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Ecotoxicity tests in the environmental analysis of wastewater treatment plants: Case study in Portugal

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ABSTRACT

A global evaluation of wastewaters should include ecotoxicological tests to complement the chemical characterization, with advantages especially in the case of complex wastewaters. A European project developed in Trancão River Basin (Portugal), integrated the ecotoxicological and physicochemical studies of wastewater samples from two municipal sewer networks and respective wastewater treatment plants. Wastewater samples were analysed for physicochemical parameters, ecotoxicological acute and chronic tests performed and the potential for endocrine disruption evaluated. Organic load parameters and total suspended solids showed significant correlations with Microtox and ThamnoToxKit test results. Data analysis showed that treated treatment plant effluent samples are associated with less organic contamination and less toxicity in ThamnoToxKit test. Chronic toxicity test and endocrine disruption assay of treatment plant effluent samples indicated that, in a long term, potential population effects could arise in the receiving waters. A test battery to monitor this type of wastewaters is proposed, including tests with a bacterium, an alga and a crustacean. In a screening phase the most sensitive test, Microtox, can be used. The use of an ecotoxicological approach can have added value to hazard and risk assessment of discharges to the receiving waters and can contribute to the environmental management of the treatment plant.
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1. Introduction

The integrated assessment of biological effects of wastewater discharges in the ecosystems is relevant and ecotoxicity tests are referred as extremely useful tools for the identification of environmental impacts. International, national and regional authorities can use these tools to help meet the various regulatory and legislative goals [1]. Regulatory and discharge drivers may contribute to the growing use of direct toxicity assessment, as a result of pressure coming either from current and developing legislation (e.g. IPPC [2], WFD [3], REACH [4]) or from discharger concerns in supporting decision-making. In particular, the development of thinking associated to the Water Framework Directive is raising issues about defining quality in the ecological domain. For countries to comply with good ecological status under WFD, the ecotoxicity of the WWTP effluent may play a significant role herein. Hence, the development of relevant, but reliable robust test systems will be of importance in developing appropriate and cost-effective management programs to (a) protect the environment and (b) comply with EU Directives, e.g. WFD [5]. Therefore a comprehensive assessment of wastewaters should complement the chemical characterization with ecotoxicity tests [6]. Considering ecotoxicity testing as an integral part of the toolbox to investigate the environmental impacts of effluents but knowing that it can be complex, time consuming and expensive, a tiered approach is recommended when defining a realistic assessment strategy [7]. In many countries ecotoxicity tests are already used in wastewater management [8–10]. This approach has advantages particularly in the impact assessment of complex wastewaters, for example to protect biological treatment plants from toxic influents and to monitor the effectiveness of wastewater treatment plants (WWTP) [11–13]. Measured concentrations of organic wastewater contaminants in freshwaters, namely steroids and hormones, are low [14] but little is known about the potential interactive effects in complex mixtures that may occur in the environment and about their effect on human health [15]. It is furthermore clear that industrial and municipal effluents are major sources of endocrine disrupting chemicals into the aquatic environment, thus raising concerns about the chronic toxicity.

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Desbrow et al. [16] identified natural hormones (17β-estradiol and estrone in concentrations from 1 to 80 ng/l) and, at lower levels, a pharmaceutical hormone (17α-ethynylestradiol in concentrations from below the detection limit to 7.0 ng/l) from human waste as the estrogenically active compounds present in effluents of sewage plants treating primarily domestic wastes. Exposure to complex mixtures of estrogens and estrogen mimics in concentrations observed in freshwaters has been associated with a range of reproductive impacts, namely in fish [17]. Also, difficulties in obtaining representative samples arise in the case of wastewater treatment plant effluents, whose composition is highly variable, and repeated testing is thus required [18]. A demonstration project was developed in Trancão River Basin (Portugal) and included the ecotoxicological and physicochemical study of wastewaters from two municipal sewer networks and respective WWTP. This paper reports the results obtained for the ecotoxicological assessment, discussing the issue of the chemical-based consent for discharges. Data from acute and chronic tests with different species (bacteria, an alga, crustaceans and a plant) and from an assay evaluating estrogenic activity for samples from the two systems are discussed.

2. Materials and methods

2.1. Wastewater sampling

Wastewater samples from two sewer network systems and respective WWTP-1 and 2, located in Loures (Portugal), were collected during four sampling campaigns (November 2003, March 2004, September 2004 and December 2004). These network systems receive domestic and industrial wastewaters namely from chemical, food, metal-mechanical, paper, printing, recycling, repair, and surface treatment sectors. Both WWTP started working by the end of the 90 s and the characteristics of each are detailed in Table 1. For each of the network systems, samples from influent to the WWTP (-inf) and effluent from the WWTP (-ef) as well as from two points along the network (-A and -B) were collected, making a total of 32 samples. 24-h composite samples were collected and each sample was divided into subsamples, kept refrigerated (4 °C) for physicochemical analysis for no more than 5 days and kept frozen (−20 °C) for ecotoxicological analysis for no more than 1 month.

2.2. Physicochemical analysis

pH potenciometric determination, chemical oxygen demand (COD), biochemical oxygen demand after 5 days (BOD_5), total hydrocarbons and oil and grease determinations were performed as described in the Standard Methods for the Examination of Water and Wastewater [19]. Total suspended solids (TSS) were determined according to standard NP EN 872:2000 [20].

The biological efficiency of WWTP can be evaluated by biodegradability studies of inflow wastewaters. BOD_5/COD ratio can be used as a biological treatability index to analyze the potential of wastewaters to be degraded. Fresenius et al. [21] considers $BOD_5/COD < 0.5$ indicative of low biodegradability and $BOD_5/COD \geq 0.5$ indicative of good biodegradability.

Table 1
Details of the WWTP including industrial/domestic ratio of wastewaters arriving to the WWTP, population equivalent numbers, annual flow through the WWTP (average for 2003–2004) and type of final treatment process

WWTP	Industrial/domestic (%)	Population equivalent	Annual flow (m^3)	Treatment type
1	65	130,000	4.6×10^6	Activated sludge
2	53	709,000	12.5×10^6	Tertiary treatment

2.3. Ecotoxicity tests

Ecotoxicological evaluation of the samples was performed using *Vibrio fischeri*, *Pseudokirchneriella subcapitata*, *Thamnocephalus platyurus*, *Daphnia magna* and *Lemna minor* as test organisms, as well as a blend of aerobic bacteria, to assess acute and chronic aquatic toxicity, according to the following methods.

Microtox test: Toxicity was assessed by determining the inhibition of the luminescence of *Vibrio fischeri* (strain NRRL B-11177) exposed for 15 min (Microtox® Test, Microbics, Carlsbad, U.S.A.). The test was performed according to the basic test procedure [22];

AlgalTox test: Alga toxicity was assessed by measuring the growth inhibition of *Pseudokirchneriella subcapitata* exposed for 72 h, according to AlgalToxKit F™ test procedure [23] that follow the OECD guideline 201 [24]. The inoculum is available in algal beads with immobilized cells. Optical density (OD 670 nm) of algae suspensions was determined.

ThamnoTox test: Crustacean toxicity was assessed by determining the mortality of *Thamnocephalus platyurus* exposed for 24 h according to ThamnoToxKit F™ test procedure [25]. Larvae for test were obtained by hatching of cysts.

Daphnia tests: Crustacean acute and chronic toxicity was also assessed by determining the inhibition of the mobility and reproduction of *Daphnia magna* (clone IRCHA-5) exposed for 48 h and 21 days, respectively, according to ISO 6341:1996 [26] and ISO 10706:2000 [27]. Juveniles for testing were obtained from cultures maintained in the laboratory.

Lemna test: Plant toxicity was assessed by determining the growth inhibition of *Lemna minor* (clone ST) exposed for 7 days, according to ISO 20079:2005 [28]. Plants for testing were obtained from cultures maintained in the laboratory. Total frond area was used as growth parameter, quantified by an image analysis system—Scanalyzer (LemnaTec, Würselen, Germany).

Polytox test: Bacteria toxicity was also evaluated by the determination of the inhibition of respiration on a mixture of specialized aerobic bacterial cultures after an exposure of 21 min, by measuring the dissolved oxygen consumed under defined conditions (POLYTOX® Test, InterLab, The Woodlands, Texas, U.S.A.). The test was performed according to the procedures of InterLab [29]. Endocrine disruption potential of the samples was assessed by Estrogen Responsive – Chemically Activated Luciferase Expression (ER CALUX®) assay (BioDetection Systems – BDS, Amsterdam, the Netherlands) determining estrogenic activity, analysis performed by BDS. This assay uses a human breast carcinoma cell line (T47D) which has been stably transfected with a plasmid containing the luciferase gene of *Photinus pyralis* coupled to three estrogen responsive elements, so it can act as a reporter for the presence of compounds that activate the estrogen receptor. Prior to analysis wastewater samples were extracted using methyltertbutylether (MTBE) and the evaporated extracts were dissolved in dimethyl sulfoxide (DMSO). Cells, previously grown in a medium supplemented with hormone-stripped serum, were exposed for 24 h and the response measured in a luminometer. Estrogenic activity of the samples is benchmarked against the reference estrogen 17β-estradiol and the results given in a quantitative way. This bioassay can be used for assessing net estrogenic activity of mixtures of estrogens.

All samples were tested with Microtox, AlgalTox, ThamnoTox, *Daphnia*, *Lemna* and Polytox tests. *Daphnia* chronic and ER CALUX tests were performed for effluent wastewater samples collected in the last sampling campaign in December 2004.

2.4. Data analysis

Ecotoxicity test results are expressed in EC_{50} or LC_{50}, the effective concentration responsible for the inhibition or lethality in 50% of tested population, after the defined exposure time. These values were calculated by Probit analysis. Results are presented as EC_{50}-15 min for Microtox test, EC_{50}-72 h for AlgalTox test, LC_{50}-24 h for ThamnoTox test, EC_{50}-48 h for *Daphnia* acute test, and EC_{50}-7 days for *Lemna* test. Lower values of EC_{50} indicate higher toxicity to the tested organism.

For the 21 days chronic test with *D. magna* significant differences between the results in the control and in tested concentrations were analysed by the application of Mann–Whitney test [30]. Results are presented as NOEC-21d, the no observed effect concentration, i.e. highest concentration for which reproduction is not significantly different from the control.

Polytox results are expressed as percent inhibition level (% I) at 100% of sample concentration, which is determined taking into account the dissolved oxygen uptake rate of the bacteria exposed to the sample, corrected for any background oxygen depletion, and the baseline dissolved oxygen uptake rate of the bacteria. Microbial populations exposed to an initial inhibition level of less than 30% can adapt to the presence of the inhibitory pollutant at the concentration level tested and the relative toxicity in the sample is negligible. If the Percent Inhibition Level falls between 30% and 50% the sample is "slightly toxic". If the Percent Inhibition Level is higher than 50% the sample is "toxic to very toxic" to the microorganisms [29].

Aiming to include all raw data for statistical analysis, EC_{50} values not determined due to low effect levels were considered as 100%. The acute tests sensitivity was assessed by Slooff's index [31]: each single test result (expressed as EC_{50} or LC_{50}) is divided by the arithmetic mean of all test results for each sample, and the geometric mean of these ratios for each test is calculated. The smaller value stands for the more sensitive test. The Slooff's index was calculated for Microtox, AlgalTox, ThamnoTox, *Daphnia* and *Lemna* tests.

Pearson correlations were determined using statistical analysis software (JMP® 5.0.1) for 23 samples on the following 9 variables, all except pH previously log transformed:

- physicochemical data from pH, COD, BOD_5 and TSS analysis;
- ecotoxicological data from Microtox, AlgalTox, ThamnoTox, *Daphnia* acute and *Lemna* tests.

In order to provide a graphical description of the correlated data and to understand its multivariate structure a principal component analysis (PCA) was also performed (see e.g. [32]) based on the Pearson correlation matrix (JMP®). PCA allows identifying the major discriminating variables associated with a given principal component (PC).

3. Results and discussion

3.1. Physicochemical parameters

The range of results from the physicochemical analysis of the samples is presented in Table 2. The results show variation (coefficient of variation (cv) > 80%) among samples for the physicochemical parameters, except for pH.

Concerning chemical characteristics of the wastewater samples collected in the sewer network system (-A and -B) and at the "input" of the WWTP (-inf), and according to the Municipal Regulation [33], emission limit values (ELV) for COD and BOD_5 were exceeded in 12.5% of the samples, and the ELV for TSS and total hydrocarbons were not exceeded. For influent wastewater samples, BOD_5/COD ratio varied between 0.41 and 0.64, with only one of the values lower than 0.5, accounting for a good biological degradation of organic substances entering the WWTP.

Concerning chemical characteristics of the wastewater samples collected at the discharge of the WWTP (–ef), and according to the Portuguese Legislation on Water Quality [34], ELV for COD and BOD_5 were exceeded in most of the cases (50% and 62.5% of the samples, respectively). ELV for TSS was also exceeded in 37.5% of the samples, but ELV for oil and grease was not exceeded. The ratio BOD_5/COD has lower values for effluents, varying between 0.22 and 0.53, than for influents. Since the values are lower than 0.5 in 75% of the effluent samples for Treatment Plant 2 that incorporates tertiary treatment, it suggests that the treatment was effective in biodegradation processes.

3.2. Acute toxicity tests

Direct toxicity assessment of influent and effluent wastewaters showed a heterogeneous group of samples (Table 3), being acute toxicity values dependent on the sample and the species tested. EC_{50} values lower than 10% were obtained for Microtox test in all sampling points, and for AlgalTox and Daphnia acute tests in sampling point 2-A. The sensitivity of Microtox test and the reliability of this test in monitoring toxicity of treatment plant wastewaters have also been observed by other authors [12,35].

Despite the frequent use of Microtox in assessing toxicity of wastewaters discharged into WWTP, Polytox test was also used to compare the performances. Polytox test results from all the input wastewater samples showed Percent Inhibition Level values lower than 30% foreseeing the good biodegradability of these samples in the WWTP, except for one sample from sampling point 1-B that can be said to be slightly toxic. Gutiérrez et al. [36] in a study comparing Microtox and activated sludge oxygen uptake inhibition found that it is impossible to state a relationship between these tests for the wastewaters toxicity. It seems that toxicity towards *Vibrio fischeri* does not imply toxicity to a blend of bacteria simulating the microbial population present at the WWTP.

Table 2
Range of values for physicochemical parameters obtained for sewage network system samples

Sampling points	pH	COD ($mg\,l^{-1}$)	BOD_5 ($mg\,l^{-1}$)	TSS ($mg\,l^{-1}$)	Total Hydrocarbons ($mg\,l^{-1}$)	Oil and Grease ($mg\,l^{-1}$)
1-A	7.2–8.2	496–2915	303–1538	200–600	0.12–2.3	4.3–59
1-B	7.7–8.3	241–637	127–294	12–400	0.22–1.8	13–31
1-inf	6.3–6.9	518–1139	264–649	119–800	0.23–0.55	19–70
1-ef	7.8–7.9	38–634	20–186	39–300	0.02–0.13	0.39–1.0
2-A	7.5–8.2	178–647	77–372	45–144	0.50–2.4	2.2–25
2-B	8.1–9.0	455–1030	303–531	103–500	0.76–1.2	27–47
2-inf	7.5–7.7	356–965	146–609	200–500	1.3–2.0	48–72
2-ef	6.8–7.8	60–158	29–83	19–41	0.02–0.17	0.16–0.64

Table 3
Range of values for ecotoxicological tests obtained for sewage network system samples.

Sampling Points	Microtox EC_{50}-15 min (%)	AlgalTox EC_{50}-72 h (%)	ThamnoTox LC_{50}-24 h (%)	*Daphnia* acute EC_{50}-48 h (%)	*Lemna* EC_{50}-7 days (%)	Polytox (% I)
1-A	0.90–4.2	72–n.d.	22–43	48–78	47–n.d.	0–24
1-B	1.5–18	41–n.d.	25–64	28–n.d.	78–n.d.	12–35
1-inf	3.7–54	20–n.d.	14–59	72–n.d.	n.d.	13–28
1-ef	5.2–n.d.	55–n.d.	46–n.d.	21–n.d.	n.d.	0–35
2-A	5.5–28	5.6–n.d.	16–n.d.	6.9–n.d.	39–86	0–14
2-B	1.8–7.1	23–n.d.	22–47	76–n.d.	59–89	8.0–21
2-inf	1.6–n.d.	n.d.	27–64	55–n.d.	73–n.d.	0–11
2-ef	1.2–n.d.	n.d.	n.d.	40–n.d.	n.d.	0–17

n.d.: not determined.

Slooff's sensitivity index calculated for the acute tests shows that the bacterium *Vibrio fischeri* is the most sensitive species, and allows to establish the following gradient of test sensitivity, Microtox > ThamnoTox > *Daphnia* = AlgalTox > *Lemna*, from the corresponding Slooff's index values 0.2 < 0.7 < 1.1 = 1.1 < 1.4.

Using a wastewater classification proposed by Tonkes et al. [37] that considers samples with an EC_{50} value for the most sensitive species lower than 10% as toxic and samples with an EC_{50} value lower than 1% as very toxic, all sampling points in this study had toxic wastewater samples and in one of the interceptors a very toxic sample was collected.

From the analysis of distribution of samples in classes of toxicity for each toxicity test in Fig. 1, it can be seen that Microtox test detects four classes of toxicity discriminating among tested samples and that ThamnoTox and *Lemna* tests detect only two classes of toxicity in the group of samples.

Concerning correlation analysis between acute toxicity tests and physicochemical parameters, significant correlations were obtained ($p < 0.05$). Organic load parameters and TSS showed significant negative correlations with Microtox (vs. COD, $r = -0.58$; vs. BOD_5, $r = -0.56$) and ThamnoTox (vs. COD, $r = -0.69$; vs. BOD_5, $r = -0.70$; vs. TSS, $r = -0.51$) results.

The principal component analysis allowed the representation of the data set on a bivariate plot. The two principal axes, PC1 and PC2, explain 66% of the total variance (43% and 23%, respectively). The plot in Fig. 2 shows the positions of the 23 samples on the plane spanned by the two principal axes together with the projections of the nine variables on those axes. The most discriminative variables in differentiating among the WWTP samples were defined as those which had at least 40% of their variance explained by PC1 or PC2 (i.e. eigenvector (v) >0.40). The first component is positively associated with the organic load parameters and negatively associated with the results of ThamnoTox test (COD, $v = 0.45$; BOD, $v = 0.46$; ThamnoTox, $v = -0.43$); the second component is positively associated with the results of AlgalTox and *Daphnia* acute tests (AlgalTox, $v = 0.57$; *Daphnia*, $v = 0.58$). The distribution of the samples in this bidimensional plot, mainly along PC1, allow to group the samples into two major clusters, the untreated and the treated wastewaters, with WWTP effluent samples associated with less organic contamination and less toxicity in ThamnoTox test.

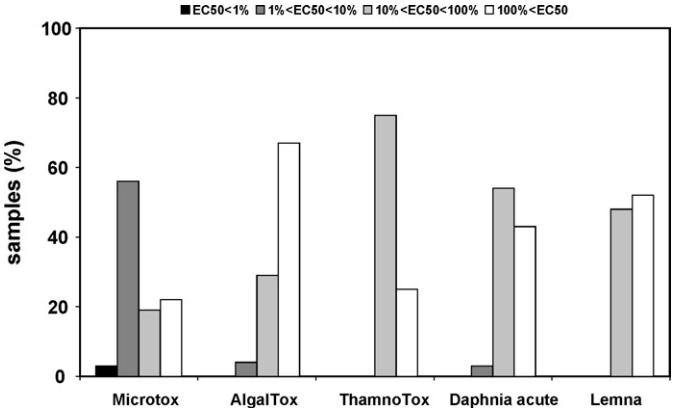

Fig. 1. Distribution of samples in classes of toxicity for each toxicity test.

3.3. Chronic toxicity test and endocrine disruption assay

Concerning *Daphnia* chronic toxicity, no mortality of adult females occurred in the control and in the lowest concentrations of effluent samples after an exposure of 21 days (Table 4). Mortality of adult females was registered in *Daphnia* groups exposed during 21 days to the higher concentrations, attaining 100% mortality at 22% concentration of sample 1-ef and at 5% concentration of sample 2-ef. First brood in all females occurred between the 7th and the 9th day, but cumulative number of juveniles after 21 days exposure was lower for highest concentrations in both tests. NOEC-21d values were 5% for sample 1-ef and 0.4% for sample 2-ef. Acute to chronic ratios (ACR = EC_{50}-48 h/NOEC-21d) were 4 and 250 for samples 1-ef and 2-ef, respectively ($ACR_{1\text{-}ef}$ = 21%/5% = 4.2 and $ACR_{2\text{-}ef}$ = 100%/0.4% = 250). In the case of sample 2-ef, the observation of chronic effects at low concentrations is particularly relevant since the sample did not show acute toxicity for any of the tested organisms. Kosmala et al. [38] in a WWTP survey using the chronic toxicity test with the crustacean *Ceriodaphnia dubia* also detected constant chronic toxicity of the effluent that revealed no acute toxicity to different organisms.

For ER CALUX a quantifiable amount of estrogenic activity could be observed in both samples: 2407 ± 2.9 pg 17β-estradiol EEQ/l water for sample 1-ef and 4868 ± 10.0 pg 17β-estradiol EEQ/l water for sample 2-ef. Sample 2-ef showed higher estrogenic activity than

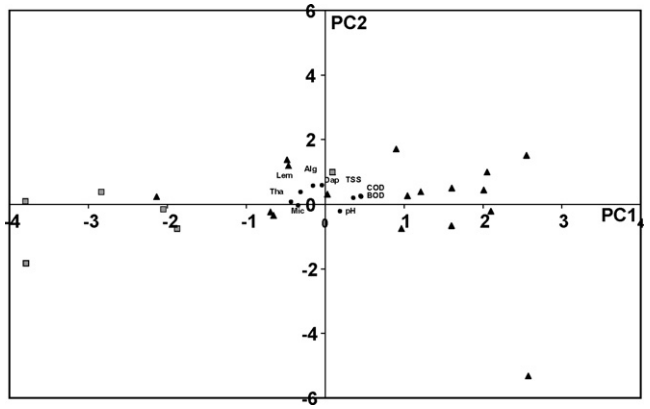

Fig. 2. Graphical representation of the two principal axes (PC1 and PC2) from a principal component analysis in a correlation matrix for the 23 samples on the 9 chemical and ecotoxicological variables (■ : treated effluent samples, ▲: other samples, ●: variables).

Table 4
Female mortality and reproduction exposure effects of wastewater treatment plant effluents in 21 days *Daphnia* reproduction test (mean ± S.E.M.: mean number of juveniles per female and standard error of the mean; Sig.: significance of differences between concentrations in number of juveniles)

Concentration (%)	Female mortality (%)	Number juveniles (mean ± S.E.M.)	Sig.[a]
Sample 1-ef			
0	0	74.5 ± 3.4	A
5	0	64.3 ± 10.0	A
10	20	57.0 ± 1.4	B
15	40	53.7 ± 7.5	B
22	100	–	–
33	60	41.5 ± 4.9	C
Sample 2-ef			
0	0	74.0 ± 8.2	A
0.2	0	61.6 ± 14.6	A
0.4	0	66.4 ± 11.9	A
0.9	20	61.0 ± 5.7	B
2.1	40	33.0 ± 8.7	C
5.0	100	–	–

[a] Concentrations with the same letter are not significantly different ($p > 0.05$).

sample 1-ef. *In vitro* bioassays can estimate the total endocrine disrupting activity of a WWTP sample because they may account for the additive and synergistic effects of the endocrine disrupting chemicals present. In a study using other assays to detect estrogen activity, E-Screen and YES bioassays, *in vitro* bioassays were considered very useful in a strategy to analyze WWTP samples for estrogens or endocrine disrupting chemicals [39]. Chronic toxicity and estrogenic activity were observed in the effluents of both WWTP, with relevant data for the WWTP effluent sample that showed no acute toxicity.

4. Conclusions

The methodologies for ecotoxicity evaluation are available and should be used in the control of complex wastewaters. In this study the alert to potential problems by chemical evaluation of samples did not always correspond to effects in tested organisms. The reverse situation was also observed, some samples presented effects in tested organisms with no indication of potential hazard by chemical evaluation. This stresses the importance of complementing the chemical approach with the ecotoxicological approach, to maximize environmental protection. The validity of the use of acute tests to drive environmental improvement has been demonstrated, but methodologies for chronic toxicity, persistence and bioaccumulation need further development [7].

When considering ecotoxicity testing as part of the toolbox to investigate the environmental impacts of effluents, a tiered approach is recommended, both for economical or technical reasons. Concerning WWTP systems and considering the relative sensitivity of the organisms used in wastewater testing and the importance to consider effects at different trophic levels, the test battery proposed would include tests with a bacterium, an alga and a crustacean to monitor this type of wastewaters. In an ecotoxicity screening phase, we propose the use of a single test, Microtox, which turned out to be the most sensitive test. In this study Polytox test, mentioned as providing rapid information on the potential toxicity of wastewaters to the biological community of wastewaters treatment systems, did not differentiate samples of wastewaters arriving to the WWTP. Effluent samples collected at the discharge of the WWTP, showed lower ratio BOD_5/COD than influent samples, proving its efficiency in reducing the organic load discharged. The principal component analysis of physicochemical and ecotoxicological data allowed to group the samples into untreated and treated wastewaters, with WWTP effluent samples associated with less organic contamination and less toxicity. Nevertheless chronic toxicity and estrogenic activity were observed in the effluents from both WWTP, with relevant data for the WWTP effluent from Treatment Plant 2, showing no acute toxicity but with higher estrogenic activity and higher acute to chronic ratio.

Chronic toxicity and endocrine disruption evaluation allowed obtaining additional information, indicative of potential population effects induced by low concentrations in a long time exposure.

The use of the ecotoxicological approach has an added value to hazard and risk assessment of discharges to the receiving waters, and environmental management can use this tool with advantages. Ecotoxicity tests identify the hazard and can be used in ecological risk assessment. In Water Framework Directive, direct toxicity assessment of WWTP discharges can contribute to attain or keep ecological quality objectives in water masses.

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References

[1] D.R. Grothe, K.L. Dickson, D.K. Reed-Judkins, Whole Effluent Toxicity Testing: An Evaluation of Methods and Prediction of Receiving System Impacts, Society of Environmental Toxicology and Chemistry (SETAC), Pensacola, FL, USA, 1996.
[2] EC, Integrated Pollution Prevention and Control Directive (IPPC), Directive 96/61/EC, 1996.
[3] EC, Water Framework Directive (WFD), Directive 2000/60/EC, 2000.
[4] EC, Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), Regulation (EC) No 1907/2006 of the European Parliament and of the Council, 2006.
[5] P. Calow, Effluent ecotoxicology: important link in the environmental protection chain, in: Effluent ecotoxicology: A European Perspective, Meeting of the Society of Environmental Toxicology, Chemistry (SETAC), Edinburgh, UK, 1999, p. 20.
[6] P.M. Chapman, Whole effluent toxicity testing—usefulness, level of protection, and risk assessment, Environ. Toxicol. Chem. 19 (2000) 3–13.
[7] ECETOC, Whole Effluent Assessment, Technical Report No. 94, European Centre for Ecotoxicology and Toxicology of Chemicals, Brussels, Belgium, 2004.
[8] E. Vindimian, J. Garric, P. Flammarion, E. Thybaud, M. Babut, An index of effluent aquatic toxicity designed by partial least squares regression, using acute and chronic tests and expert judgments, Environ. Toxicol. Chem. 18 (1999) 2386–2391.
[9] E.A. Power, R.S. Boumphrey, International trends in bioassay use for effluent management, Ecotoxicology 13 (2004) 377–398.
[10] USEPA, NPDES Compliance Inspection Manual, EPA 305-X-03-004, 2004.
[11] M. Daniel, A. Sharpe, J. Driver, A.W. Knight, P.O. Keenan, R.M. Walmsley, A. Robinson, T. Zhang, D. Rawson, Results of a technology demonstration project to compare rapid aquatic toxicity screening tests in the analysis of industrial effluents, J. Environ. Monit. 6 (2004) 855–865.
[12] G. Libralato, C. Losso, A. Arizzi Novelli, F. Avezzù, A. Scandella, A. Volpi Ghirardini, Toxicity bioassays as effective tools for monitoring the performances of wastewater treatment plant technologies: SBR and UF-MBR as case studies, in: Proceedings of 4th MWWD and 2nd IEMES, Antalya, Turkey, 2006.
[13] E. Emmanuel, Y. Perrodin, G. Keck, J.-M. Blanchard, P. Vermande, Ecotoxicological risk assessment of hospital wastewater: a proposed framework for raw effluents discharging into urban sewer network, J. Hazard. Mater. A117 (2005) 1–11.
[14] D.W. Kolpin, E.T. Furlong, M.T. Meyer, E.M. Thurman, S.D. Zaugg, L.B. Barber, Pharmaceuticals, hormones and other organic wastewater contaminants in U.S. streams, 1999–2000: a national reconnaissance, Environ. Sci. Technol. 36 (2002) 1202–1211.
[15] A.L. Filby, T. Neuparth, K.L. Thorpe, R. Owen, T.S. Galloway, C.R. Tyler, Health impacts of estrogens in the environment considering complex mixture effects, Environ. Health Perspect. 115 (2007) 1704–1710.
[16] C. Desbrow, E.J. Routeledge, G.C. Brighty, J.P. Sumpter, M. Waldock, Identification of estrogenic chemicals in STW effluents. Chemical fractionation and in vitro biological screening, Environ. Sci. Technol. 32 (1998) 1549–1558.
[17] K.A. Kidd, P.J. Blanchfield, K.H. Mills, V.P. Palace, R.E. Evans, J.M. Lazorchak, R.W. Flick, Collapse of a fish population after exposure to a synthetic estrogen, Proceedings of the National Academy of Sciences (PNAS) 104 (2007) 8897–8901.
[18] P.M. Chapman, Determining when contamination is pollution—weight of evidence determinations for sediments and effluents, Environ. Int. 33 (2007) 492–501.

[19] L.S. Clesceri, A.E. Greenberg, A.D. Eaton (Eds.), Standard Methods for the Examination of Water and Wastewater, 20th ed., APHA, American Water Works Association, Water Environment Federation, Washington, USA, 1998.
[20] IPQ, Qualidade da Água – Determinação dos sólidos em suspensão – Método por filtração com filtros de fibra de vidro, NP EN 872, 2000.;
IPQ, Monte da Caparica, 2001.
[21] W. Fresenius, W. Schneider, B. Böhnke, K. Pöppinghaus, Technologie des eaux résiduaires – Production, collecte, traitement et analyse des eaux résiduaires, Springer-Verlag, Berlin, 1990.
[22] Microbics, Microtox Manual—A Toxicity Handbook, vol. I–IV, Microbics Corporation Inc., Carlsbad, CA, 1992.
[23] MicroBioTests, AlgalToxKit F™—Freshwater Toxicity Test with Microalgae. Standard Operational Procedure, MicroBioTests Inc., Nazareth, Belgium, 2004.
[24] OECD, Alga Growth Inhibition Test. Guidelines for the Testing of Chemicals, Test Guideline 201, OECD, Paris, France, 1984.
[25] MicroBioTests, ThamnoToxKit F™—Freshwater Toxicity Screening Test. Standard Operational Procedure, MicroBioTests Inc., Nazareth, Belgium, 2003.
[26] ISO, Water Quality – Determination of the Inhibition of the Mobility of *Daphnia magna* Straus (Cladocera, Crustacea) – Acute toxicity Test, ISO 6341, International Standard Organization, 1996.
[27] ISO, Water Quality—Determination of Long Term Toxicity of Substances to *Daphnia magna* Straus, ISO 10706, International Standard Organization, 2000.
[28] ISO, Water Quality – Determination of Toxic Effect of Water Constituents and Wastewater to Duckweed (*Lemna minor*) – Duckweed Growth inhibition test, ISO 20079, International Standard Organization, 2005.
[29] InterLab, Polytox™ Rapid Toxicity Test—Application Procedure, InterLab®, The Woodlands, TX, 2005.
[30] E.J. Duderwicz, S.N. Mishra, Modern Mathematical Statistics, Series in Probability and Mathematical Statistics, John Wiley, New York, 1988.
[31] W. Slooff, Benthic macroinvertebrates and water quality assessment: some toxicological considerations, Aquat. Toxicol. 4 (1983) 73–82.
[32] R.A. Johnson, D.W. Wichern, Applied Multivariate Statistical Analysis, 3rd ed., Prentice Hall, Englewood Cliffs, NJ, 1992.
[33] SMAS-Loures, Regulamento de descarga de águas residuais industriais nas redes de colectores municipais do concelho de Loures, SMAS-CM Loures, 1993.
[34] Decreto-Lei 236/98, Diário da República, Série I-A no. 176, Lisboa, 1998.
[35] C.V.M. Araújo, R.B. Nascimento, C.A. Oliveira, U.J. Strotmann, E.M. da Silva, The use of Microtox® to assess toxicity removal of industrial effluents from the industrial district of Camaçari (BA, Brazil), Chemosphere 58 (2005) 1277–1281.
[36] M. Gutiérrez, J. Etxebarria, L. de las Fuentes, Evaluation of wastewater toxicity: comparative study between Microtox® and activated sludge oxygen uptake inhibition, Water Res. 36 (2002) 919–924.
[37] M. Tonkes, P.J.F. de Graaf, J. Graansma, Assessment of complex industrial effluents in the Netherlands using a whole effluent toxicity (or WET) approach, Water Sci. Technol. 39 (1999) 55–61.
[38] A. Kosmala, S. Charvet, M.-C. Roger, B. Faessel, Impact assessment of a wastewater treatment plant effluent using instream invertebrates and the *Ceriodaphnia dubia* chronic toxicity test, Water Res. 33 (1999) 266–278.
[39] J. Nelson, F. Bishay, A. van Roodselaar, M. Ikonomou, F.C.P. Law, The use of in vitro bioassays to quantify endocrine disrupting chemicals in municipal wastewater treatment plant effluents, Sci. Total Environ. 374 (2007) 80–90.