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Profiling the biological effects of wastewater samples via bioluminescent bacterial biosensors combined with estrogenic assays

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Abstract Various water samples were successfully evaluated using a panel of different recombinant bioluminescent bacteria and estrogenic activity analysis. The bioluminescent bacteria strains induced by oxidative (superoxide radical or hydroxyl radical), protein damage, cell membrane damage, or cellular toxicity were used. Estrogenic activities were examined by using the yeast strain BY4741, which carries the β-galactosidase reporter gene under the control of the estrogen-responsive element (ERE). A total of 14 samples from three wastewater treatment plants, one textile factory, and seawater locations in Tunisia were analyzed. A wide range of bio-responses were described. Site/sample heterogeneity was prevalent, in combination with generally high relative bioluminescence scores for oxidative stress (OH•). Estrogenic activity was detected at all sites and was particularly elevated at certain sites. Our perspectives include the future exploration of the variation detected in relation to treatment plant operations and environmental impacts. In conclusion, this new multi-experimental method can be used for rapid bio-response profile monitoring and the evaluation of environmental samples spanning a wide range of domains. This study confirms that bio-reactive wastewater treatment plant (WWTP) effluents are discharged into seawater, where they may impact coastal populations.

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Introduction

Assessing the biological effects of wastewater discharged into ecosystems is of key importance, and toxicity tests identifying ecological hazardousness should be mandatory for the identification of their environmental impacts. The various chemicals issuing from different industrial areas can be found in corresponding wastewater treatment plant (WWTP) samples. The resulting pollutant cocktail (including pesticides, PPCPs, pharmaceuticals, and so on) may contain elements capable of inducing biological effects such as mutagenicity, oxidative damage at the DNA and/or membrane levels, neurotoxicity, or modifications of hormonal function. Though crucial for driving both regulatory and voluntary decision-making processes, the identification of wastewater toxicity remains difficult and complicated. Nevertheless, in many countries, eco-toxicity tests are already in use for wastewater

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management (Vindimian et al. 1999; EPA 2004; Power and Boumphrey 2004; Tinsley et al. 2004).

Whole cell-based toxicity screening is one method used for water sample monitoring. Screening assays based on recombinant luminescent bacteria strains are particularly useful in regard to response speed and ease-of-manipulation (Gil et al. 2000; Gu and Choi 2001; Choi and Gu 2001; Lee and Gu 2003; Kim and Gu 2005). Each recombinant luminescent bacteria strain carries a vector containing a promoter stress gene that, when exposed to a target analyte, "points towards" the bacteria luciferase gene. Thus, bacteria exposed to a targeted stress signal will fluoresce in proportion to signal strength. For example, bacteria strain DPD2511, which includes a *katG::luxCDABE* (promoter gene/bioluminescent reporter gene) fusion, is used to examine the biological effects of exposure to hydrogen peroxide, because the *katG* promoter gene is induced by hydroxyl radical (HO•) compounds. With recombinant bioluminescent strains, we can detect DNA damage via DNA cascades or DNA alkylation, oxidative stress (by superoxide radicals (O_2•$^-$) and hydroxyl radicals (HO•)), membrane damage, protein damage, and/or cellular toxicity compounds (Ahn et al. 2010).

Endocrine-disrupting compounds (EDCs) are thought to be responsible for the majority of adverse reproductive effects observed in several fish species exposed to municipal wastewater effluents (Jobling et al. 1998; Hinck et al. 2007; Kessabi et al. 2010; Kessabi et al. 2013) as well as in natural waters. In this case, the issue of estrogenic EDCs (eEDCs) in wastewater has been recognized by scientists in the European community and worldwide. Recombinant yeast assays (RYA) (or yeast estrogen screens (YES)) based on the use of vertebrate estrogen receptors are a convenient means for evaluating the endocrine disruption potential of a substance (García-Reyero et al. 2001). Recombinant strains of *Saccharomyces cerevisiae* have been developed that incorporate the human estrogen receptor (hER) gene into the main chromosome of the yeast. These strains also have expression plasmids carrying the reporter gene *lac-Z* encoding for the enzyme β-galactosidase (Routledge and Sumpter 1996). Upon activation of the hER receptor, β-galactosidase is secreted into the medium and may be assayed using a chromogenic substrate. The advantages of using yeast for eEDC detection include ease-of-manipulation, rapid attainment of stable transformants, ability to process large samples, and a limited metabolic capacity compared to more elaborate bioassay systems (Gaido et al. 1997). The use of this tool for determining WWTP effluent impacts on hormonal dysfunction have resulted in a multitude of publications over the last 5–10 years.

Different ecotoxicology studies report screening results for the discharge of toxic and estrogenic substances from urban and industrial wastewater treatment plants into the aquatic environment (Kessabi et al. 2010; Kessabi et al. 2013). Moreover, in many studies, authors used a combined assessment of toxicity and estrogenicity for specific compounds (Chen et al. 2002) and effluents, in order to evaluate their impact on overall fish health (Hemming et al. 2001).

In this study, we present a new combination of bioluminescent bacteria-based toxicity screening and a yeast-based estrogenic activity assay for the monitoring of water samples. In other words, we can detect specific, functional stress signals for bacteria by using recombinant bioluminescent bacteria responses and endocrine disruption via β-galactosidase activity in recombinant yeast with yeast estrogen screening assays (YES). By integrating these two different methods, we were able to detect the increasing or decreasing trends of complementary biological responses to influent and effluent samples from WWTPs. The advantages of this integrative method include its promptness and easy conduct; it can be performed in 96-well plates, within 6 h. As a useful, preliminary example, we applied this bio-sensing method to a limited number of influents and effluents from three WWTPs in a specific area of Tunisia, one nearby textile factory, as well as seawater samples.

Material and methods

Wastewater treatment plant and seawater sample sites

Water samples originate from three WWTPs located in urban, industrial, or agricultural areas in Tunisia and one textile factory rejection site (see map in Fig. 1). The characteristics of the three studied WWTPs that receive domestic and industrial wastewaters are presented in Table 1. WWTPs were sampled at both the input and output levels (except for the site at Sousse, where we were also able to obtain samples from an intermediate purification step) and differ from each other namely in the magnitude of flow (daily flow ranges from 1500 to 15,000 m^3/day), the treatment level implemented (from preliminary treatment to tertiary treatment), and the site of discharge (river, estuary, or coastal area). The Frina WWTP and the textile factory were sampled at two different dates, once at the beginning of March 2010 (Frina 3 and Textile 3) and once at the beginning of April 2010 (Frina 4 and Textile 4). Seawater samples at three localities were also collected (see map in Fig. 1).

Sample collection and extraction

Raw water samples were centrifuged (2000 g, 15 min) to separate suspended solids. Supernatants were then extracted by solid-phase extraction as described by (Pillon et al. 2005). Briefly, aqueous samples were concentrated on reverse-phase C18 (5 g, 20 mL) cartridges (Sigma-Aldrich, Saint-Quentin-Fallavier, France) preconditioned with methanol. Compound elutions from the column were triggered using methanol

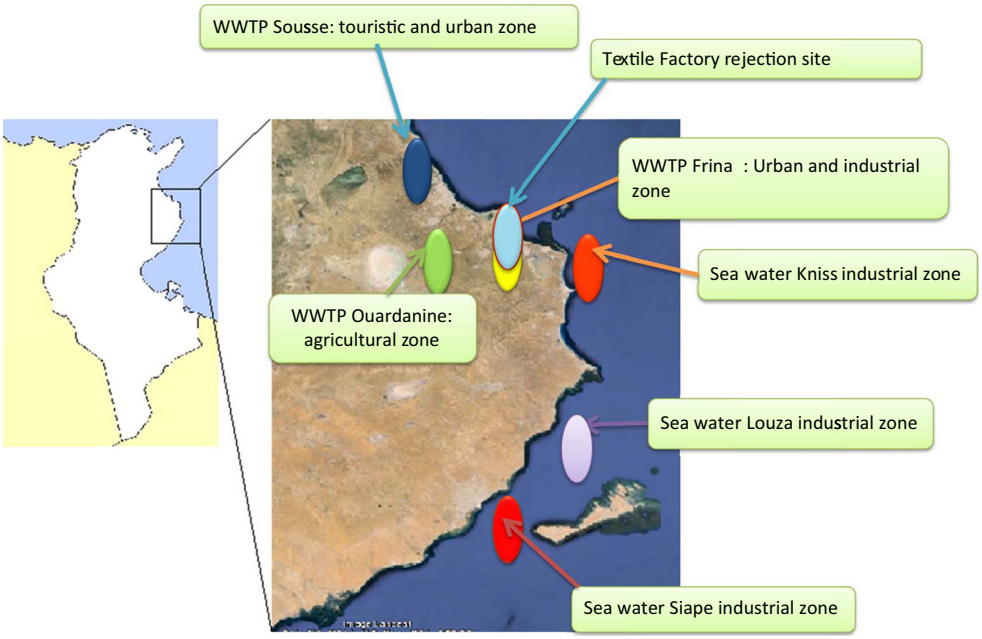

Fig. 1 Map of the water sampling sites in Tunisia. *WWTP* wastewater treatment plant

followed by hexane. Eluates were dried at 37 °C in a rotary evaporator, and residues were taken up in 2 mL methanol (concentration factor, 500).

Suspended solids

Suspended solids were homogenized and then lyophilized. They were extracted via 15 min of agitation with a mixture of dichloromethane/methanol solvent (2/1; 80 ml). After centrifuging (2 min at 3.500 rpm), the supernatant was filtered on GF/C and then dried with anhydrous sodium sulfate. Extracts were then evaporated at 40 °C using a rotary evaporator. Two milliliters of methanol was added, and extracts were then stored at −20 °C until analysis.

Bacterial toxicity assays

Five bacteria strains [EBHJ2 (pSodALux); DPD2511 (pKatGLux); TV1061 (pGrpELux); DPD2540 (pFabALux); GC2 (pLITE2)] representing five complementary functions (oxidative stress via $O_2^{\bullet-}$, oxidative stress via HO•, protein damage, cell membrane damage, and cellular toxicity, respectively) were used. All strains were streaked on agar plates containing ampicillin (50 μg/ml). After obtaining isolated colonies from agar plates, a single colony was inoculated in 5 ml Luria-Bertani medium (LB) containing ampicillin (50 μg/ml) in a falcon tube. The bacteria were incubated in a rotary incubator at 200 rpm and 37 °C until the optical density reached an early stationary phase (OD_{600} = 0.8~1.0). A subculture was then conducted in an autoclaved flask for collecting concentrated cells in a rotary incubator at 200 rpm and 37 °C, until an exponential phase (OD_{600} = 0.65~0.8) was reached. The samples were then diluted to different concentrations (4× to 0.0625×, 50 μl) and subculture bacteria solutions added (50 μl). Relative (as compared to a control blank) bioluminescence responses were measured by a plate reader (victor3, 1420 multilabel counter, PerkinElmer Co.) in 96-well plates after 4 h. Table 2 provides a list of the recombinant

Table 1 General information for studied wastewater treatment plants (WWTP)

WWTP (north to south)		Sousse	Ouardanine	Frina
Population		148,000	165,000	17,000
Surroundings		Touristic and urban	Agricultural	Urban and industrial
Discharge		Sea	River and estuary	Sea
Flow (m^3/day)		15,500	1500	13,500
BOD kg/day	Inlet	3350	600	3125
	Outlet	16.25	120	10.5
COD kg/day	Inlet	9040	780	8440
	Outlet	53.95	90	29.75

BOD biological oxygen demand, *COD* chemical oxygen demand

Table 2 Recombinant luminescent bacteria strains used in this study

	Strain	Plasmid	Abbreviation in text	*E. coli* host	Induced by	Reference
1	EBHJ2	*pSodALux*	SodA	RFM443	Oxidative stress via superoxide radicals ($O_2^{\bullet-}$)	(Lee and Gu 2003)
2	DPD2511	*pKatGLux*	KatG	RFM443	Oxidative stress via hydroxyl radical (HO•)	(Belkin et al. 1996)
3	TV1061	*pGrpELux*	GrpE	RFM443	Protein damage	(Van Dyk et al. 1995)
4	DPD2540	*pFabALux*	FabA	RFM443	Cell membrane damage	(Choi and Gu 2001)
5	GC2	*pLITE2*	GC2	RFM443	Cellular toxicity	(Marincs and White 1994)

bioluminescent bacteria used in this study, along with their characteristics.

Estrogenic activity

Yeast strain BY4741 (MATa ura3D0 leu2D0 his3D1 met15D0) was obtained from Euroscarf, Frankfurt, Germany. The expression plasmid pH5HE0 contains the human estrogen hormone receptor cloned into the constitutive yeast expression vector pAAH5 (García-Reyero et al. 2001). Plasmid pVITB2x encompasses the reporter gene β-galactosidase from *Escherichia coli* fused to the yeast CYC1 promoter and is under the control of the pseudopalindromic estrogen-responsive element ERE2 from the *Xenopus laevis* vitellogenin B1 gene (50-AGTCACTGTGACC-30, two copies) (García-Reyero et al. 2001). The test measures β-galactosidase activity with 4-methylumbelliferone β-D-galactopyranoside (fluorescence at 460 nm and exCitation at 355 nm) with a fluorimeter (Fluoroskan Twinkle LB 970, BERTHOLD Technologies) after 6 h of exposure to analyte solutions. Tests were performed in 96-well plates. To determine the estrogen activity of the studied samples, E2 was used as a positive control and distilled water was used as a negative control. Stock solutions of 17 β-estradiol (10^{-3} M) were prepared in methanol and serially diluted in distilled water ($1x10^{-12}$, $1x10^{-11}$, $1x10^{-10}$, $1x10^{-9}$, $1x10^{-8}$, $1x10^{-7}$, $1x10^{-6}$, $1x10^{-5}$, $1x10^{-4}$M). For effluent samples, a series of dilutions (5×, 2×, 0.5×, 0.05×) were tested for their estrogenic activity.

The transformed yeast strains were grown overnight at 30 °C in a non-selective medium (YPD: 5 g/l yeast extract, 10 g/l peptone, 20 g/l glucose, all from Sigma-Aldrich, France). The following day, 15 µl of saturated culture was added to 15 ml of selective media (SD, 6.7 g/l yeast nitrogen base without amino acids, DIFCO, Basel, Switzerland; 20 g/l glucose, supplemented with 0.1 g/l of prototrophic markers as required). The final culture was diluted in selective media and adjusted to an optical density (DO) between 0.1 and 0.2 at 600 nm. Forty-five-microliter aliquots were transferred into a 96-well polypropylene microtiter plate. Five-microliter aliquots of each sample were then dispensed into wells in quadruplicate. This experimentation was carried out three times in order to obtain representative statistical data. After 6 h of incubation, 50 µl of YPER (Pierce, Rockford, IL, USA) was added to each well and incubated at 30 °C for 30 min. Fifty microliters of buffer Z (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0 plus 0.5 % 2-mercaptoethanol (Fluka), 1 % Triton X-100 (Sigma), 4-methylubelliferyl β-D-galactoside (Sigma)) was added to the cell lysate, then incubated at room temperature for 30 min after centrifugation at 1000 rpm for 1 min. Fluorescence was measured at 460 nm (exCitation at 355 nm and emission at 460 nm).

Data and error analysis

All experiments were performed in triplicate.

Relative bioluminescence scores (RBs) were calculated as A/B, where A is the raw bioluminescence measure for the test water sample and B is the raw bioluminescence measure for the reference sample. "Bounded ratios" (BRs) were also calculated as the form $A/(A+B)$ (see "Results and discussion" section) for both bacterial bioluminescence results and for estrogenic activity results. Maximum BRs were reported per site and sample type.

For estrogen activity, the half maximal effective concentration (EC_{50}) was calculated based on the sigmoidal dose–effect curve of E2 (see Fig. 2). EC_{50} is herein defined as the concentration where the transcriptional response reaches 50 % of its value at saturating ligand concentrations. The EC_{50} for the samples is expressed as estradiol equivalents.

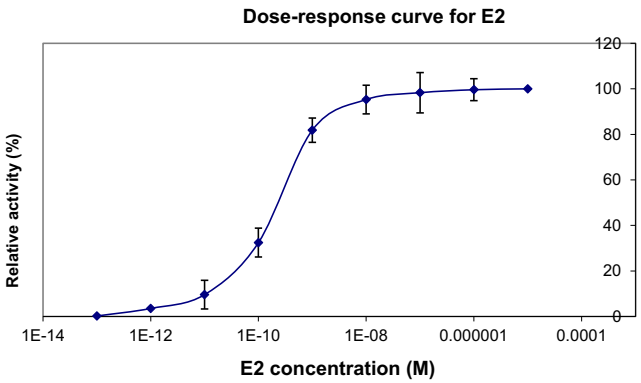

Fig. 2 The dose–response curve for E2: E2 concentration (M) versus relative activity (%)

Measured estradiol equivalents (Eqv) were determined as follows: Estrogenic activities of samples expressed as absorbance at 460 nm were expressed in estradiol concentration using the maximum response of the positive control E2 from the same microtiter plate and were plotted to logarithmically transformed estradiol equivalents.

Mathematical modelling For the description of the in vitro dose response relationships during sample analysis, a regression model (curve fitting using MicroSoft Excel) was used (Eq. 1):

$$F(x) = Cmin + \left((Cmax - Cmin)/\left(1 + e^{-b-a*logx}\right)\right) \quad (1)$$

where x = concentration and $F(x)$ = mean effect. The parameter C_{min} describes the minimal mean effect of the control response (17β-estradiol), and C_{max} describes the asymptotical maximal effect.

Coefficients a and b were determined on the basis of Eq. 2:

$$S(x) = (F(x) - Cmin)/(Cmax - Cmin) = 1/\left(1 + e^{-b-a*logx}\right) \quad (2)$$

The response of the test system towards the different samples investigated was determined by comparing experimental fluorescence obtained in yeast culture and expressed in estradiol equivalents (Eqv) with the theoretical fluorescence obtained with the predicted model.

Raw mean sample RBs and estrogen activity measures (EAs) for dilutions at 4–5×, 2×, 0.5×, and 0.05 to 0.07× were centered and reduced and then submitted to principal component analysis (PCA) in order to explore differences between sample profiles in multivariate space. For the latter, GC2 values were multiplied by −1 so that they could be interpreted "in the same direction" as other variables. PCA was performed using RStudio Version 0.98.978 – © 2009-2013 RStudio, Inc.

Results and discussion

Combined toxicity and YES screening for water samples

With the perspective of developing a rapid method for bioresponse profiling of liquid samples, we combined two different toxicity testing methods, one using recombinant bioluminescent bacteria and the other one using yeast estrogen screening. By combining these two methods, we seek to analyze water samples from various, but complementary, viewpoints: oxidative stress ($O_2^{\bullet-}$ and HO•), membrane damage, protein damage, cellular toxicity, and estrogenic activity of any given water sample. All five recombinant bacteria strains listed in Table 2, as well as the YES test, gave positive results during the course of the study, indicating that the targeted bioresponses were all present in the studied samples, despite the restricted sampling available for this preliminary study.

Water sample monitoring

Relative bioluminescence scores varied by several orders of magnitude. Minimums were close to 0, but maximums ranged from 1.06 (GC2) to 163.93 (KatG) (see Table 3), making comparisons between the different markers difficult using the same scale. To overcome this constraint, we also calculated relative bioluminescence scores for KatG, SodA, FabA, and GrpE using a ratio of the form $A/(A+B)$, where A is the raw *bioluminescence measure for the test sample and B is the raw bioluminescence measure for the reference* sample. For GC2, where bioluminescence decreases with increasing toxicity, the inversed form $B/(B+A)$ was used so that all new ratios could be interpreted "in the same direction." In a similar fashion, for estrogenic activity, we also calculated the ratio EC_{50} sample water / (EC_{50} sample water + EC_{50} E2) (Table 4).

Here forward, we will call these new ratios "bounded ratios" (BRs), because they are bounded by 0 and 1. When a test sample has the same level of bioluminescence as the reference sample, $A = B$ and the BR = 0.5. When $A > B$, the ratio A/B tended towards infinity, but the BR tends towards 1. When $A < B$, the ratio A/B tended towards 0, and the BR also tends towards 0.

A radar plot ranging up to 1.0 (maximum toxicity) was used to present maximum mean BRs per site–sample combination (see Fig. 3). Oxidative stress (HO•) was ubiquitous and over 0.9 for all non-seawater samples. The most variable toxicity biomarker was protein damage, which ranged from close to 0.5 for certain WWTP and seawater samples to over 0.9 for certain Frina samples. A relatively high cellular toxicity BR was found at Ouardanine. Membrane damage BRs varied from low (near 0.5) to close to 0.8, with the highest scores found in certain seawater samples, notably at Siape. Oxidative stress ($O_2^{\bullet-}$) signals were relatively weak (alternating around 0.5), with one noticeable spike for the textile factory rejection samples. Most of the WWTP effluent samples analyzed at the original sample solution (onefold dilution) had estrogenic effects. The outlet samples from Sousse and Frina induced a maximum of 40 % of estrogenic activity, while the estrogenic activity of Ouardanine samples was increased from 30 to 60 %

Table 3 Minimum and maximum relative bioluminescence scores observed during the study for toxicity markers

	KatG	SodA	FabA	GrpE	GC2
Minimum	0.49	0.17	0.12	0.07	0.35
Maximum	163.93	3.33	4.74	10.12	1.06

Table 4 Estrogenic activity expressed in EEQ (m) (estradiol equivalent)

Site	Sample type	EC_{50}	
		Water	Suspended solids
Sousse WWTP	Inlet	2.50E−11	1.50E−11
	Purification	3.00E−11	4.00E−13
	Outlet	1.80E−09	5.00E−12
Ouardanine WWTP	Inlet	3.00E−10	5.00E−10
	Outlet	1.00E−08	1.00E−11
Frina 3 WWTP	Inlet	2E−10	1.50E−11
	Outlet	1.80E−09	1.00E−11
Frina 4 WWTP	Inlet	5.00E−11	1.50E−11
	Outlet	9.00E−11	2.00E−12
Textile 3	Rejection	2.00E−10	4.00E−11
Textile 4	Rejection	4.50E−11	1.00E−11
Kniss	Seawater	2.80E−10	1.50E−11
Louza	Seawater	1.80E−10	1.50E−11
Siape	Seawater	3.00E−10	6.00E−12

EC_{50} values, defined as the concentration where transcriptional response reaches 50 % of its value at saturating concentration of ligand, were calculated from dose–response assays, using nine concentrations for 17β-estradiol

of the E2 estrogenic activity after treatment. This is reflected in the BR spikes shown in Fig. 3. In general, bio-reaction profiles are difficult to generalize and are rather site and sample dependent. Comparisons between Textile 3 versus Textile 4 as well as Frina 3 versus Frina 4 also suggest that they can vary with time, though this requires confirmation by a properly designed study.

Curiously, the bio-responses of effluent samples were often higher than the responses of influent samples, especially as concerns oxidative stress (HO•) and estrogenic activity. Though this first descriptive preliminary study is not designed for the statistical comparison of samples, this observation is unexpected and suggests that HO• oxidative stress and estrogenic activity are increased even after wastewater is treated. The highest oxidative stress (HO•) or estrogenic activity response was observed from the agricultural wastewater samples (Ouardanine). The effects of wastewater treatment on bio-response profiles merits thorough investigation over a wide range of WWTPs. Explanations include a random finding (due to limited sampling), or wastewater treatment processes that increase ligands during the organic molecule degradation process, or other unknown factors. The stations are properly functional, as

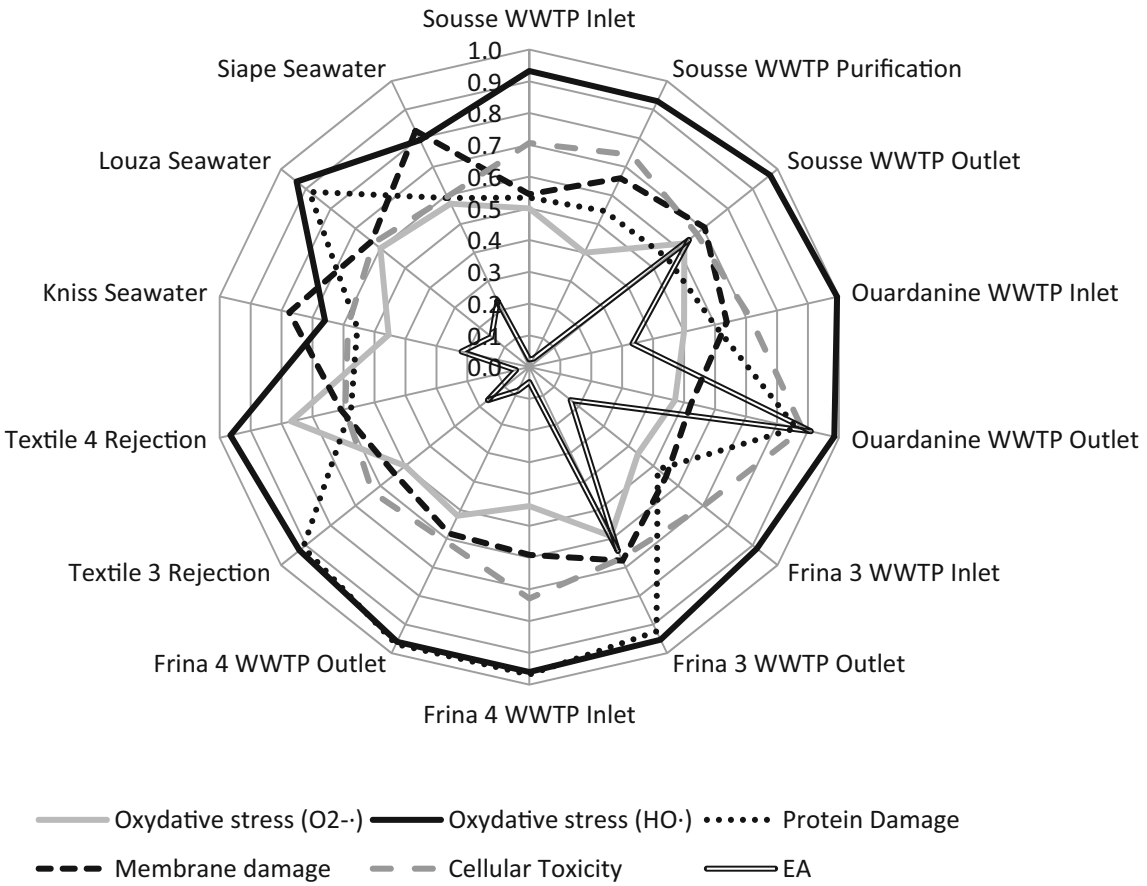

Fig. 3 Radar plot of maximum BRBs (bounded relative bioluminescence scores; see text) per site–sample combinations. *WWTP* wastewater treatment plant

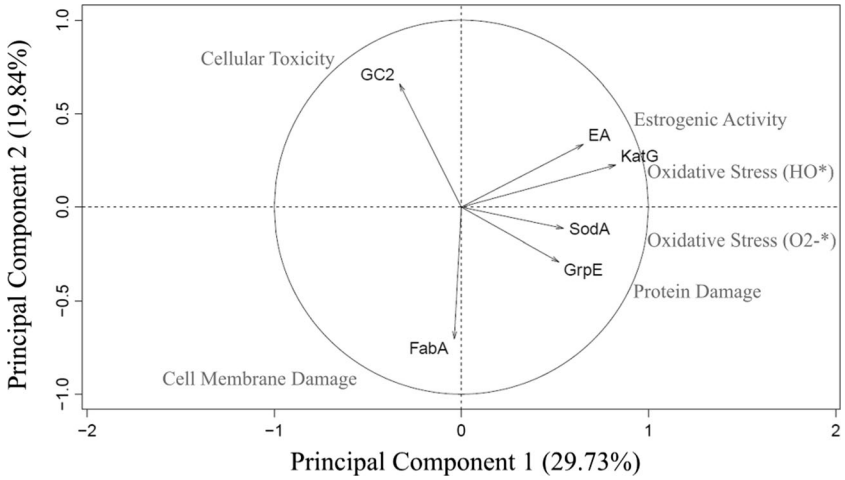

Fig. 4 Variable factor map for a PCA incorporating relative bioluminescence scores for toxicity markers (five bacteria strains [EBHJ2 (pSodALux); DPD2511 (pKatGLux); TV1061 (pGrpELux); DPD2540 (pFabALux); GC2 (pLITE2)] representing five complementary functions (oxidative stress via $O_2^{\bullet -}$, oxidative stress via HO•, protein damage, cell membrane damage, and cellular toxicity, respectively) and estrogenic activity. *Arrows* that are close to each other tend to represent variables that are positively correlated. *Arrows* that point towards opposite directions tend to represent variables that are negatively correlated. *Arrows* that are at right angles tend to represent variables that are orthogonal

demonstrated by the decreases in BOD and COD mentioned in Table 1.

Relationships between toxicity and estrogenic activities for different water samples

Raw replicate means per sample and dilution were submitted to principal component analysis (PCA). For the latter, GC2 values were multiplied by −1 so that they could be interpreted "in the same direction" as other variables. The first two components determined by the PCA represented 29.73 and 19.84 % of the variation present in the data, for a cumulative representation nearly reaching a majority at 49.57 %. Principal component 1 was positively associated with KatG, EA, SodA, and Grp3 and, to a lesser extent, negatively associated with GC2. Principal component 2 was dominated by a negative correlation with FabA and a positive correlation with GC2 (see Fig. 4). The variables cover a large range of the unit circle and are not packed together in one multiple-correlated group, demonstrating their complementarity. Nevertheless, the couple "EA and KatG" as well as the couple "SodA and Grp3" appear correlated in the dimensions 1 versus 2 biplot (see Fig. 5). Further investigations for significant correlations (Pearson's correlation test) between toxicity variables were insignificant. The PCA placed many of the samples around the origin (see Fig. 5); the latter space represents highly

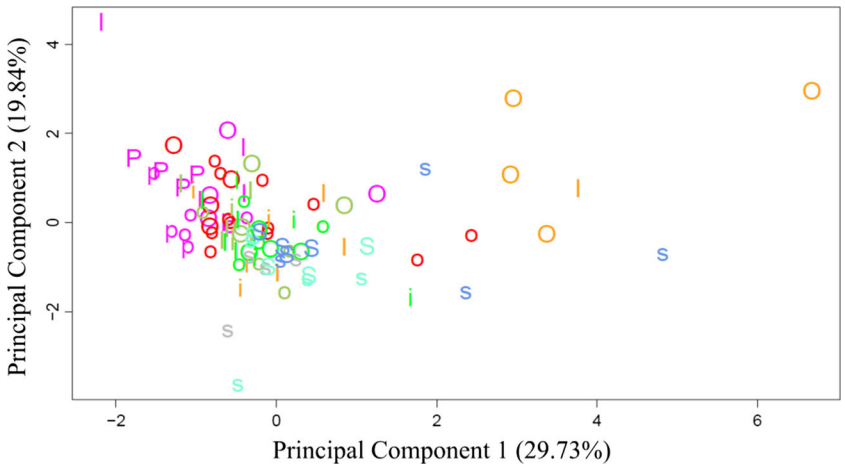

Fig. 5 Individual sample scores for a PCA incorporating relative bioluminescence scores for toxicity markers (five bacteria strains [EBHJ2 (pSodALux); DPD2511 (pKatGLux); TV1061 (pGrpELux); DPD2540 (pFabALux); GC2 (pLITE2)] representing five complementary functions (oxidative stress via $O_2^{\bullet -}$, oxidative stress via HO•, protein damage, cell membrane damage, and cellular toxicity, respectively)) and estrogenic activity. Site color coding: *magenta* Sousse, *red* textile factory, *light green* Frina 4, *olive green* Frina 3, *orange* Ouardanine, *gray* Kniss, *aquamarine* Siape, *blue* Louza. Sample type letter coding: *uppercase letters* supernatants, *lowercase letters* suspended solids, *I* inlets, *P* purification, *O* outlets, *S* seawater

diluted samples which are naturally similar to each other due to their dilute nature. There is also a great deal of overlap between sites. However, samples from the Sousse WWTP and the textile factory can be almost completely separated from the Ouardanine WWTP or from most seawater samples based on the PCA sample biplot (Fig. 5), indicating that there is some clustering by site. Otherwise, the variation present is site and sample dependent, with samples from the Ouardanine WWTP and the Sousse WWTP (as well as certain seawater samples) being quite different from the majority of samples. No similarity with a geographical gradient is found.

Perspectives

The present study demonstrates a wide variety of bio-responses among only a limited set of water samples. Now that we have a first idea of the range of responses that can be expected, we hope to explore this variation for a greater number of WWTPs, especially as concerns before versus after wastewater treatment procedures, and associated variables.

The site and sample specificity of bio-response profiles demonstrated in the present study underline the complex mosaic nature of pollution and toxicity environments that can be found in the field. Our perspectives include explorations of this variation, with the goal of studying correlations with environmental impacts. In particular, we would like to call attention to the studies by Kessabi involving the Mediterranean killifish, *Aphanius fasciatus* (Kessabi et al. 2010; Kessabi et al. 2013). Spinal deformation rates for this species are about eight times higher near the Siape site (Sfax area) as compared to the Louza and Kniss seawater sites (see (Kessabi et al. 2013)). Deformed fish had CYP1A mRNA levels about 10 times higher than that of normal fish (Kessabi et al. 2010). The activity of the latter gene is classically triggered by dioxin or dioxin-like pollutants and contributes to the formation of reactive oxygen species that can subsequently lead to cellular and oxidative stress (Barouki and Morel 2001). Casalegno et al. (2015) suggest that due to their hydrophobic character, dioxins can quickly penetrate into a lipid membrane, both as single molecules and as aggregates. They "find clear evidence for their ability to accumulate in cell membranes." In the present study, the Siape site is distinguished from other sites by a particularly high membrane damage score with a BRB of 0.83. We wonder if this signal, or other toxicity signals, may help explain the high spinal deformation rates in the area. The present multivariate toxicity profile will also allow us to explore "bio-response cocktail" effects, which might be important in the *A. fasciatus* case. The latter fish populations are also deficient in males (Kessabi et al. 2010), suggesting endocrine perturbation. In the current studies, all seawater samples had estrogenic activity over 20 %, which may help explain very high levels of vitellogenin transcripts (a biomarker for estrogenic activity) present in all fish groups studied by (Kessabi et al. 2010).

Conclusion

In this study, we implemented a new combined bio-sensing platform for toxicity monitoring of wastewater samples. The procedures used result in the rapid and relatively easy acquirement of multivariate toxicity and estrogenic perturbation information. Though this preliminary study analyzed a limited number of samples, a wide range of bio-responses were described. Heterogeneity among sites and samples was evident, in combination with ubiquitously high relative bioluminescence scores for oxidative stress (OH•). This new combined method can be used for toxicity profile monitoring and the evaluation of environmental samples spanning a wide range of domains. This study confirms that bio-reactive WWTP effluents are discharged into seawater, where they may impact coastal populations.

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