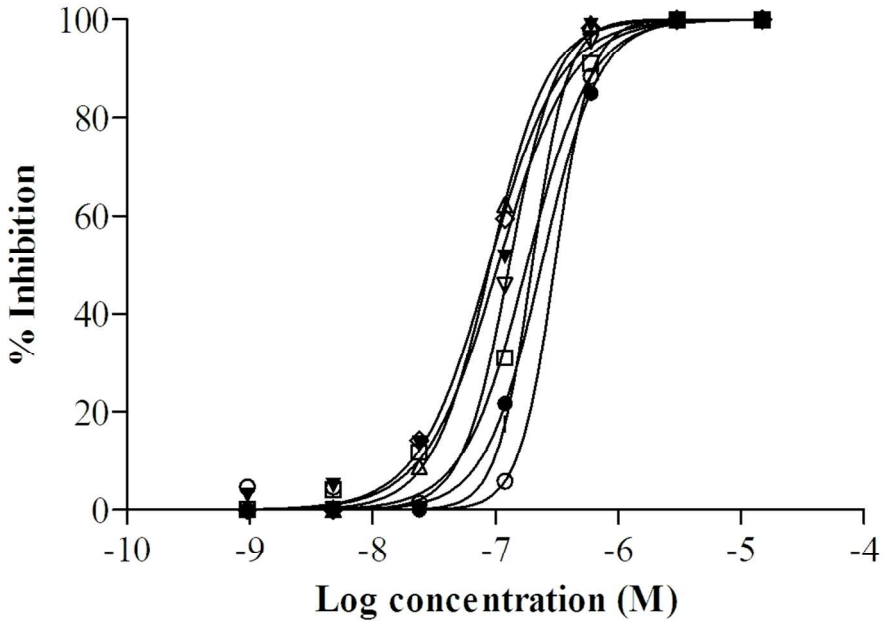

Mean ± SD luminescence inhibition (%) vs log concentration (M) of pentachlorophenol analyzed in the BLT-Screen using experimental medium and media diluted with ultra-pure laboratory grade water from 10 to 50%.
103x72mm (300 x 300 DPI)




Mean ± SD luminescence inhibition (%) vs. log relative enrichment factor (REF) of two samples that were analyzed in the BLT-Screen as both raw water and solid-phase extracts.
102x72mm (300 x 300 DPI)




Mean (± SD) luminescence inhibition (%) vs. log relative enrichment factor (REF) of ultra-pure, river, drinking and STP effluent water extracts analyzed in the BLT-Screen. The vertical dotted line at log REF = 0 indicates the point where samples have essentially not been enriched. Only the STP effluent produces an inhibition of luminescence >10% at this point.
127x71mm (300 x 300 DPI)




The % inhibition vs. log concentration (M) for the pentachlorophenol standard analyzed in the assay on eight separate occasions.
103x72mm (300 x 300 DPI)

Chemical pollutants are entering aquatic system globally at increasing rates, highlighting the need for fast, economical and effective assays for measuring their toxicity. This paper presents the Bacterial Luminescence Toxicity Screen (the BLT-Screen): an assay measuring inhibition of bacterial bioluminescence in response to chemical exposure that is rapid, high throughput and highly sensitive to a wide range of contaminants (organic and inorganic). The BLT-Screen is a novel and cost effective analytical tool with a wide range of research, monitoring and operational applications, particularly in the areas of contaminant exposure and impacts in aquatic environments.