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10 **A multi-pumping flow system for acute toxicity**
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13 **bioassay using the *Vibrio fischeri* bacteria**
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Abstract

A multi-pumping flow system was developed for automation of the acute toxicity bioassay using the *Vibrio fischeri* bacteria. Solenoid micro-pumps were exploited to improve mixing conditions, and to accomplish the required in-line dilutions of the tested compounds by modifying the sample volume and exploiting the partial overlap between the sample zone and the bacterial suspension. A spiral flow cell, placed at the emission window of a spectrofluorimeter, was used for signal measurements and a lab-made water bath based on the Peltier effect was used for temperature control of the bioassay. A 120- μ L of the bacterial suspension was selected in order to minimize its consumption and, consequently, the costs of the assay. The contact time between the bacterial suspension and sample was reduced to 5 min to increase the sampling throughput and to avoid luminescence fading due to the short bacteria lifetime. The coefficients of variation were estimated at 2.4 and 2.0 % (n=10), in the absence and in the presence of 0.60 mg L⁻¹ Zn(II), respectively. The EC₅₀ values for emerging contaminants (parabens, caffeine, acetaminophen, diclofenac, and salicylic acid) agreed at the 95% confidence level with those obtained with the commercial BioTox™ kit performed in micro plate. The proposed flow system is then a simple, fast, robust, and accurate alternative for acute toxicity determination, using low sample and bacterial suspension volumes. Furthermore, it presented other advantages in relation to batch and previously proposed flow-based bioassays, such as in-line osmotic adjustment and sample dilutions, and the evaluation of toxicity kinetic for every assayed sample.

Keywords: Flow analysis, multicommutation, multi-pumping flow system, in-line dilution, ecotoxicity, emerging contaminants.

Introduction

Ecotoxicological bioassays are important to assess the bioavailability and toxicity of pollutants in the environment. A test organism (*e.g.* microalgae or bacteria) is exposed to different pollutant concentrations in order to estimate the toxic effects. The bioassay is carried out under experimental conditions defined by specific norms, including controlled temperature, salinity and pH.¹ A negative control (*i.e.* the response in the absence of the pollutant) and a positive control (*i.e.* the test with a chemical species with a well-known effect) are required to evaluate the effect of other factors on the test-organism and the effectiveness of its response, respectively.

One of the most applied ecotoxicological bioassays is based on the marine photobacterium *Vibrio fischeri*. Under favourable conditions, these bacteria show natural bioluminescence, which is directly proportional to the mean metabolic activity and the integrity of the bacterial cells. Thus, parameters that affect the enzymatic activity (mainly of NAD(P)H dehydrogenase enzyme, EC 1.6.8.1), such as a pollutant species in the medium, diminish the bioluminescence. Optical measurements can then be performed to estimate the acute toxicity of the pollutant,² usually expressed as the effective concentrations that diminishes the bioluminescence in 20 or 50% (EC₂₀ or EC₅₀ values). These parameters can be estimated from the gamma (Γ) values, defined in equation 1,³ where I_0 is the initial luminescence intensity at the established contact time and I_x is the corresponding value in the presence of the pollutant. Gamma values of 0.25 or 1.00 correspond to EC₂₀ or EC₅₀, respectively. The estimative can be done either directly or, more properly, from the linearized relation $\ln \Gamma$ versus $\ln C$ (equation 2), which express the dose response curve for a defined exposition time; C is the percentage of the initial concentration.

$$\Gamma = (I_0 / I_x) - 1 \quad (1)$$

$$\ln C = a + b \ln \Gamma \quad (2)$$

Although simple, sensitive and cost-effective in comparison with other bioassays, the toxicological test with *Vibrio fischeri* is time-consuming, requires skilled analysts and the exposing time is difficult to control accurately, thus hindering the precision. Aiming at to circumvent these limitations, two flow-based systems designed in the (FIA)⁴ or (SIA)⁵ modalities were proposed. The former was designed in the single line configuration and comprised a lab-made detection system (flow cell in a wall-jet configuration coupled to a photomultiplier); by injecting the bacterial suspension, the effect of toxic metal ions was evaluated, but results were not compared with those attained by the batchwise ecotoxicological bioassay. The later was exploited for evaluation of the toxicity of ionic liquids, but the EC₅₀ values did not agree with those obtained by using the batch assay. This agreement is essential for the replacement of the conventional procedure by novel approaches with reliable results. In addition, the flow-based procedures also presented some disadvantages, such as the need for off-line sample dilutions and osmotic (*i.e.* salinity) adjustment, then hindering the sample throughput and increasing the risks of systematic errors.

Solenoid micro-pumps have been efficiently used for flow delivery, sample and reagent insertion as well as commutation in flow analysis. The inherent pulsed flow causes sudden and repetitive changes in the linear velocities of the fluid elements that improve the radial mass transference and the mixing conditions.⁶ Minimization of sample and reagent consumption, portability and facilities to modify the flow-rate or to implement the stopped-flow approach are other advantages. Multi-pumping flow systems (MPFS) have also been successfully applied to sample dilutions exploiting different approaches.⁷

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3 In order to circumvent the disadvantages of the abovementioned systems, a multi-
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5 pumping flow system was developed to accomplish in-line dilutions by modifying the
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7 sample volume or by exploiting the partial overlap between the gradient of
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9 concentration of the pollutant in the sample zone and the bacterial suspension, similarly
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11 to a previous approach for dilutions in flow injection analysis.⁸ Furthermore, the
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13 solenoid micro-pumps improve the mixing conditions, thus the efficiency of the contact
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15 between sample and bacterial suspension.
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20 21 **Experimental**

22 *Apparatus*

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25 The flow system was built-up with four solenoid micro-pumps (Biochem Valve
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27 Inc., Boonton NJ, USA; model 120SP) with nominal volumes per stroke of 20 (P₁, P₂
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29 and P₃) and 10 μL (P₄). The calibrated volumes released by the micro-pumps were
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31 20.9±0.9 (P₁), 20.9±0.7 (P₂), 21.1±0.8 (P₃) and 10.3±1.6 (P₄). A three-way solenoid
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33 valve (NResearch, West Caldwell, NJ, USA), 0.8-mm i.d. polyethylene tubes and
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35 acrylic joint points were also used. The active devices were computer-controlled
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37 through a parallel port of a Pentium IV microcomputer by using a power drive based on
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39 a ULN2803 integrated circuit, similar to one previously described.⁹ Commercially
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41 available current drives (*e.g.* NResearch CoolDrive® Valve Driver,
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43 <http://www.nresearch.com>) can also be used. The control software was developed in
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45 Microsoft Visual Basic 6.0 (Microsoft, Redmond, WA, USA) and is available upon
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47 request to the authors.
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52 Bio and chemiluminescence measurements were carried out in a
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54 spectrofluorimeter (Varian-Eclipse, Mulgrave, VIC, Australia) equipped with a lab-
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56 made polyethylene spiral flow cell (100-cm long, 0.8-mm i.d. and 500-μL internal
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3 volume) positioned in front of the emission window of the spectrofluorimeter. The
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5 integration time and the voltage applied to the photomultiplier tube (PMT) were 5 and
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7 100 ms and 600 and 800 V for the chemiluminescence and bioluminescence
8
9 measurements, respectively. The emission slit was adjusted to yield a 20-nm resolution
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11 and the software provided by the manufacturer was used for data acquisition.
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14 The temperature of the bioassay was controlled by immersing both the bacterial
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16 suspension and the reaction coil in a portable lab-made water bath based on the Peltier
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18 effect with the temperature controlled at (13.0 ± 0.1) °C. Ambient temperature was
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20 maintained at (17 ± 1) °C during the experiments through air-conditioning facilities.
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23 24 25 *Reagents and solutions* 26

27 All solutions were prepared with analytical grade chemicals and deionized water
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29 (resistivity > 18.2 MΩ cm). For the model system, a 0.975 mmol L⁻¹ hydrogen peroxide
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31 stock solution was daily prepared from a 30% (w/w) solution previously standardized
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33 with potassium permanganate. Reference solutions (20-100 nmol L⁻¹ H₂O₂) were
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35 prepared by dilutions of the stock in water. The reagents were 12.5 and 25.0 mmol L⁻¹
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37 potassium hexacyanoferrate(III) solutions prepared in water and a 4.50 mmol L⁻¹
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39 luminol solution in 0.2 mol L⁻¹ potassium carbonate buffer, pH 10.5.
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43 For the *Vibrio fischeri* system, 4.0 and 2.0 % (m/v) NaCl solutions were used for
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45 osmotic adjustment and as diluent/carrier stream, respectively. Lyophilized bacteria
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47 (*Vibrio fischeri*, NRRL B-11177) and reconstitution solution were obtained from
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49 Biolux[®] Lyo test kit (Umwelt, Blumenau, SC, Brazil). A diluent solution for bacterial
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51 suspension was prepared according to official procedure,² containing 20.000 g NaCl,
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53 2.035 g MgCl₂·6H₂O and 0.3000 g KCl in 1.00 L of water. ZnSO₄·7H₂O, K₂Cr₂O₇ and
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55 CuSO₄·5H₂O were used to prepare solutions used as positive controls for the *Vibrio*
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3 *fischeri* test. Parabens (methyl, ethyl, propyl and butyl), caffeine, acetaminophen,
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5 diclofenac, salicylic and acetylsalicylic acids solutions were prepared in water or in
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7 2.0 %(m/v) NaCl for the toxicity assays by the proposed and reference procedures,
8
9 respectively. EC₅₀ values obtained with the proposed procedure were compared with
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11 those attained in a batch bioassay based on the BioTox™ kit performed in micro plate.²
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14 15 16 *Reconstitution of lyophilized Vibrio fischeri*

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18 The lyophilized bacteria were mixed with 1.00 mL of the reconstitution solution
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20 immediately before the assay. The suspension was equilibrated at 5 °C for 5 min before
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22 adding 10 mL of the diluent solution for bacterial suspension, also at 5 °C. This
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24 suspension contained *ca.* 10⁷ cells mL⁻¹ and it was stabilized at 13 °C for at least
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26 15 min. During the assay, the bacterial suspension was kept at the same temperature
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28 with periodical stirring to avoid deposition of the bacterial cells.
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32 33 34 *Flow system*

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36 The multi-pumping flow system in Figure 1 was operated according to the
37
38 switching course in Table 1. All measurements were based on peak heights and carried
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40 out in triplicate.
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43 A model system was designed to simulate the bacterial assay and to evaluate the
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45 response of the detection system and the experimental conditions for in-line dilutions. It
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47 was based on the oxidation of luminol to aminophthalate by H₂O₂, using potassium
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49 hexacyanoferrate(III) as catalyst. Hydrogen peroxide, luminol, and potassium
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51 hexacyanoferrate(III) were used to simulate the pollutant solution, bacterial suspension
52
53 and solution used for osmotic adjustment, respectively. A potassium
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55 hexacyanoferrate(III) solution was also used as carrier and diluent. The sample (S) was
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3 inserted simultaneously with R₁ (potassium hexacyanoferrate(III)) in the analytical path
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5 (step 1), and the sample zone was diluted by the carrier stream when flowing inside the
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7 coil B (step 2). The extent of sample dilution was defined by the volumetric fraction
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9 established in steps 1 and 2, with the total volume kept constant. The sample zone was
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11 transported by the actuation of micro-pump P₃ (step 3) and the concentration gradient
12
13 was exploited by inserting a single aliquot of luminol at different points (step 4). The
14
15 sample zone was transported through the spiral flow cell (step 5) and then towards
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17 waste (step 7) by the actuation of micro-pump P₃. The light emission was monitored at
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19 460 nm.
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23 For the bioassay, the MPFS in Fig. 1 was also operated according to the
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25 switching course in Table 1. The pollutant solution (S) and the salinity adjuster solution
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27 (R₁, 4.0 %(m/v) NaCl) were simultaneously inserted into the analytical path (step 1),
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29 and the sample zone was diluted by the carrier stream inside coil B (step 2). The sample
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31 zone was transported by the actuation of micro-pump P₃ (step 3). A single aliquot of the
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33 bacterial suspension was inserted at different points on the sample zone, exploiting the
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35 concentration gradient as aforementioned (step 4). Thereafter, the sample zone was
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37 carried through the spiral flow cell (step 5) and the flow was stopped in order to
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39 increase the sample residence time and the contact time between sample and bacterial
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41 suspension (step 6). The bacterial bioluminescence was monitored at 490 nm. For
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43 estimating the EC₅₀ values, these measurements were based on peak heights for 5 min
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45 of contact time between sample and bacterial suspension. Then, the sample zone was
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47 directed towards waste by pump P₃ (step 7).
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52 The solenoid valve (V) was used for sample replacement (step 8) in order to
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54 direct solutions to waste, thus minimizing risks of contamination.
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Results and discussion

General aspects

Essential steps of the bioassay with the *Vibrio fischeri* bacteria (*i.e.* osmotic adjustment and sequential dilutions of the pollutant solutions) were not in-line implemented in the previously described flow systems,^{4,5} thus hindering sample throughput and increasing the risks of systematic errors. In addition, mixing of sample and the bacterial suspension occurred by dispersion under conditions of laminar flow, which shows limited efficiency. In the present work, the multi-pumping approach was selected to circumvent these drawbacks. Micro-pumps with similar volumes were selected for handling sample and R₁ solutions aiming at the osmotic adjustment in every sample. Modification of the sample volumetric fraction and exploitation of the gradient of the pollutant solution allowed sample dilutions. The efficient turbulent mixing provided by the pulsed flows improved the interaction of the pollutant with the bacterial suspension. The stopped-flow approach, easily implemented in MPFS, was adopted to increase the contact time, thus improving sensitivity of the bioassay. A model system was designed for preliminary studies as well as to define the experimental conditions for the controlled dilutions of the pollutant.

Model system

Different flow cell geometries (spiral and “U”-shaped) and detection systems (photodiode-based luminometer and PMT of a spectrofluorimeter) were evaluated for luminescence measurements. The detectors yielded similar detection limits (0.2 and 0.3 nmol L⁻¹ for PMT and the photodiode) as well as linear response ranges (up to 100 nmol L⁻¹) for the model system. Although the luminometer is advantageous in view of the low-cost and portability, the spectrofluorimeter was preferred because sensitivity

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3 can be improved by modifying the integration time and the voltage applied to the PMT.
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5 Indeed, this was required for the measurement of the low intensity bioluminescence of
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7 the *Vibrio fischeri*. Minimization of light scattering is important to avoid spurious
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9 signals that could affect the bioassay accuracy. Light scattering was evaluated by using
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11 a suspension of silica particles (mean diameter $\leq 60 \mu\text{m}$) in H_2O_2 medium to simulate
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13 the bacterial suspension. The effect was less pronounced when the spiral flow cell was
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15 used instead of the “U”-shaped commercial cell, being the former selected.
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19 The model system was also exploited to define the conditions for in-line
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21 dilutions with complementary sample and diluent volumes (total volume = $502 \mu\text{L}$;
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23 pulses of $P_1 + P_2 + P_3 = 24$). When the reactor length was fixed at 100 cm, up to 25-fold
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25 sample dilution was achieved by varying the sample/carrier volumetric ratio (Table 1,
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27 steps 1 and 2). The luminescence intensity (I) increased linearly with the sample volume
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29 (V_s , μL) according to the equation $I = 4.72 V_s + 31.0$ ($r = 0.993$; $n=6$).
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33 The partial overlap of the dispersed sample and luminol aliquot was exploited
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35 for higher dilutions. Results obtained by inserting the luminol solution in different
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37 portions of the dispersed sample zone (condition achieved by changing the number of
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39 pulses of P_3 in step 3) are shown in Figure 2a. As expected, linear responses were also
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41 obtained when the volumetric fractions were changed (steps 1 and 2) and measurements
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43 were carried out in different portions of the dispersed zone (Figure 2b). It was then
44
45 possible to choose some dilution factors to work with the *Vibrio fischeri* system (Table
46
47 2), by taking into account the dilutions required in the batch procedure. For the model
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49 system, the slopes of the calibration curves (luminescence intensity *versus* H_2O_2
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51 concentration) obtained by in-line dilutions agreed with those achieved in batch
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53 (deviations $< 3.6\%$) and similar results were obtained when the experiment was
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3 repeated on different working days. This demonstrates the reliability of the proposed
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5 approach.
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8 9 *Vibrio fischeri* bioassay

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11 The stability of bacterial bioluminescence was evaluated in function of time,
12 during a 40-min stopping period and the variations in the emission intensity were lower
13 than 1.0 %. The contact time was then defined as 5 min aiming at to increase the sample
14 throughput, also making feasible to use the same bacterial suspension in a full working-
15 day (*ca.* 80 measurements). Variations of the negative control due to the stability of the
16 bacterial suspension were not significant in this short contact time. The luminescence
17 signal (I) increased linearly with the volume of the bacterial suspension (V_{R2}) up to
18 250 μL ($I = -12.1 + 3.90 V_{R2}$, $r = 0.995$; $n=6$), due to minimization of the dispersion.
19 However, despite the response to the negative control was reduced to half, 120 μL was
20 selected to minimize the consumption of the bacterial suspension and, consequently, the
21 cost of the bioassay. The coefficients of variation were estimated as 2.4 and 2.0% for
22 the negative and positive ($0.60 \text{ mg L}^{-1} \text{ Zn}^{2+}$) controls, respectively.
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39 The flow-based bioassay was applied to the evaluation of the toxicity of toxic
40 metals and emerging contaminants, with Zn(II) as positive control. Figure 3 shows the
41 signal profiles and the linear relation $\ln C$ versus $\ln r$ obtained in the evaluation of
42 Zn(II) toxicity and Table 3 shows the corresponding equations obtained for Cu(II),
43 Cr(VI) and different emerging contaminants. For most of the evaluated substances, the
44 EC_{50} values agreed at the 95% confidence level with those obtained by the reference
45 procedure with the same contact time (Table 4). In addition, a good correlation was
46 observed between the results obtained by both procedures: $\text{EC}_{50}(\text{MPFS}) = (1.11 \pm 0.05)$
47 $\text{EC}_{50}(\text{batch}) + (1 \pm 4)$, $r = 0.989$. The slope close to 1.00 and the interval of the linear
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3 coefficient that includes zero reinforce the agreement between the results. This confirms
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5 that suitable mixing conditions and effective temperature control were achieved and that
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7 the calibration defined by the model system successfully yielded correct concentrations
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9 in the in-line dilutions. It should be recalled that results achieved in the SIA procedure⁵
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11 did not agree with those obtained in the batch reference one and that this comparison
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13 was not done in the FIA procedure.⁴ As a consequence, and in contrast to the present
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15 proposed approach, these procedures can be used only for screening purposes.
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19 According to the EC₅₀ values, the emerging contaminants were classified¹⁰ as
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21 toxic, *i.e.* EC₅₀ from 1 to 10 mg L⁻¹ (parabens, diclofenac and salicylic acid); harmful,
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23 *i.e.* EC₅₀ within 10 - 100 mg L⁻¹ (acetylsalicylic acid) and non-toxic, *i.e.* EC₅₀ > 100 mg
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25 L⁻¹ (caffeine and acetaminophen). None of the compounds was classified as very toxic
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27 (EC₅₀ < 1 mg L⁻¹).
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30 Although the estimated EC₅₀ values cannot be directly compared with those
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32 reported in the literature in view of different incubation times, a similar trend was
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34 observed. For example, a previous study¹⁰ reported that methylparaben is less toxic for
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36 *V. fischeri* than the analogous species with ethyl, propyl and butyl groups and the
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38 reported EC₅₀ values were lower than in the present study due to the higher contact time
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40 (15 min), except for ethylparaben. The four parabens evaluated in this work have also
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42 been classified as toxic.¹⁰ High EC₅₀ values and uncertainties were also reported for
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44 acetaminophen.¹¹ The same effect was observed for caffeine in the present study. This
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46 can be related to the lower absorption of the species by the bacteria (thus resulting in
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48 high EC₅₀ values), which is hindered by the low lipophilicity of these substances
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50 (caffeine and acetaminophen shows the lower partition octanol water among the
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52 evaluated species: log K_{OW} = -0.01 and 0.34, respectively).¹² It is possible that the effect
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3 is not observed even in high concentrations and this can vary for different populations
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5 of bacteria.
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8 Similar slopes of the concentration-response curves were obtained for methyl
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10 and ethylparaben, which indicates that the same toxicity mechanism is involved,
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12 although ethylparaben shows higher toxicity in lower concentrations. The curves for
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14 these species showed higher slopes than the observed for the other parabens, which
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16 indicate a fast absorption and toxic action on the test-organism. On the other hand,
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18 lower slopes can indicate deficient absorption, fast excretion or that the substances show
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20 the toxic effects after a longer contact time.¹³
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24 In-line sample dilution and osmotic adjustment are advantages of the proposed
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26 procedure in comparison to batch and previously described flow based systems. The
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28 batch procedure is also more time-consuming and requires a skilled analyst to assure
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30 reliable results. Other characteristics of different acute bioassays with *V. fischeri* are
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32 compared in Table 5. In the proposed procedure, *ca.* 1.3 mL is needed for the full test,
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34 which is considerably lower than the required in batch and flow procedures when the
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36 required dilutions are taken into account. This is an important aspect when the sample
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38 volume is limited as well as to minimize the waste volume. The volume of the bacterial
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40 suspension affects both the bioluminescence intensity and the cost of the assay. The
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42 amount consumed in the present procedure is 2.4-fold higher than in the SIA
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44 procedure,⁵ but 4-fold lower than in FIA⁴ and the batch procedure.¹⁴ In the proposed
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46 procedure, the volume of the bacterial suspension could be further reduced by
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48 increasing the integration time of the PMT, but the representativeness of the lower
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50 aliquot of the bacterial suspension should be critically evaluated in order to assure
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52 reproducible results. The contact time affects directly the sample throughput, but lower
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54 values are not necessarily an advantage for bioassays because sensitivity is critically
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1 hindered. The increase in sample throughput in the proposed procedure becomes evident
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3 when the preliminary steps (osmotic adjustment and sample dilutions) are taken into
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5 account. For example, a full test requires at least 1 h in the SIA procedure (twice as the
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7 required in the proposed procedure). In addition, sample replacement is time-consuming
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9 in the SIA and single line FIA procedures. Table 5 also shows the improvement in
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11 precision provided by the flow-based procedures.
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16 Differently of the previously described approaches, the present proposal allows
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18 the continuous monitoring of the bioluminescence in the presence of the sample
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20 (stopped-flow with the sample zone in the flow cell), as shown in Figure 4. This kinetic
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22 information is useful for the evaluation of the mechanism of toxicity. Among the
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24 investigated species, three different kinetic profiles were observed: (i) a gradual
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26 diminution of the bioluminescence in the presence of Zn(II), Cu(II) and Cr(VI) – Fig. 4I
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28 shows the results observed for Zn(II); (ii) an initial attenuation of the bioluminescence
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30 followed by an emission plateau – Fig. 4II shows the results observed for
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32 methylparaben, but the same pattern was observed for the other parabens, caffeine,
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34 acetaminophen and salicylic acid; (iii) a significant attenuation of bioluminescence from
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36 the first contact with the pollutant, followed by a gradual decrease of the emission. This
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38 was observed for diclofenac (Fig. 4III) and acetylsalicylic acid.
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45 **Conclusions**

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47 The proposed flow-based procedure is a simple, fast, robust and accurate alternative
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49 for evaluation of the acute toxicity with the *V. fischeri* bacteria, using low sample and
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51 bacterial suspension volumes. In-line osmotic adjustment and sample dilutions
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53 improved sample throughput as well as minimized risks of systematic errors. In
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55 addition, the proposed approach effectively allowed the determination of EC-values
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rather than sample screening. Results for emerging contaminants agreed with those obtained in the batch reference procedure, being also consistent with values reported in the literature. Evidences for understanding of the toxicity mechanism are also provided by the continuous monitoring of the bioluminescence in the presence of the pollutant.

Acknowledgements

The authors acknowledge the fellowships and financial support from the Brazilian agencies CAPES, CNPq and FAPESP (proc. 2011/23498-9) and from *Núcleo de Pesquisa em Tecnologia e Inovação para Sustentabilidade da Agricultura* (NAPTISA). Prof. E.A.G. Zagatto and Ms. Alex D. Batista are thanked for critical comments. This is a contribution of the National Institute of Advanced Analytical Sciences and Technology (INCTAA). The research group REQUIMTE from Universidade do Porto is thanked for the infrastructure provided to the batch ecotoxicological bioassay as well as profitable discussions.

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Figure captions

Figure 1. Flow diagram of the multi-pumping flow system for acute toxicity bioassay with *Vibrio fischeri*. P₁–P₄: solenoid micro-pumps; V: three-way solenoid valve; x and y: confluence points; B: 100-cm tubular reaction coil; D: spiral flow cell; S: sample; C: carrier; R₁ and R₂: reagents; W: waste vessels. **Model system:** S: hydrogen peroxide; C: 12.5 mmol L⁻¹ potassium hexacyanoferrate(III); R₁: 25.0 mmol L⁻¹ potassium hexacyanoferrate(III) and R₂: 4.50 mmol L⁻¹ luminol solution (buffered at pH 10.5). **Vibrio fischeri system:** S: sample; C: 2.0 % (m/v) NaCl; R₁: 4.0 % (m/v) NaCl and R₂: bacterial suspension. Dashed lines indicate that temperature is controlled (13.0±0.1 °C) in a water bath.

Figure 2. Strategies for sample dilutions referent to the model system. (I) profile of the sample zone obtained by changing the portion of the gradient in which the luminol aliquot is inserted. The selected portions of the dispersed zone are indicated as D₁–D₃, which correspond to 100.0, 59.8 and 9.0% of the maximum concentration. (II) Responses obtained by changing the sample volumetric fractions with measurements at the portions of the gradients previously defined: (a) D₁, (b) D₂, and (c) D₃. The inset shows the expanded graph referent to the condition of higher dispersion (c).

Figure 3. I. Influence of the contact time for (a) negative control; (b) 0.42; (c) 1.08, (d) 3.46, (e) 5.95, (f) 12.65, (g) 25.77 and (h) 31.05 mg L⁻¹ Zn(II). II. Corresponding ln C versus ln r graph. Zinc concentrations achieved by in-line dilutions of a 100 mg L⁻¹ solution.

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3 **Figure 4.** Kinetic monitoring of the toxicity effects for (I) Zn(II); (II) methylparaben
4 and (III) diclofenac. Curves correspond to: (a) negative control (black lines); (b) 0.42;
5 and (III) diclofenac. Curves correspond to: (a) negative control (black lines); (b) 0.42;
6 and (III) diclofenac. Curves correspond to: (a) negative control (black lines); (b) 0.42;
7 (c) 1.08; (d) 3.46; (e) 5.95; (f) 12.65; (g) 25.77 and (h) 31.05 % of the initial
8 concentrations (100 mg L⁻¹ Zn(II) or diclofenac and 250 mg L⁻¹ methylparaben).
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Tables

Table 1. Switching course of solenoid micro-pumps for the model and *Vibrio fischeri* systems.

Step	Description	Active device	Pulses	Total Volume (μL)
1	Insertion of S and R ₁	P ₁ and P ₂	0 – 12	v ₁ ^a
2	In-line dilution	P ₃	0 – 24	v ₂ ^a
3	Transport of sample zone	P ₃	0 – 57	0 – 1203
4	Insertion of R ₂	P ₄	12	124
5	Transport to detection	P ₃	30	633
6 ^b	Stopped-flow for 300 s	—	—	—
7	Washing	P ₃	75	1582
8	Sample replacement	P ₁ , V	25	522
		P ₃ , V	10	211

^av₁ + v₂ = 502 μL ; ^bStep required only for the bioassay.

Table 2. In-line dilution factors for the flow-based bioassay.

Volumetric fraction ($N_{p1}+N_{p2}$)/(N_{p3})^a	Portion of the gradient^b	Equivalent concentration (%)^c	Dilution factor^c
24/0	D ₁	31.0	3.22
18/6	D ₁	25.8	3.88
10/14	D ₁	12.6	7.90
6/18	D ₁	5.95	16.8
10/14	D ₂	2.54	39.4
24/0	D ₃	3.46	28.8
18/6	D ₃	1.08	92.8
12/12	D ₃	0.42	236

^a N_{p_n} = number of pulses of the pump n ($N_{p1}+N_{p2}+N_{p3} = 24$); ^bD₁, D₂ and D₃ refer to 27, 42 or 57 pulses of the carrier in step 3 (please see Table 1 and Figure 2); ^cestimated in relation to the initial concentration of the pollutant.

Table 3. Linear equations (n=3) for the estimative of the EC₅₀ values by the flow-based bioassay.

Chemical species	Equation ^a	r		Equation ^a	R
Methylparaben	$\ln C = 1.08 \ln r + 1.82$	0.984	Acetaminophen	$\ln C = 1.89 \ln r + 2.19$	0.988
	$\ln C = 1.06 \ln r + 1.67$	0.982		$\ln C = 2.51 \ln r + 2.33$	0.992
	$\ln C = 1.00 \ln r + 1.55$	0.974		$\ln C = 1.18 \ln r + 2.58$	0.996
Ethylparaben	$\ln C = 1.16 \ln r + 1.50$	0.989	Diclofenac	$\ln C = 3.00 \ln r + 2.09$	0.994
	$\ln C = 1.02 \ln r + 1.06$	0.996		$\ln C = 2.76 \ln r + 2.37$	0.983
	$\ln C = 1.16 \ln r + 1.03$	0.989		$\ln C = 2.72 \ln r + 1.36$	0.999
Propylparaben	$\ln C = 1.47 \ln r + 1.81$	0.990	Salicylic acid	$\ln C = 0.55 \ln r + 3.75$	0.999
	$\ln C = 1.05 \ln r + 1.97$	0.982		$\ln C = 1.46 \ln r + 2.12$	0.999
	$\ln C = 1.78 \ln r + 1.54$	0.983		$\ln C = 1.59 \ln r + 2.48$	0.995
Butylparaben	$\ln C = 0.92 \ln r + 2.52$	0.989	Cu(II)	$\ln C = 1.13 \ln r + 1.52$	0.992
	$\ln C = 0.98 \ln r + 2.31$	0.977		$\ln C = 0.71 \ln r + 1.06$	0.995
	$\ln C = 0.86 \ln r + 2.51$	0.978		$\ln C = 1.30 \ln r + 1.55$	0.991
Caffeine	$\ln C = 2.65 \ln r + 2.03$	0.998	Cr(VI)	$\ln C = 3.09 \ln r + 2.31$	0.991
	$\ln C = 2.73 \ln r + 1.93$	0.999		$\ln C = 2.17 \ln r + 1.73$	0.984
	$\ln C = 2.99 \ln r + 1.29$	0.995		$\ln C = 1.57 \ln r + 2.61$	0.997
Acetylsalicylic acid	$\ln C = 1.36 \ln r + 2.10$	0.991	Zn(II)	$\ln C = 1.07 \ln r + 1.34$	0.991
	$\ln C = 0.53 \ln r + 2.25$	0.990		$\ln C = 1.48 \ln r + 1.88$	0.979
	$\ln C = 1.24 \ln r + 2.08$	0.987		$\ln C = 1.38 \ln r + 1.49$	0.981

Table 4. EC₅₀ values (means and standard deviations) estimated by the proposed flow-based bioassay and the reference batch procedure with a 5-min contact time.

Species	EC ₅₀ (mg L ⁻¹)	
	Proposed procedure	Batch reference ²
Methylparaben	6.8±0.9	6.20±0.11
Ethylparaben	2.9±0.4	2.66±0.16
Propylparaben	3.0±0.6	3.67±0.10
Butylparaben	2.9±0.3	2.18±0.04
Caffeine	181±63	135±26
Acetylsalicylic acid	12.8±1.2	5.8±0.2
Acetaminophen	216±42	210±10
Diclofenac	3.8±1.7	3.40±0.01
Salicylic acid	5±4	6.4±0.9
Cu(II)	2.0±0.5	5.1±0.5
Cr(VI)	10±4	10.7±0.5

Table 5. Comparison of different acute bioassays with *V. fischeri* bacteria.

Parameter	This work	FIA⁴	SIA⁵	(BioTox™)¹⁴
Sample volume (μL)	21 – 252	> 1000	50	500
Volume of the bacterial suspension (μL)	120	500	50	500
Contact time (min)	5	< 1	3	30
Coefficient of variation (%), n=10	2.0	0.7	< 1.1	> 10

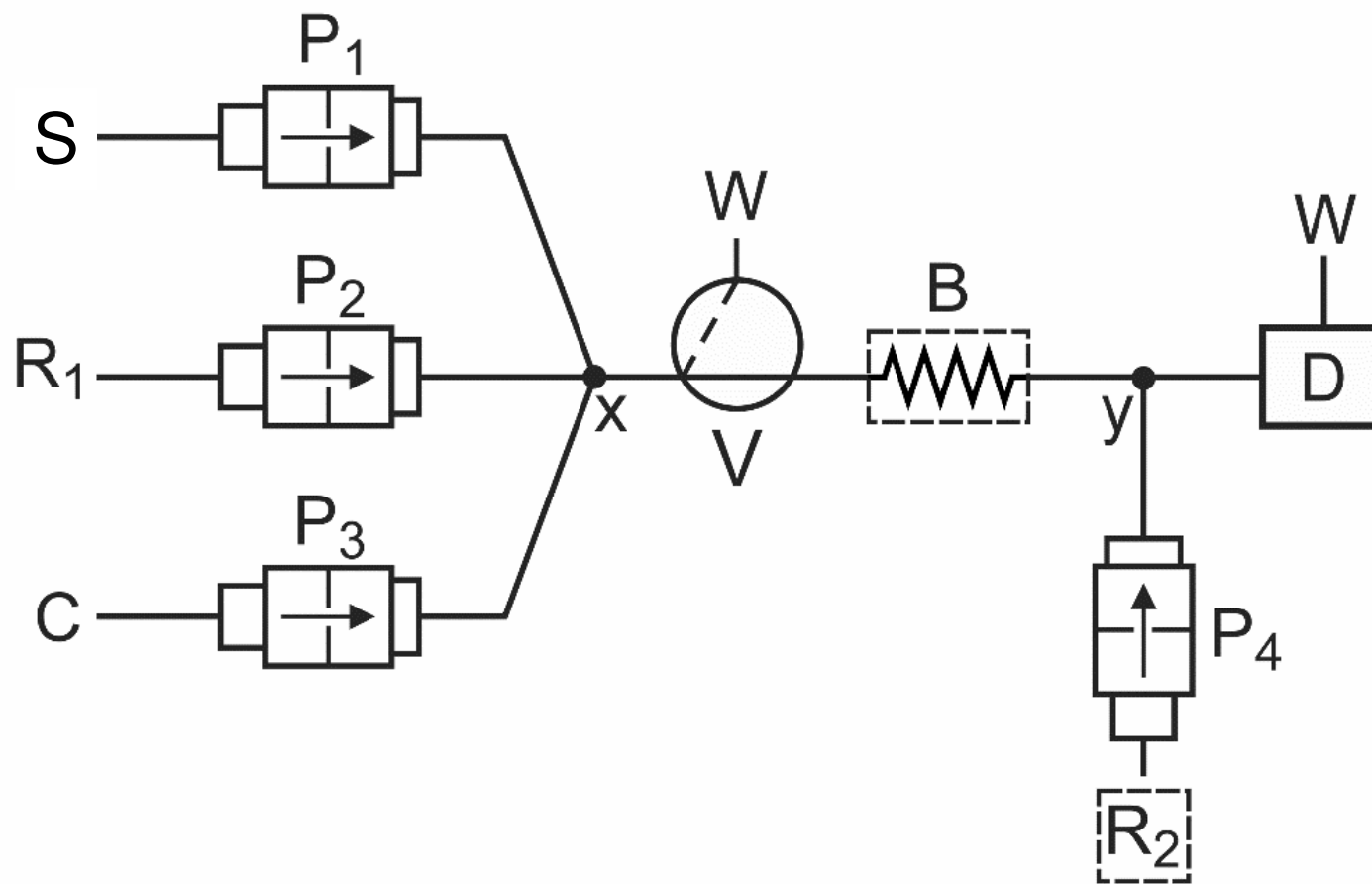


Figure 1

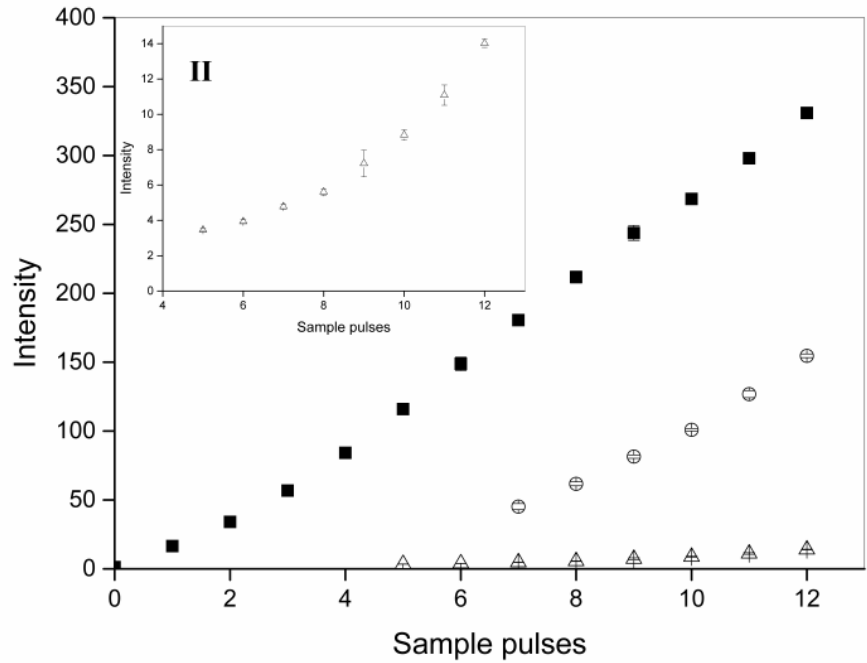
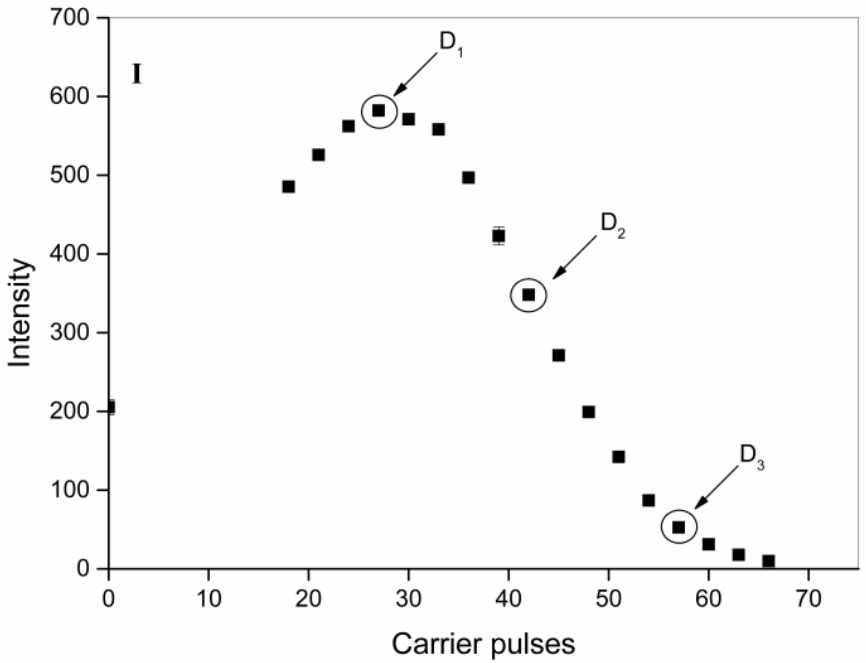


Figure 2

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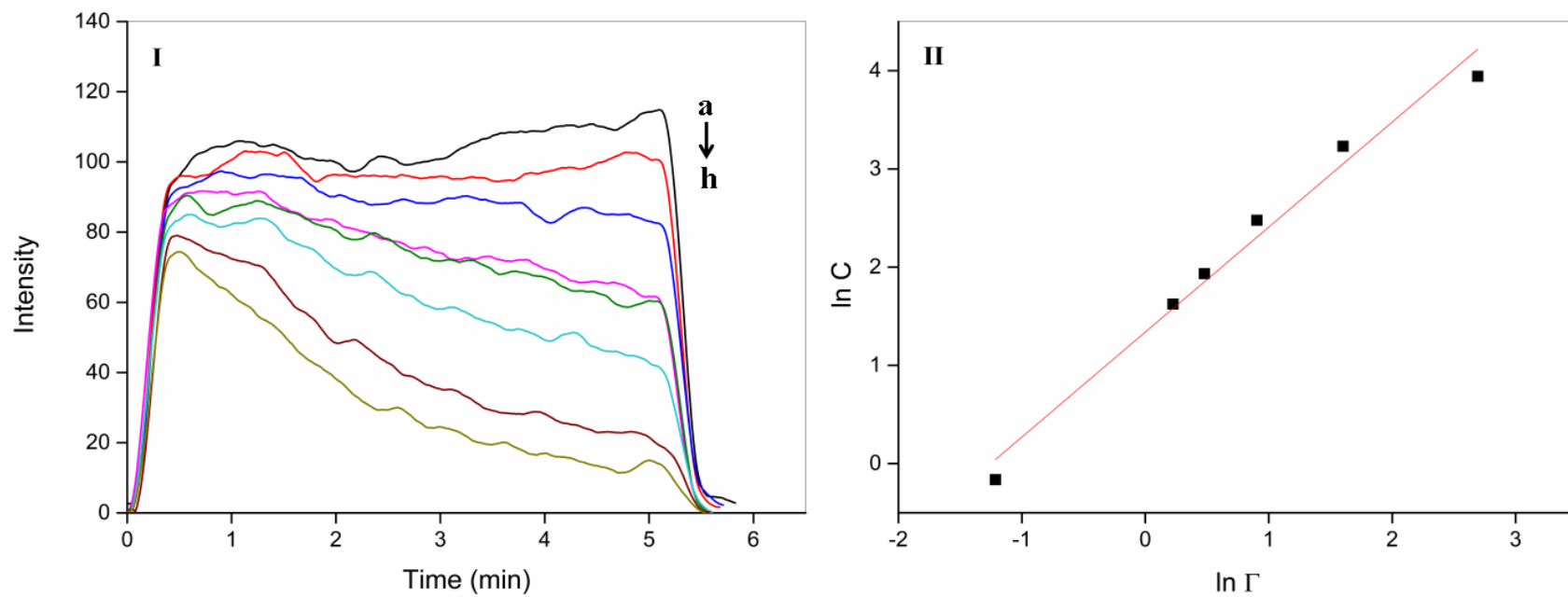


Figure 3

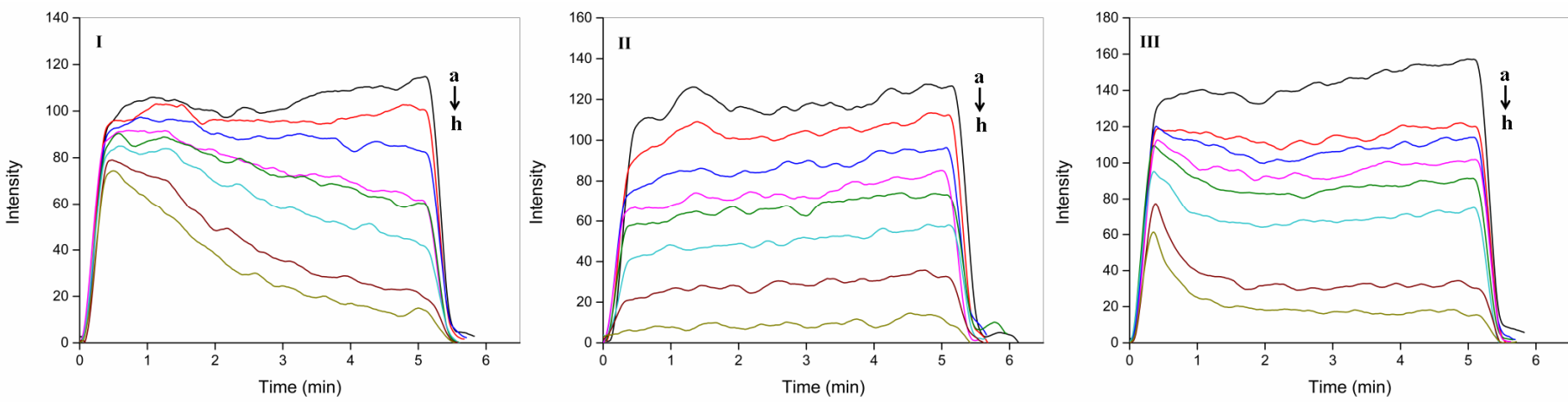


Figure 4

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