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RESEARCH ARTICLE

Rapid in situ toxicity testing with luminescent bacteria *Photorhabdus luminescens* and *Vibrio fischeri* adapted to a small portable luminometer

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Abstract The present study demonstrates development of a rapid testing protocol based on a small portable luminometer using flash kinetic assessment of bacterial bioluminescence. The laboratory comparisons based on six model organic toxicants and two metals showed significant correlations between responses of freshwater bacteria *Photorhabdus luminescens* and standard marine bacterial species *Vibrio fisheri*. While *P. luminescens* was less sensitive in standard arrangements, the responses of both organisms were comparable in the newly introduced portable luminometer setup. The applicability and reproducibility of the portable luminometer protocol was further demonstrated in the assessment of 43 European wastewater effluents that were simultaneously tested for toxicity and analysed for 150 organic and 20 inorganic contaminants grouped into 13 major chemical classes. Clear association between the toxic responses in both compared bacterial species and the elevated levels of inorganic compounds (toxic metals), chlorophenols and benzotriazole anticorrosives was observed. The new protocol with a portable luminometer provides a fast (30 s) response and may be used as a tool for rapid in situ toxicity evaluation of freshwater environmental samples such as effluents.

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Introduction

Large amounts of contaminant mixtures of varied composition enter the environment as—for example—industrial waste, field runoff, municipal and domestic waste and effluents (Farré and Barceló 2003). Despite of intensive treatment of wastewater, residual contamination still has a significant impact on aquatic biota as well as on human health (Heberer 2002). The most studied groups of aquatic pollutants include halogenated persistent organic pollutants (POPs), polycyclic aromatic hydrocarbons (PAHs), toxic metals, pesticides and other micropollutants such as pharmaceuticals and personal care products (Deblonde et al. 2011). Despite of existing diversity and documented adverse effects, current legislation such as the European Water Framework Directive (EU WFD) monitors and regulates only a limited number—currently 45—of so-called priority pollutants (Directive 2008/105/EC 2008).

Routine monitoring efforts of water quality in Europe currently combine two approaches, namely chemical analyses of target compounds (the priority pollutants) to evaluate chemical status and in situ assessment of biota to evaluate the biological status (Directive 2000/60/ES). Direct assessment of potential adverse (toxic) effects of water or effluents by applying bioassays is currently lacking in the EU water legislation despite of scientific recommendations and running effect-based monitoring efforts (Schulze et al. 2015; Wernersson et al. 2015; Escher et al. 2013).

While the chemical analyses inform about the identity of the chemical substances in the sample, they provide only minimum information about the overall toxicity (Wolska

et al. 2007). This limitation can be overcome by applying effect-based tools and bioassays. A number of aquatic bioassays have been evaluated for testing of complex samples including diverse model organisms such as bacteria, algae, plants, invertebrates, embryonal and adult fish or amphibians as well as cell-based in vitro models relevant for human health endpoints (Rosal et al. 2010). For example, a recent international study (Escher et al. 2014) compared a series of bioassays for wastewater assessment that included acute (cyto)toxicity endpoints, biomarkers of chronic effects (such as metabolic pathways activation or receptor modulations) as well as rapid bacterial luminescence tests. Among the other assays, bacterial bioluminescence tests employing marine *Vibrio fischeri* (now reclassified as *Aliivibrio fischeri*; Urbanczyk et al. 2007) have been found highly sensitive (Escher et al. 2014). Their low costs and fast responses make them suitable for broader use in water quality assessment (Nohava et al. 1995; Wolska et al. 2007).

Bacterial bioluminescence tests have been used for decades. The most widely used model employs *V. fischeri* and it has been routinely employed in testing of chemicals as well as environmental samples like wastewater effluents or contaminated soil (Rodriguez-Ruiz et al. 2015, Pandard et al. 2006). The original *V. fischeri* test can be used for clean and transparent samples but it has limitations when testing coloured or turbid materials that interfere with the emitted bacterial bioluminescence. To overcome this limitation, a solid-phase variant of the test has been developed (Volpi Ghirardini et al. 2009) but it has also been shown to provide false positives due to the protocol, where a fraction of bacteria is adsorbed on the studied matrix and removed from the test (Lappalainen et al. 1999; Campisi et al. 2005). A suitable alternative for testing of coloured samples is based on kinetic bioluminescence measurements known as the Flash assay (Lappalainen et al. 1999; 2001). The immediate flash luminescence (measured during seconds after the injection of bacteria into the sample) is affected only by the colour or turbidity, while the actual toxic effect is recorded after prolonged exposure. This kinetic approach, in which each sample serves as its own control for colour or turbidity, has also been standardized by ISO (ISO 21338:2010). Another limitation of using marine bacterial species such as *V. fischeri* is the need to add relatively high concentrations of salt to the studied samples. This manipulation of the sample can change its properties and alter the toxicity (Dunlap 1985, Cook et al. 2000; Deheyn et al. 2004).

Previous research addressing the above-described problems has explored various approaches such as insertion of luminescence Lux operon into the freshwater bacteria *Escherichia coli* (Kurvet et al. 2011), *Pseudomonas putida* (Stewart and Williams 1992), or the cyanobacteria *Anabaena* spp. (Rosal et al. 2010). In addition to genetically modified bacteria, other naturally bioluminescent species such as *Photorhabdus luminescens* could be used as an alternative for toxicity testing (Tabei et al. 2013). This species was isolated from the gastrointestinal tract of nematodes and it can be cultured in general microbiological media, i.e. without a need for elevated salt concentrations (Thomas and Poinar 1979). During the environmental monitoring, it is also important to minimize sample manipulations such as additions of stabilizers, drying, sieving and prolonged storage or extractions, which could lead to volatilization, transformation or degration of chemicals. Therefore, rapid portable protocols for direct field in situ toxicity assessment have been developed, such as the assessment of algal chlorophyll fluorescence (Kumar et al. 2014; Muller et al. 2008).

The aim of the present study was to assess a rapid screening bacterial luminescence test for field in situ toxicity assessment using a freshwater species *P. luminescens* and compare its performance and sensitivity with marine *V. fischeri* using a set of model toxicants and 43 wastewater effluents collected and chemically characterized in the course of pan-European monitoring campaigns.

Material and methods

Design of the study

First, the sensitivity of *P. luminescens* was compared with *V. fischeri* using a set of model chemicals and a standardized protocol (ISO 11348-3, 2009). In addition, the sensitivities of both *P. luminescens* and *V. fischeri* were tested also in the kinetic flash format (comparing the laboratory microwell plate arrangements vs. portable luminometer). Finally, the toxicity of 43 wastewater effluents collected during a pan-European monitoring study was evaluated by both microorganisms using the portable luminometer protocol, and the toxicity was compared with the results of chemical analyses of 150 organic and 20 inorganic contaminants.

Chemicals

Potassium dichromate (K2Cr2O7, purity 99.5 %); phenol (purity 99 %); and mercury chloride (HgCl2 purity 99.5 %) were purchased from Lach–Ner s.r.o (Neratovice, Czech Republic). 3,5-dichlorphenol (purity 97 %), 2-4-dichlorphenol (99 % GC purity grade), 2,3-dichloranilin (purity 99 %), and ethanol (purity 97 %) were purchased from Sigma-Aldrich (Steinheim, Germany). Sodium dodecyl sulphate (SDS, research grade purity) was from Serva GmbH (Heidelberg, Germany).

Environmental samples

Wastewater treatment plant (WWTP) effluents were collected during a pan-European study of the Joint Research Centre (EC-JRC) in Ispra, Italy (Loos et al. 2012). The 43 samples investigated in the present study covered WWTPs utilizing different treatment technologies from 13 European countries in different climatic regions. Each sample was thoroughly characterized for a range of 150 organic and 20 inorganic contaminants. Detailed information on the samples, WWTPs and methods of chemical analyses can be found in the publically available report and accompanying research paper (Loos et al. 2012, 2013). The present study used the same coding of the samples as described in the original report (Loos et al. 2012). Wastewater samples were kept frozen at −20 °C, thawed prior to toxicity testing, homogenized by vortexing and tested for toxicity as described below (direct testing of 1× concentrated sample without dilutions).

Bacterial strains

Vibrio fischeri (lyophilized NRRL-B-11177, LUMIStox, Hach Lange, Düsseldorf, Germany) were rehydrated according to the manufacturer's instructions prior to analysis and kept on ice until the test. *P. luminescens* CCM7077 was obtained from the Czech collection of microorganisms (Masaryk University, Brno, Czech Republic), and was cultured on standard Tryptic soy agar in Petri dishes at 30 °C. Ready-to-use batch of bacterial suspensions were prepared as follows: after 24-h incubation on agar, five colonies were transferred into 1.5 mL of freezing medium (tryptic soy broth with 5 % of glycerol) containing several sterile glass beads and gently agitated. Glass beads coated with bacteria were aliquoted and kept at −80 °C as inoculum for the experiments. Twenty four hours before the experiment, one glass bead from the freezer was transferred into the liquid media containing 7.5 mL of phosphate buffer salt solution (PBS) and 2.5 mL of liquid tryptone soy broth (TSB, Gibco, prepared according to the manufacturer's instructions), and then incubated for 18 h at 30 °C (Erlenmeyer flask, shaking at 120 RPM). The resulting bacterial suspension was directly used for toxicity testing. The composition of the testing media was assessed during the optimization experiments with *P. luminescens* with the aim to achieve good bacterial viability and stability of the luminescence signal, and at the same time, minimizing the content of TSB that might interfere with the outcome of the test. The ratio 3:1 PBS/TSB was found optimal and used in the experiments.

Microplate bioluminescence protocol

A setup according to ISO (ISO 11348-3, 2009) was employed using both *V. fischeri* and *P. luminescens*. Bacterial suspensions were transferred into a white 96-well microplate and initial luminescence was recorded using a Biotek Synergy microplate reader (500-ms integration). Immediately after the initial luminescence recording, the tested samples (dilutions prepared in a separate microwell plate) were added using the multichannel pipette. The plate was gently agitated and the bacteria exposed for 30 min at 15 °C and room temperature for *V. fischeri* and *P. luminescens*, respectively. Then, the final luminescence was recorded. Each plate contained negative controls (2 % NaCl solution adjusted to pH 6.5–7.5 for *V. fischeri* and 20 % PBS for *P. luminescens*) as well as a positive control (potassium dichromate at concentration 1 mg/mL). The results were evaluated according to ISO 11348-3 as follows:

$$Ht = \left(\frac{(ICt - It)}{Ict}\right) \times 100 \qquad \mathrm{IC_t} = I0 \times f_{\mathrm{kt}} \qquad f_{\mathrm{kt}} = \mathrm{I_{kt}}/\mathrm{I_0}$$

where *Ht* is the inhibition of luminescence, *It* is the luminescence intensity after the exposure time *t*, IC_t is the corrected value of bacterial luminescence of the tested sample, *I0* is the luminescence intensity before addition of the samples, f_{kt} is the correction factor to eliminate natural attenuation of luminescence during the exposure, I_{kt} is the luminescence of the negative control sample after the exposure and I_0 is the luminescence of the negative control in the beginning of exposure.

Microplate flash kinetic protocol

A miniaturized version of the previously published protocol (Lappalainen et al. 1999, 2001) included preparation of the studied samples (concentration dilutions; positive and negative controls as described above) directly in the white 96-microwell plate and brought to the appropriate temperature (15 °C or room temperature for *V. fischeri* and *P. luminescens*, respectively). The final volume of the sample in each microplate well was 80 μL. Bacterial suspension (20 μL) was then injected into the well using an automated injector, and the initial bioluminescence was immediately recorded (Biotek Synergy microplate reader with Gen 5 software employing the "Flash mode"). The microplate was then shaken inside the microplate reader at corresponding temperature for 30 s when the final luminescence was recorded. The inhibition of luminescence was calculated as previously described (Bláha et al. 2010):

$$inh\% = 100 - \frac{ITt}{CF \times IT0} \times 100 \qquad CF = \frac{ICt}{IC0}$$

where *CF* is the correction factor controlling for the natural attenuation of luminescence, *ICt* is the luminescence intensity of the control sample after the contact time, *IC0* is the initial luminescence intensity of the control sample, *ITt* is the luminescence intensity of the test sample after 30 s of

the contact time and *IT0* is the initial luminescence intensity of the tested sample.

Portable luminometer protocol

The 30-s Flash protocol has been adapted to commercially available cuvette luminometer (Biofix Lumi 10, Macherey-Nagel, Germany). A small hole was drilled into the original lid of the luminometer that allowed insertion of a needle with an inner diameter of 0.9 mm. This served as a holder for the second syringe needle used for direct manual injections of bacterial suspensions into the samples positioned in the measuring cell of the luminometer. The supplementary Fig. S1 shows a schematic presentation of the adapted portable luminometer. The test procedure used similar volume ratios as in the microplate arrangement. First, 0.5 mL of the studied sample (i.e. dilutions of the chemicals, wastewater samples, negative or positive controls) was prepared in the glass test tube, inserted into the luminometer, and the lid closed. Then, 0.2 mL of the bacterial suspension (either *V. fischeri* or *P. luminescens*) was injected directly into the sample using the syringe needle inserted through the lid hole. Immediately with the injection, the initial luminescence was recorded for 2-s light integration. The test tube was then removed from the luminometer and manually shaken at room temperature for 30 s. The tube was then placed back into the luminometer and the final luminescence was recorded. The luminescence inhibition was calculated as described in the previous paragraph (Microplate flash kinetic protocol).

Statistical analyses

Model chemicals (dilution series) were tested in three replicates. At least three independent experiments were performed for each compound and each of the three compared protocols (Microplate bioluminescence protocol, Microplate flash kinetic protocol, Portable luminometer protocol). Inhibitions and dose-response data (ECx calculations) were evaluated using Microsoft Excel and GraphPad Prism (GraphPad software, San Diego, USA). Statistical analyses were done in Statistica (StatSoft, Tulsa, OK, USA). The relationships between toxic responses and chemical contamination in the effluent samples were assessed by non-parametric Spearman correlation. Concentrations of chemicals below the limit of detection (LOD) were replaced by 1/2 of LOD value (Jarošová et al. 2014). Principal component analysis (PCA) was run with log-transformed contaminant data, which improved symmetry and normality. Toxicity data for both *V. fischeri* and *P. luminescence* were originally symmetric and were not log-transformed for PCA. Toxicity results (EC50 values) were inverted (1/EC50) for PCA to better represent actual effects (higher toxicity indicated by higher 1/EC50 value). For PCA, all data were standardized to mean = 0 and SD = 1 (i.e. mean subtracted from each individual value and then divided by the standard deviation, SD).

Results

To evaluate the suitability of *P. luminescens* for toxicity testing and to compare its sensitivity with *V. fischeri*, the experiments were performed with a range of model chemicals (Table 1) using all three compared protocols.

When comparing the results from laboratory microplate arrangements (upper four rows in Table 1), the Flash kinetic protocol (30 s) showed lower sensitivity at most of the toxicants in both *V. fischeri* and *P. luminescens*. The exception was 3,5-chlorophenol, which was comparably toxic in *V. fischeri* at both variants (30 min vs. 30-s Flash). Ethanol was the least toxic compound and its EC50 values were comparable in both species and evaluated protocols. In the microplate-based protocols *P. luminescens* seemed to be less sensitive (higher EC50 values). Using the portable luminometer protocol (results in the two bottom lines in Table 1), the trend in the toxicity of individual chemicals remained the same as for the laboratory microplate protocols but both *V. fischeri* and *P. luminescens* showed comparable responses and sensitivities. The quality and reproducibility of the P. luminescens assay was confirmed by repeated testing of the standard chemical potassium dichromate in the portable luminometer protocol during eight repeated experiments covering a period of 9 months. The resulted EC50 values ranged from 0.26 to 0.68 mg/mL, with the mean EC50 = 0.5 ± SD = 0.14.

After the experiments with model chemicals, the toxicity of 43 wastewater effluents were tested with both bacterial species using the portable luminometer protocol (Fig. 1). For *V. fischeri*, most of the samples caused weak inhibitions of luminescence (grey columns in Fig. 1). One undiluted sample (code no. 159) caused an inhibition of around 50 %, while strong 50 % stimulations were recorded for sample no. 233. The responses of *V. fischeri* to effluents were not significantly correlated with the responses of *P. luminescens* (Fig. 1), where most of the samples caused luminescence stimulations, and one sample (no. 184) caused inhibition greater than 20 %.

Statistical analyses were performed with the effluent samples to disclose relationships between the toxic responses of *V. fischeri* and *P. luminescens* and chemical contamination. Summary data on contamination, i.e. groups of analysed compounds, are presented in Table 2 (for full details, see Loos et al. 2012).

For *V. fischeri*, there were no statistically significant correlations between the toxic responses and the concentrations of pollutant groups (Spearman's R, $p > 0.05$). On the other hand, comparison of contamination and responses (% of bioluminescence change) of *P. luminescens* showed significant correlations. When considering all

Table 1 Comparison of *V. fischeri* and *P. luminescens* sensitivity (EC50 values ± SD) to model chemicals in various experimental setups. (Note: EC50 values are in mg/mL with the exception of HgCl2 where μg/mL concentrations are used)

	Potassium dichromate (mg/mL)	3,5-dichlorophenol (mg/mL)	3,4-dichloroaniline (mg/mL)	Sodium dodecyl sulphate (mg/mL)	Ethanol (mg/mL)	Phenol (mg/mL)	Mercury chloride (μg/mL)	2,4-dichlorophenol (mg/mL)
Microplate bioluminescence protocol								
Standard 30-min exposure								
V. fischeri	0.0056 ± 0.0019	0.0043 ± 0.0007	0.00092 ± 0.0003	0.0057 ± 0.0011	31.3 ± 4.4	0.053 ± 0.014	0.0737 ± 0.0025	0.003 ± 0.0024
P. luminescens	0.019 ± 0.012	0.0074 ± 0.0017	0.15 ± 0.028	0.099 ± 0.029	44.1 ± 18.2	0.62 ± 0.16	3.66 ± 0.38	0.037 ± 0.0047
Flash kinetic 30-s exposure								
V. fischeri	1.72 ± 0.69	0.008 ± 0.00031	0.017 ± 0.0049	0.029 ± 0.0049	36.3 ± 6.54	0.73 ± 0.129	0.61 ± 0.098	0.015 ± 0.00027
P. luminescens	1.69 ± 0.17	0.022 ± 0.005	0.14 ± 0.012	0.096 ± 0.03	32.3 ± 1.64	0.41 ± 0.697	7.92 ± 1.36	0.042 ± 0.0034
Portable luminometer protocol (flash kinetic 30-s exposure)								
V. fischeri	3.22 ± 2.19	0.017 ± 0.004	0.20 ± 0.04	0.045 ± 0.011	39.8 ± 9.2	>0.6	0.23 ± 0.024	0.061 ± 0.0087
P. luminescens	0.54 ± 0.057	0.039 ± 0.027	0.18 ± 0.021	0.032 ± 0.023	28.4 ± 2.71	0.37 ± 0.13	11.3 ± 0.60	0.047 ± 0.003

available data, i.e. both inhibitions and stimulations of luminescence, statistically significant (Spearman rank, $p < 0.05$) were correlations of the toxicity with the sum of pesticides (Spearman's Rs = 0.33), the sum of pharmaceuticals (Rs = 0.32) and the sum of musk fragrances (Rs = 0.44).

The multivariate presentation by PCA is shown in Fig. 2. *V. fischeri* toxicity seemed to be associated with inorganic contaminants (Inorg), nitrophenols and benzotriazoles for both "all data" (Fig. 2a) and inhibitions only (Fig. 2b). Alignments of a vector of toxic response of i (Fig. 2c, d) with vectors of chemical contaminants were more variable but—similar to *V. fischeri*—associations of toxicity with inorganics, nitrophenols and benzotriazoles was observable as the major general trend.

Discussion

The present work aimed to develop a simple portable battery-supplied luminometer tool for field in situ toxicity assessment with freshwater bacterium *Photorhabdus luminescens*. The initial investigation of sensitivity of *P. luminescens* with model compounds and employing standardized assays in microplates indicated rather lower sensitivity of this strain in comparison with the standard marine bacteria *V. fischeri*. Interestingly, in the portable luminometer, protocol sensitivities of both bacterial strains were similar indicating thus good applicability of *P. luminescens*. Significant correlations were observed between the responses of *V. fischeri* and *P. luminescens* (e.g. Spearman's Rs = 0.97, $p < 0.001$ for portable luminometer protocol). The interspecies differences in sensitivities had also been observed before for studied bacterial species (Jennings et al. 2001; Schmitz et al., 1999). Nevertheless, statistical analyses of larger datasets often showed systematic correlations between different model organisms (Kaiser 1998).

Our further comparisons focused on protocols with different exposure durations, namely comparing standard 30-min microplate luminometer protocol with *V. fischeri* (ISO standard) with fast Flash kinetic 30-s exposure, which allows for the assessment of turbid or coloured samples (Lappalainen et al. 1999, 2001). As it could be expected, the shorter exposure in the Flash kinetic protocol resulted in lower sensitivity (higher EC50 values) and this was apparent for both *V. fischeri* and *P. luminescens*. Interestingly, both dichlorophenols tested (3,5- and 2,4-dichlorophenol) appeared to be comparably toxic to both *V. fischeri* and *P. luminescens* irrespectively of the protocol or exposure duration. This may be related to their known rapid mechanism of toxicity affecting membranes via polar narcosis (Zhao et al. 1998). On the other hand, large differences between the 30-min and 30-s exposures were observed at metals, particularly at potassium dichromate. This

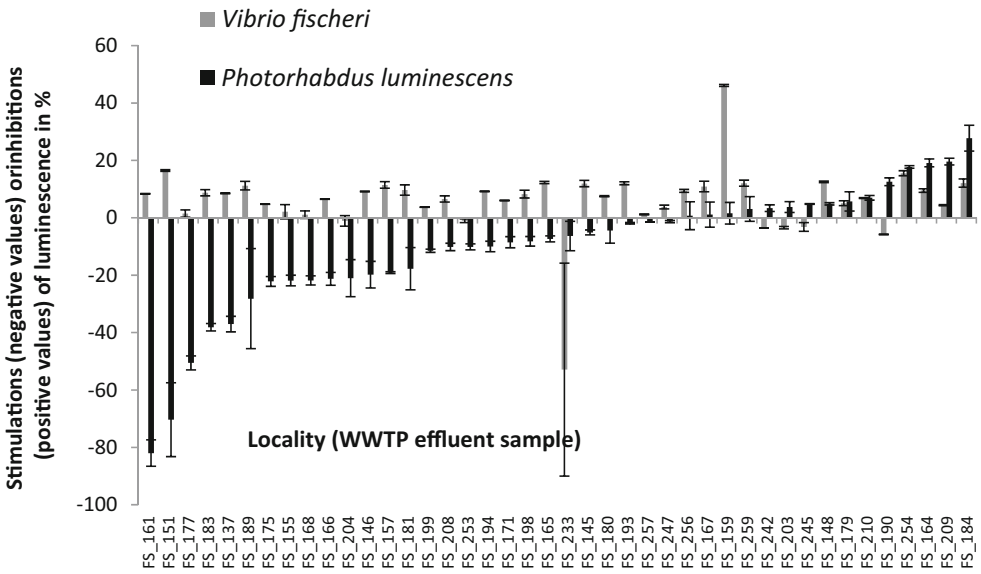

Fig. 1 Effects of wastewater effluents on *V. fischeri* and *P. luminescens* as determined with a novel portable luminometer flash kinetic protocol. Codes of samples on *X*-axis correspond to a previously published report (Loos et al. 2012). *Columns* represent mean ± SD of three replicated analyses

could be linked to known interactions of metals with proteins and other macromolecules, which may manifest only after longer exposures (van Assche and Clijsters 1990).

The comparisons showed some non-systematic trends where explanation is not straightforward. For example, 3,4-dichloroaniline was 10 times less toxic to *V. fischeri* in the portable luminometer (EC50 = 0.2 mg/mL) compared to microplate mode (EC50 = 0.017 mg/mL) while for all the other model compounds, the sensitivity of *V. fischeri* in both 30-s arrangements was similar. This difference was confirmed by repeated independent experiments, and we can only hypothesise about possible causes such as different volumes-to-surface ratios in different arrangements affecting the sorption/bioavailability or differing oxygen content that might aid to fast transformation of the studied compound.

As mentioned, one of the limitations of the assays with marine *V. fischeri* is the need to add high concentrations of sodium chloride into the test media. Previous studies showed that elevated osmomolarity interfered with luminescence emission in other bacteria such as *Photobacterium leiognathi* (Dunlap 1985) or lux-transfected *Pseudomonas fluorescens* (Cook et al. 2000), and it also has substantial effects on metal toxicity (Deheyn et al. 2004). To overcome this limitation, some authors evaluated freshwater bioluminescent bacteria such as *Vibrio qinghaiensis* (Ma et al. 1999) or lux genes-transfected non-luminous species such as *P. putida* or *E. coli* (Stewart & Williams 1992; Kurvet et al. 2011). Another viable approach, as shown also in the present study, is the use of naturally bioluminescent freshwater species *P. luminescens*.

In the standardized microplate protocols, lower sensitivity of *P. luminescens* was observed in comparison with *V. fischeri*. This could be caused by possible species-specific differences (Jennings et al. 2001; Schmitz et al. 1999) but also by other factors such as composition of the exposure media. With this respect, both compared organisms had specific limitations. *V. fischeri* requires additions of high salt concentrations that might interfere with toxicity of metals (Deheyn et al. 2004; Rüdel et al. 2015). On the other hand, stable luminescence signal at *P. luminescens* was achieved only in the presence of 25 % organic media TSB, which could decrease bioavailability and lower thus toxicity of both inorganic (Thavamani et al. 2015) and organic toxicants (Beesley et al. 2010). Interestingly, these differences the species diminished in the Flash cuvette protocol in portable luminometer, where the influence of media could be minimized during very short 30-s exposures.

Further assessment of the assay included testing of complex environmental samples, i.e. waste water effluents. Various whole effluent toxicity (WET) tests have been explored in the past including, e.g. *Daphnia magna*, fathead minnow, zebrafish embryos (Chapman 2000) as well as bacterial bioluminescence tests (Mendonça et al. 2009). The importance of WET testing has been highlighted especially for arid areas, where insufficient dilution of the effluent may directly affect biotic communities within the recipient stream (Diamond and Daley 2000). Recently, toxicity limits for WET have been discussed (Huybrechts et al. 2014).

With respect to bacterial assays, Kováts et al. (2012) employed *V. fischeri* Flash kinetic testing of effluents, and reported EC50 values around the 50 % dilution of the original effluent. In contrast, the present study found weaker luminescence inhibitions in both *V. fischeri* and *P. luminescens* with maxima around 20 % effect at undiluted effluents. These observations are in general agreement with a previously published European interlaboratory study (Farré et al. 2006), where generally minor responses were detected in a number of *V. fischeri*-based protocols (Microtox, Tox alert, Biofix lumi and Tox tracer tests). Despite of weaker effects of

Table 2 Chemical analyses of WWTP effluents—all concentrations are in nanograms per litre. Presented are the sum concentrations of groups of chemicals (see at the bottom of the table; values below the limit of quantification LOQ were replaced by 1/2 of LOQ, ng/L), empty cells - samples not analysed

Sample	Sweeteners	Perfluoalkyl substances	Pharmaceuticals*	Pesticides	PCPs	Contrast media	Gadolinium	Nitro phenols	Anticorrosives	Veterinary antibiotics	Siloxanes	Musk fragrances	OPEs	Inorganic ions
FS_137	21,700	43	6661	394	798	3080	41	58	2860	253	68	30	2535	69,270,000
FS_145	97,110	156	4097	442	400	720	2	74	4410	1059	68	16	1241	16,750,000
FS_146	75,200	89	2626	282	264	125	6	39	4310	1036	68	30	811	28,120,000
FS_148	41,190	166	4174	2833	282	125	56	5	5810	498	68	30	1667	21,580,000
FS_149	63,940	81	3242	1084	1072	15,840	115	162	10,440	1440	68	30	1901	15,350,000
FS_151	410	7181	129	300	89	125	84	5	7430		68	30	2626	56,380,000
FS_155	300	120	366	161	55	125	54	5	380		68	30	6421	37,050,000
FS_157	55	59	177	435	8	125	35	5	10		68	30	740	61,850,000
FS_159	55	74	241	682	135	125	34	606	24,970		68	30	471	11,000,000
FS_161	55	15	440	193	785	125	16	5	221,460		68	30	1073	57,309,000
FS_164	2360	571	1444	896	40	5200	219	5	90		68	73	6210	30,450,000
FS_165	15,870	8197	2836	472	1490	1700	154	5	3950		68	35	1235	19,930,000
FS_166	12,840	16,752	3454	487	325	860	419	5	3770		68	46	3222	8,350,000
FS_167	21,200	60	5652	1115	783	3790	389	5	10,070		68	25	3347	12,830,000
FS_168	12,400	4806	3717	165	425	640	155	576	5070		68	21	1516	6,520,000
FS_171	10,580	83	4269	18	16,178	750	24	14	5440		68	31	8339	10,870,000
FS_175	2,299,200	22	1622	161	3964	6640	65	69	6590		68	18	887	83,140,000
FS_177	2,482,440	24	1919	61	208	840	56	1529	4740		68	30	824	53,130,000
FS_179	39,500	79	9082	145	65	150	100	12	16,610		68	55	992	11,400,000
FS_180	32,390	140	3750	539	2939	230	95	5	8790		68	24	2885	11,590,000
FS_181	37,680	287	4683	168	1121	300	232	5	4420		68	30	5868	7,090,000
FS_183	11,810	37	3238	55	287	2010	789	5	6140		68	96	2118	144,080,000
FS_184	9460	33	1142	551	953	2690	204	5	2100		68	22	1375	87,360,000
FS_189	14,500	65	1143	736	380	125	3	5	250		68	30	1009	9,940,000
FS_190	14,130	111	1497	521	719	230	92	5	5440		68	84	1006	24,640,000
FS_193	12,140	95	1342	268	145	125	11	58	9370		68	20	1821	14,820,000
FS_194	16,180	64	2175	234	55	14,450	66	5	8990	1252	68	14	3490	15,220,000
FS_198	6890	173	2260	436	1203	360	21	5	12,690		68	30	46,532	11,750,000
FS_199	5620	306	1802	466	274	3450	118	5	12,280		68	63	9860	13,690,000
FS_203	7710	68	1474	4150	516	18,160	25	98	18,500	622	68	30	769	28,620,000
FS_204	23,260	767	2235	1063	172	8750	275	5	8050	634	68	30	1227	19,830,000
FS_208	21,410	95	5272	961	129	14,300	19	5	17,370	550	68	30	1820	24,540,000
FS_209	16,140	36	11,092	3044	505	380	34	5	4780	655	68	112	7870	29,040,000
FS_210	6120	30	3450	5242	222	2000	4	183	12,470	2510	68	30	3174	15,050,000
FS_233	41,880	136	3652	722	95	180	122	5	900		68	620	2335	10,950,000
FS_242	18,860	39	3495	1528	598	1000	223	5	8510		68	71	4105	8,920,000
FS_245	102,400	33	4714	495	552	125	227	82	10,560		68	173	3242	7,830,000
FS_247	55,130	175	4167	206	239	125	84	5	1320		68	95	4337	13,620,000
FS_253	17,770	8	2353	20	197	125	16	22			68	50	781	8,540,000
FS_254	44,430	29	7855	284	10,030			55	920	345	68	407	16,748	8,330,000

Table 2 (continued)

Sample	Sweeteners	Perfluoalkyl substances	Pharmaceuticals*	Pesticides	PCPs	Contrast media	Gadolinium	Nitro phenols	Anticorrosives	Veterinary antibiotics	Siloxanes	Musk fragrances	OPEs	Inorganic ions
FS_256	20,960	112	2818	80	1162	1600	59	5	1920		68	12	740	30,420,000
FS_257	18,590	24	6473	1981	976	125	146	5	8730		68	110	909	6,320,000
FS_259	12,290	11	7914	83	639	152,900	50	5	6750	165	68	183	1845	6,440,000

Sweeteners include: sucralose and acesulfam

Perfluoralkyl substances: PFHxA, PFHpA, PFOA, PFNA, PFDA, PFBS, PFHxS and PFOS

Pharmaceuticals (* without veterinary antibiotics): Alfuzosin, Alprazolam, Amitryptiline, Atorvastatin, Azelastine, Biperiden, Bisoprolol, Bromocriptin, Buprenorphin, Bupropion, Chloprothixen, Chlorpromazine, Cilazapril, Citaprolam, Clemastine, Clomipramine, Clonazepam, Clotrimazol, Codeine, Cyproheptadine, Dicycloverin, Diltiazem, Diphenhydramin, Duloxetin, Eprosartan, Etonogestrel, Fenofibrate, Fentanyl, Fexofenadine, Flecainide, Fluconazole, Fluoxetin, Flupetixol, Fluphenazine, Flutamid, Glibenclamide, Glimepirid, Haloperidol, Hydroxyzine, Ibersartan, Loperamide, Maprotilin, Meclozine, Memantin, Mianserin, Miconazole, Nefazodon, Orphenadrin, Oxazepam, Paroxetin, Perphenazine, Pizotifen, Promethazin, Ranitidine, Repaglinide, Risperidone, Rosuvastatin, Sertraline, Tamoxifen, Telmisartan, Terbutalin, Tramadol, Trihexyphenidyl, Venlavafaxin, Verapamil and Zolpidem

Pesticides: MCPA, Mecoprop, 2,4-D, Bentazone, Dichlorprop, Diuron, Terbutylazine, DET, Atrazine, Isoproturon, Diazinon, DEA, Simazine, Metolachlor, Carbaryl, Linuron, Hexazinon, Chlortoluron, Methabenzthiazuron and 2,4,5-T

Personal care products (PCPs): sum of Triclosan, DEET and caffeine

Nitrophenols: Nitrophenol and Dinitrophenol

Anticorrosives: Benzotriazole and Methylbenzo-triazole

Veterinary antibiotics: Oxytetracycline, Doxycycline, Penicilline V, Penicilline G, Amoxilline, Ampicilline, Tilmicosine, Clindamycine, Lincomycine, Tiamuline, Sulfamethoxazole, Sulfadiazine, Sulfadoxine, Trimethoprim, Flumequine, Enrofloxacine, Ciprofloxacine and Levamisol

Siloxanes: Decamethylcyclopentasiloxan (D5), Decamethyltetrasiloxan (MD2M), Dodecamethylcyclohexasiloxan (D6), Dodecamethylpentasiloxan (MD3M), Octamethylcyclotetrasiloxan (D4), and Octamethyltrisiloxan (MDM)

Musk fragrances: Cashmeran, Celestolid, Galaxolid, Phantolid, Tonalid and Traesolid

Organophosphate ester (OPEs) Flame Retardants: tri-iso-butylphosphate (TIBP), tributylphosphate (TBP), tris(2-chloroethyl)phosphate (TCEP), tris(2-chloro-isopropyl)phosphate (TCPP), tris(1,3-dichloro-2-propyl)phosphate (TDCP), tris(2-butoxyethyl)phosphate (TBEP), triphenylphosphate (TPP), 2-ethylhexyldiphenyl-phosphate (EHDPP), tris(2-ethylhexyl)phosphate (TEHP) and tricresylphosphate (TCP)

Inorganics: Hg, Ag, Al, As, Ba, Cd, Co, Cu, Mg, Mn, Mo, Ni, Pb, Sb, Se, Tl and Zn

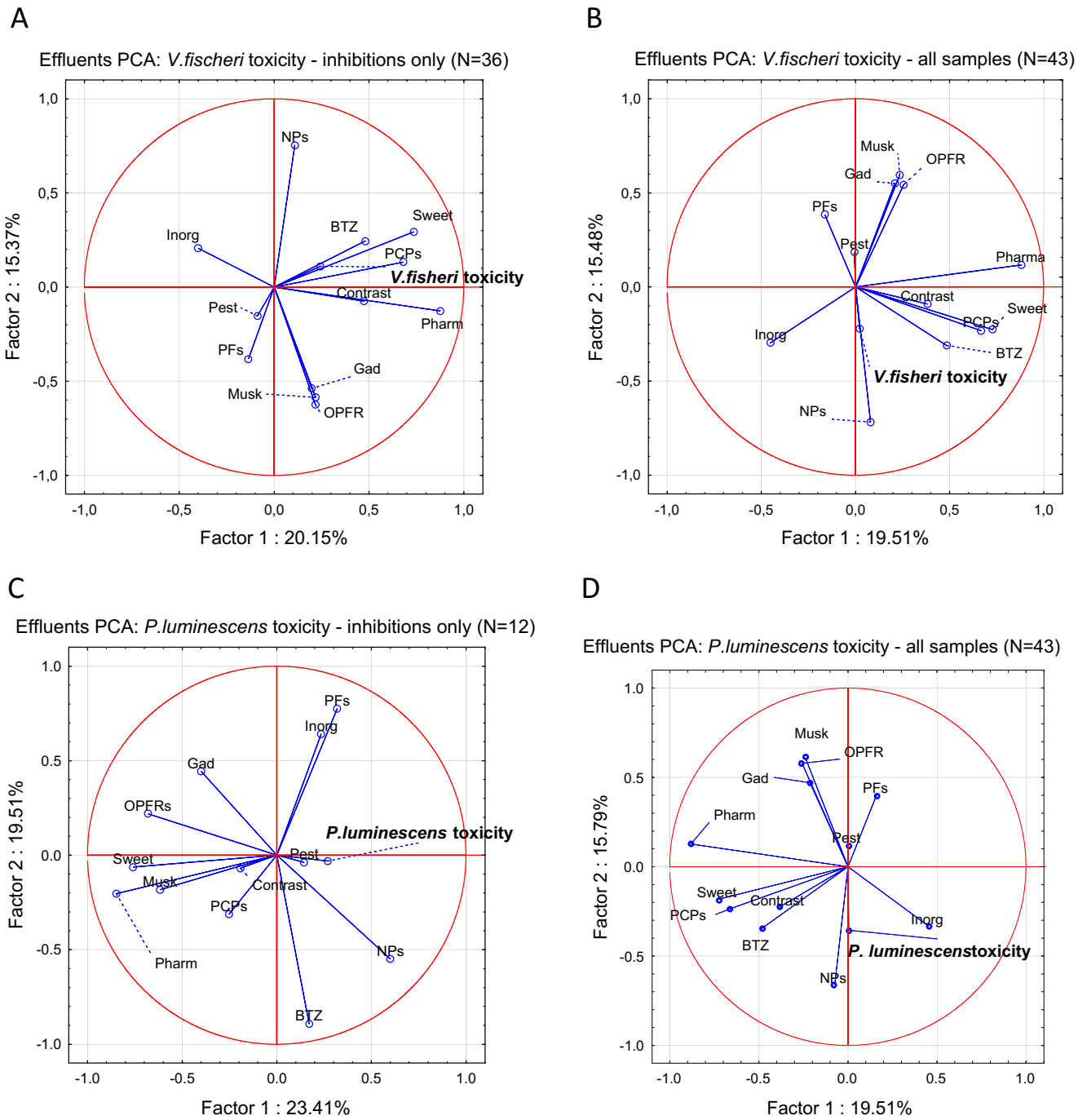

Fig. 2 Principal component analyses (PCA) relating the toxic effects of wastewater effluents to concentrations of 13 groups of chemical contaminants. **a**, **b** *V. fischeri* toxicity - inhibitions and all samples, respectively; **c**, **d** *P. luminescens* toxicity - inhibitions and all samples, respectively. *Sweet* sweeteners; *PFs* perfluoralkyl substances; *Pharm* pharmaceuticals (without vet. antibiotics); *Pest* pesticides; *PCPs* personal care products (sum of triclosan, DEET, caffeine); *Contrast* contrast media; *Gad* gadolinium; *NPs* nitrophenols; *BTZ* benzotriazoles; *Musk* musk fragrances; *OPFR* organophosphate flame retardants (for details on chemical groups see Table 2)

effluents in the present study, multivariate PCA analysis revealed clear associations between toxicity and elevated levels of inorganic compounds, chlorophenols and benzotriazole anticorrosives. Effect-based monitoring thus provides additional value to routine chemical analyses and may serve for sample prioritization or identification of major drivers of toxic effects (Wernersson et al. 2015; Escher et al. 2013). However, limitations also exist in the interpretation of bacterial bioluminescence assays in WET testing. For example, the inhibition of luminescence is the expected and evaluated endpoint but practical testing often shows both inhibitions and stimulations (Dizer et al. 2002; Pessala et al. 2004). This was also

confirmed in the present study. Although some of the factors affecting luminescence in complex matrices have been suggested such as pH, potassium and calcium ions or salinity (Berglind et al. 2010; Cook et al. 2000), further research and debate on the interpretation of stimulatory responses in bacterial bioluminescence is needed.

Conclusions

The present study demonstrated the development of a rapid bioluminescence-based testing tool employing a small battery-supplied luminometer. The sensitivity of freshwater *P. luminescens* was comparable with standard marine bacterium *V. fischeri* using the portable luminometer protocol. The applicability and reproducibility of the assay was further confirmed in the study of 43 European effluents, where elevated levels of inorganic compounds (metals), chlorophenols and benzotriazoles were the main drivers of toxicity in both compared bacterial species. The use of *P. luminescens* in combination with a portable luminometer brings several advantages such as applicability at room temperatures, fast 30-s response and a freshwater setup.

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