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Ecotoxicological evaluation of wastewater in a municipal WWTP in Lisbon area (Portugal)

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ABSTRACT

Wastewater management has a central role in sustainable development, and, in this context, an integrated management of wastewater treatment plants (WWTP) can be important. WWTP discharge complex effluents and for a new strategy in environmental protection ecotoxicological evaluation should complement the usual chemical evaluation. The EU project WW4Environment was set up for a WWTP located in Lisbon area and discharging into Tagus estuary (Portugal). One of the main objectives of the project is to optimize the management of the WWTP in terms of environmental impact. A battery of toxicity tests with organisms bearing different functions at the ecosystem level (the bacterium *Vibrio fischeri*, the alga *Pseudokirchneriella subcapitata*, the crustaceans *Thamnocephalus platyurus* and *Daphnia magna*, and the plant *Lemna minor*) was used to characterize the wastewater in the different treatment phases. *V. fischeri*, test organism for Microtox test, was the most sensitive species in WWTP samples evaluation. Microtox, Alga, and Daphnia tests were able to distinguish two levels of treatment and to assess toxicity removal efficiency. The results demonstrated not only that the treatment efficiently reduced wastewater toxicity, but also that the use of an ecotoxicological approach can contribute to the environmental management of the treatment plant.

Keywords: WWTP; Environmental management; Wastewater; Ecotoxicity; Treatment efficiency

1. Introduction

Half of the world's population lives in cities, most of which have inadequate infrastructure and resources to address wastewater management in an efficient and sustainable way. Inadequate infrastructure and management systems for the increasing volume of wastewater that we produce are at the heart of the wastewater crisis. Finding appropriate solutions will require innovation both to reduce the volume and

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contamination of wastewater produced and to treat or even reuse the waste, in an affordable sustainable way. The quality of water is important for the well-being of the environment, society, and the economy. It is recognized that wastewater management has a central role in sustainable development [1].

The major problem in controlling wastewater discharges is related to its environmental toxicity. Chemical analysis of wastewater is usually insufficient to provide the information on water quality as a high number of chemical compounds are present and some in concentrations lower than detection/quantification limits. It is also impossible to predict the toxicity of complex wastewater using the physicochemical approach, due to antagonistic and synergistic effects of chemicals in mixtures. Repeated testing is required to overcome the difficulties in obtaining representative samples, whose composition is highly variable [2]. So, it is essential to use biological test systems that give a global response. Direct toxicity assessment can be an added value strategy when we face complex effluents for which many chemicals cannot be quantified and/or interactive effects are likely to be significant, for example [3–7].

As stated by Lofrano and Brown [8] "…with greater understanding of the impact of the wastewater on the environment and more sophisticated analytical methods, advanced treatment is becoming more common". Despite the fact that the adoption of this approach is still not global, the validity of the use of ecotoxicity tests to drive environmental improvement has been demonstrated [9]. Biological evaluation became as relevant to the protection of ecological systems as chemical specific evaluation after the implementation of the water framework directive (WFD), 2000/60/EC [10].

Wastewater treatment plants (WWTP) discharge complex effluents to the receiving waters raising special concern. In many countries, ecotoxicity tests are in use for wastewater management via site-specific risk assessment or hazard-based standards definition through the promotion of "best available technology" for specific industry sectors [11,12]. Ecotoxicological evaluation in the different stages of the wastewater treatment process has also advantages to protect biological treatment plants from toxic influents [13] and to monitor the effectiveness of WWTP [10,14–20].

When assessing effects in WWTP and controlling complex wastewaters, it is important to consider effects at different trophic levels due to differences in relative sensitivity of the organisms. The sensitivity of the *Vibrio fischeri* test and the reliability of this test in monitoring bacterial toxicity of treatment plant wastewaters have been observed [17,21,22]. Concerning microalgae use in wastewater toxicity testing, although several authors use algal tests to assess toxicity removal [16,23–25], the alga test is not considered the most appropriate test for nutrient-rich wastewaters because of the complex relationship of inhibition and promotion of algae growth [12]. Related to the crustacean toxicity, several authors concluded that *D. magna* acute test can be a useful analytical tool for early warning system to monitor the different operational units of WWTP [26,27]. Concerning phytotoxicity, the test with the aquatic plant *Lemna minor* is considered suitable for surface water quality assessment and adequate for biomonitoring of municipal effluents [28]. In a previous work, Mendonça et al. [29] proposed a test battery to monitor WWTP wastewaters, including tests with a bacterium, an alga, and a crustacean.

The EU project WW4Environment ("Integrated approach to energy and climate changes: changing the paradigm of wastewater treatment management", LIFE08 ENV/P/000237, 2010–2012) was set up for a WWTP located in Lisbon area and discharging into Tagus estuary (Portugal). The main objectives of the project are to implement a tool to optimize the management of WWTP in terms of energy efficiency and environmental impact and to reduce the environmental costs of the treatment process. The aim of the ecotoxicological survey is the evaluation of the effectiveness of the treatment process in reducing the toxicity and the protection of the biological treatment through the setting up of adequate ecotoxicological methodologies and the selection of a battery of tests to be used in the characterization of WWTP wastewaters.

In this study, to characterize the wastewater in the different treatment phases, a battery of tests with organisms bearing different functions at the ecosystem level was used. Aquatic toxicity tests were performed using the bacterium *V. fischeri*, the alga *P. subcapitata*, the crustaceans *Thamnocephalus platyurus* and *D. magna*, and the plant *L. minor* as test organisms. Knowing that the ecological relevance of species and exposure time is questionable in routine ecotoxicological evaluation, the results of such an approach should help building an adequate testing strategy for the ecotoxicological effects of WWTP discharges. The first results of this program are reported and discussed.

2. Materials and methods

2.1. Wastewater treatment plant (WWTP)

The WWTP under study is located in Loures (Portugal), receives domestic and industrial wastewaters and discharges into Tagus estuary. It has the capacity to treat an average flow of 54,500 m^3/day corresponding to a population equivalent of about 213,500 inhabitants.

This WWTP was commissioned in 1989 with a secondary level of treatment. Between 1998 and 1999, it has undergone improvement works that include the additional line of treatment of liquid and solid phases being endowed with a tertiary treatment with final disinfection. The intervention also included the installation of a deodorization system and a process of anaerobic digestion of biosolids and therefore production of biogas.

2.2. Wastewater sampling

Wastewater 1 h-composite samples were collected during two sampling campaigns (April 2010 and January 2011) with different strategies and periodicities:

- 2010 Campaign—samples collected at the input (A) and after primary treatment (B) in different days of the week (Monday, Tuesday and Friday) at 10, 14, and 23 h.
- 2011 Campaign—samples collected every 3 h at the input (A), after primary treatment (B) and after secondary treatment (C) from Friday at 10 h to Saturday at 13 h.

A total of 47 samples were collected. Each sample was divided into subsamples, kept frozen (−20°C) for ecotoxicological analysis for no more than one month.

2.3. Ecotoxicity tests

Ecotoxicological evaluation of the samples was performed using *V. fischeri*, *P. subcapitata*, *T. platyurus*, *D. magna*, and *L. minor* as test organisms, to assess aquatic toxicity, according to the following methods:

- Microtox test: bacteria toxicity was assessed by determining the inhibition of the luminescence of *V. fischeri* (strain NRRL B-11177) exposed for 15 min (Microtox® Test, Microbics, Carlsbad, USA). The test was performed according to the basic test procedure [30];
- Alga test: alga toxicity was assessed by measuring the growth inhibition of *P. subcapitata* exposed for 72 h. A miniaturized test in microtitration plates was carried out according to ISO 8692: 2004 [31]. The inoculum used was available in algal beads with immobilized cells [32]. Optical density at 670 nm of algae suspensions was determined. To select the best methodology for this kind of samples, 2010 campaign samples were filtered by 0.45 μm pore size membranes, and 2011 campaign samples were decanted;
- ThamnoTox test: crustacean toxicity was assessed by determining the mortality of *T. platyurus* exposed for 24 h according to ThamnoToxKit F™ test procedure [33];
- Daphnia test: crustacean toxicity was also assessed by determining the inhibition of the mobility of *D. magna* (clone IRCHA-5) exposed for 48 h, according to ISO 6341: 1996 [34]. Juveniles for testing were obtained from cultures maintained in the laboratory;
- Lemna test: plant toxicity was assessed by determining the growth inhibition of *L. minor* (clone ST) exposed for seven days, according to ISO 20079: 2005 [35]. 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).

2.4. Data analysis

For each toxicity test, EC_{50}-t or LC_{50}-t, the effective concentration (% v/v) responsible for the inhibition or lethality in 50% of tested population after the defined exposure period (t), was calculated:

- EC_{50}-15 min for Microtox test by using Microtox Omni™ software (Azur Environmental, 1999);
- EC_{50}-72 h for Alga test, LC_{50}-24 h for ThamnoTox test, and EC_{50}-48 h for Daphnia test by using ToxCalc™ software (version 5.0, Tidepool Scientific software, 2002);
- EC_{50}-7 d for Lemna test by using Biostat 2.0 software (LemnaTec, 2001).

Aiming to include all raw data for statistical analysis, EC_{50} values not determined due to low effect levels were considered as 100%. Data obtained are also presented as percentage inhibition at the highest tested concentration.

The tests sensitivity was assessed by Slooff's index [36]: 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.

A wastewater classification proposed by Tonkes et al. [37] was used to classify the different samples tested. This classification is based on the EC_{50} value for the most sensitive species and considers samples with an EC_{50} higher than 100% as non-toxic, EC_{50}

Table 1
EC_{50}-t and LC_{50}-t values from ecotoxicological tests of wastewater samples of the 2010 campaign (A: WWTP input and B: after primary treatment)

	Sample	Microtox EC_{50}-15 min	Alga EC_{50}-72 h	Daphnia EC_{50}-48 h	ThamnoTox LC_{50}-24 h	Lemna EC_{50}-7 d
A	Mon-10 h	17.2	>90	>90	35.4	<90
	Mon-14 h	5.2	>90	>90	33.0	>90
	Mon-23 h	3.1	>90	67.0	28.1	90.0
	Tues-10 h	7.2	>90	>90	37.0	<90
	Tues-14 h	7.9	>90	>90	35.4	>90
	Tues-23 h	2.2	>90	52.0	29.4	>90
	Fri-10 h	5.6	>90	28.0	37.9	>90
	Fri-14 h	2.3	>90	53.0	39.7	>90
	Fri-23 h	1.1	>90	74.0	41.1	>90
B	Mon-14 h	42.6	>90	>90	36.2	>90
	Mon-23 h	9.0	>90	90.0	33.0	90.0
	Tues-10 h	34.9	90.0	>90	54.8	<90
	Tues-14 h	20.8	>90	>90	36.2	<90
	Tues-23 h	5.6	>90	67.0	42.5	>90
	Fri-10 h	6.0	>90	67.0	46.6	>90
	Fri-14 h	8.8	>90	90.0	44.5	>90
	Fri-23 h	2.8	>90	>90	43.5	>90

between 10 and 100% as slightly toxic and EC_{50} lower than 10% as toxic.

Toxicity removal of the two treatment units (primary and secondary) was calculated using values of inhibition at the highest tested concentration for samples before and after treatment as:

$$\text{Toxicity removal} = I_{\text{In}} - I_{\text{Out}}/I_{\text{In}} \times 100$$

3. Results

3.1. 2010 Campaign

Different response ranges for the input wastewater samples vs. primary treated wastewater samples can be observed for the 2010 Campaign (Table 1) on three of the tests: for Microtox [1.1% < EC50 < 17.2%] and [2.8% < EC50 < 42.6%]; for Daphnia [28.0% < EC_{50} < 90%] and [67.0% < EC_{50} < 90%]; for ThamnoTox [28.1% < LC_{50} < 41.1%] and [33.0% < LC_{50} < 46.6%]. For Alga and Lemna tests, EC_{50} are either 90% or higher, revealing no toxicity and not distinguishing treated from untreated samples.

Analyzing the results for Microtox and Daphnia tests obtained in the different days of the week, the highest toxicity was measured on Friday. A peak in toxicity was also obtained for Microtox test in all samples collected at 23 h.

Results of ecotoxicity tests presented as percentage inhibition at the highest tested concentration (Fig. 1), excluding ThamnoTox test that showed 100% effect for all samples, show that the pattern of inhibition can be different also along the day according to the test organism:

- After primary treatment, the inhibition of the bacteria luminescence gets higher along the day;
- The alga test shows growth inhibition between 18 and 50% for all samples with no pattern along the day or the week. These low inhibition values can be linked with the inclusion of filtration in the test procedure in this campaign;
- In the same day, the inhibition of the mobility in *Daphnia* ranges from 0 to 100%, both for input and after primary treatment samples;
- At the input, the growth inhibition of *Lemna* usually decreases along the day and the week.

The alga and the plant test results seem to express simultaneously growth inhibition due to wastewater contaminants and interferences from factors like shading and nutrient concentration.

3.2. 2011 Campaign

Results of the 2011 campaign (Table 2) show differences in ranges for the same tests when comparing

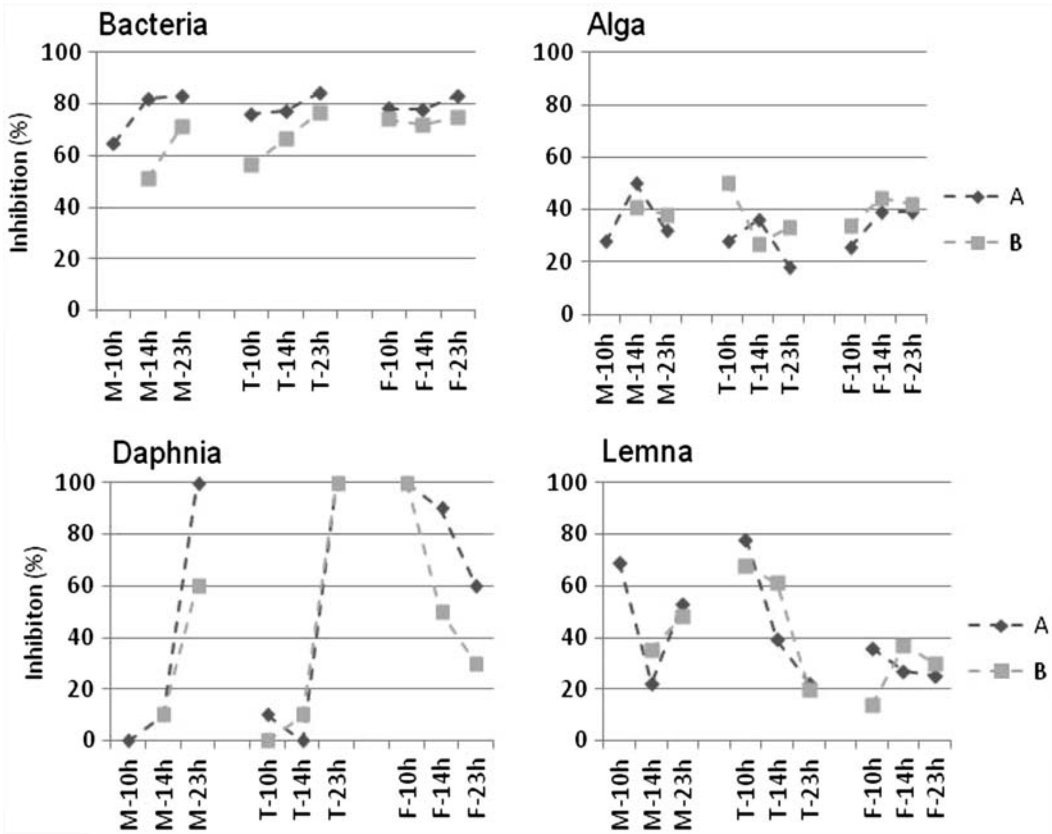

Fig. 1. Inhibition effect in test organisms at the highest tested concentration in ecotoxicological tests of wastewater samples of the 2010 campaign (A: WWTP input and B: after primary treatment).

input wastewater with primary and secondary treated wastewater samples: for Microtox [3.1% < EC_{50} < 31.0%], [2.9% < EC_{50} < 22.0%] and EC_{50} higher than 90%, respectively; for *Daphnia* [33.0% < EC_{50} < 90%], [35.0% < EC_{50} < 90%], and EC_{50} higher than 90%, respectively; for ThamnoTox [30.8% < LC_{50} < 74.9%], [35.4% < LC_{50} < 57.0%] and [65.6% < LC_{50} < 83.1%], respectively. For the Alga test, EC_{50} values range from 9.0% for a sample of primary treated effluent to values higher than 90% obtained for input samples and for the majority of secondary treated samples. For Lemna test, EC_{50} are either 90% or higher, revealing no toxicity and not distinguishing treated from untreated samples, except for the input sample on Friday at 10 h.

On the basis of EC_{50}, we can distinguish input and after secondary treatment samples, with different values in the tests for input samples and with EC_{50} higher than 90% for all treated samples in Microtox, Daphnia, and Lemna tests and the majority of treated samples in Alga test. Also for ThamnoTox, EC_{50} values show detoxification of wastewater.

In general, along these 28 h monitoring program, the lowest EC_{50} values in sites A and B, corresponding to higher toxicity, were obtained for wastewater samples collected between Friday at 19 h and Saturday at 4 h.

Results of ecotoxicity tests presented as percentage inhibition at the highest tested concentration (Fig. 2) confirm the results obtained in the 2010 campaign showing that the pattern of inhibition can be different according to the test organism and makes evidence for secondary treatment efficiency:

- After secondary treatment, the inhibition of the bacteria luminescence and *Daphnia* mobility get very low;
- The alga test shows growth inhibition between 15.7 and 100% with variation along the sampling period for the input samples, high values for the primary treated samples and lower values for the secondary treated samples. No pattern of effect can be seen;
- The inhibition of *Daphnia* mobility ranges from 0 to 100% at the input and from 15 to 100% after primary treatment;
- The growth inhibition in *Lemna* is higher on Friday morning decreasing along the sampling period for all sampling sites.

Table 2
EC_{50}-t and LC_{50}-t values from ecotoxicological tests of wastewater samples of the 2011 campaign (A: WWTP input; B: after primary treatment and C: after secondary treatment)

	Sample	Microtox EC_{50}-15 min	Alga EC_{50}-72 h	Daphnia EC_{50}-48 h	ThamnoTox LC_{50}-24 h	Lemna EC_{50}-7 d
A	Fri-10 h	26.3	50.1	>90	36.2	50.0
	Fri-13 h	5.4	16.9	80.0	41.6	<90
	Fri-16 h	3.5	25.8	59.0	40.6	>90
	Fri-19 h	4.1	42.5	33.0	32.2	>90
	Fri-22 h	2.8	47.0	59.0	32.2	>90
	Sat-1 h	3.1	>90	52.0	39.7	>90
	Sat-4 h	5.7	29.1	90.0	30.8	>90
	Sat-7 h	31.0	>90	52.2	74.9	>90
	Sat-10 h	24.2	64.4	>90	60.2	>90
	Sat-13 h	3.9	42.2	62.2	47.7	>90
B	Fri-10 h	22.0	30.2	>90	47.7	<90
	Fri-13 h	12.7	22.2	>90	40.6	>90
	Fri-16 h	9.1	64.5	>90	44.5	>90
	Fri-19 h	7.1	48.4	59.0	37.9	>90
	Fri-22 h	3.5	40.2	67.0	35.4	>90
	Sat-1 h	4.2	25.3	69.5	39.7	>90
	Sat-4 h	2.9	61.4	35.0	54.8	>90
	Sat-7 h	6.7	9.0	54.8	36.2	>90
	Sat-10 h	4.6	47.9	69.4	43.5	>90
	Sat-13 h	14.9	54.3	>90	57.0	>90
C	Fri-10 h	>90	>90	>90	65.6	<90
	Fri-13 h	>90	>90	>90	65.6	>90
	Fri-16 h	>90	>90	>90	82.5	>90
	Fri-19 h	>90	>90	>90	65.6	>90
	Fri-22 h	>90	24.1	>90	69.5	>90
	Sat-1 h	>90	>90	>90	71.7	>90
	Sat-4 h	>90	27.0	>90	75.8	>90
	Sat-7 h	>90	>90	>90	78.5	>90
	Sat-10 h	>90	>90	>90	80.3	>90
	Sat-13 h	>90	>90	>90	83.1	>90

ThamnoTox test presented the highest effect [63 and 100% mortality] in all samples, seeming to be the lowest discriminative test. Nevertheless, analyzing mean mortality values for A–C sites allow verifying that percentage effect gets lower after secondary treatment.

4. Discussion

In general, considering the usual ecotoxicity parameters EC_{50}/LC_{50}, different response ranges could be observed for the same test when comparing untreated with treated wastewater samples. EC_{50} is higher than 90% for all secondary treated samples in Microtox, Daphnia, and Lemna tests and the majority of treated samples in Alga test, and EC_{50} mean values for ThamnoTox also show detoxification of wastewater. Taking into account, wastewater composition variability over time, Microtox and Daphnia tests could always distinguish untreated from primary or secondary treated wastewater samples in the two sampling campaigns. ThamnoTox test, although responding to the treatment level was less discriminative. If only EC_{50} values are analyzed, Alga and Lemna tests did not show a pattern of response.

By direct analysis of the data on percentage inhibition/mortality at the highest tested concentration for each test, complementary considerations can be done: (1) the pattern of response varied along the week with higher effects on Friday and (2) effects got higher during the night period for untreated and primary treated wastewater.

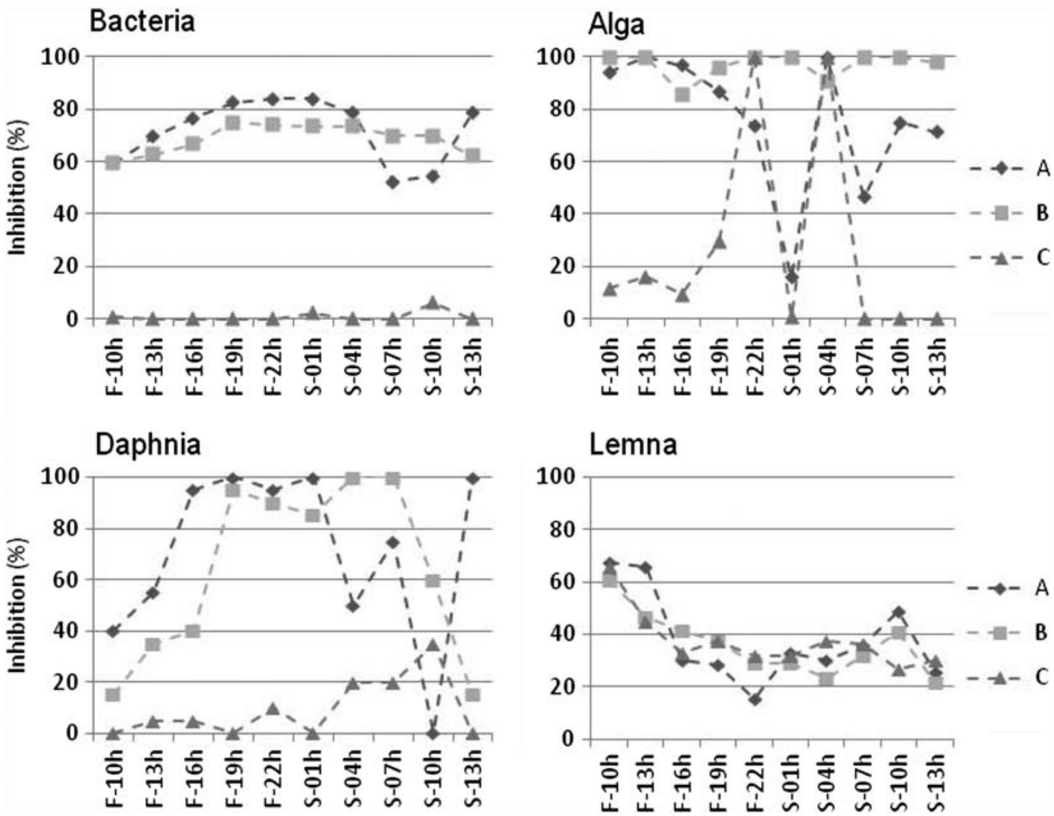

Fig. 2. Inhibition effect in test organisms at the highest tested concentration in ecotoxicological tests of wastewater samples of the 2011 campaign (A: WWTP input; B: after primary treatment and C: after secondary treatment).

Slooff's sensitivity index shows that the bacterium *V. fischeri* is the most sensitive species, and allows to establish a gradient of test sensitivity: Microtox > ThamnoTox > Alga > Daphnia > Lemna, from the corresponding Slooff's index values $0.2 < 0.7 < 1.0 < 1.2 < 1.5$. The sensitivity of Microtox test and the reliability of this test in monitoring toxicity of treatment plant wastewaters have also been referred by other authors [17,21,22].

Using a wastewater classification [37] to summarize the toxicity data results for each sample, we can say that at the WWTP input, 74% of the samples are toxic and 26% are slightly toxic, that after primary treatment, 67% of samples are toxic and 33% are slightly toxic and that after secondary treatment all the samples are non toxic.

Toxicity removal was obtained for both treatment levels when considering *V. fischeri* luminescence inhibition, *P. subcapitata* growth inhibition, and *D. magna* inhibition of mobility. For primary treatment, the mean toxicity removal values were 9% for the bacteria and 11% for the crustacean, and for secondary treatment, the mean toxicity removal values were 99% for the bacteria, 65% for the alga, and 87% for the crustacean. Tyagi et al. [27] found similar values of toxicity removal for *D. magna* after primary and secondary treatment.

5. Conclusions

The ecotoxicity of the samples analyzed shows to be dependent on the WWTP treatment level and the species tested. Microtox, Alga, and Daphnia tests were able to distinguish the two levels of treatment and to assess toxicity removal efficiency. *V. fischeri*, the bacterium used in the Microtox test, was the most sensitive species in WWTP samples evaluation.

These results demonstrated not only that the treatment efficiently reduced wastewater toxicity toward the selected test organisms, but also that the use of an ecotoxicological approach can contribute to the environmental management of the treatment plant. What needs to be stressed is the holistic approach that ecotoxicological assessment is able to perform.

Notation list

The following symbols are used in this article:

- EC_{50}-t = effective concentration (% v/v) responsible for the inhibition in 50% of tested population after the defined exposure period (*t*);
- I_{In} = inhibition at the highest tested concentration for samples before treatment;
- I_{Out} = inhibition at the highest tested concentration for samples after treatment;
- LC_{50}-t = effective concentration (% v/v) responsible for the lethality in 50% of tested population after the defined exposure period (*t*);
- WFD = water framework directive;
- WWTP = wastewater treatment plant.

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