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Is the evaluation of "traditional" physicochemical parameters sufficient to explain the potential toxicity of the treated wastewater at sewage treatment plants?

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Abstract Water scarcity is one of the most important environmental and public health problems of our century. Treated wastewater reuse seems to be the most attractive option for the enhancement of water resources. However, the lack of uniform guidelines at European and/or Mediterranean level leaves room for application of varying guidelines and regulations, usually not based on risk assessment towards humans and the environment. The benefits of complementing the physicochemical evaluation of wastewater with a biological one are demonstrated in the present study using Cyprus, a country with extended water reuse applications, as an example. Four organisms from different trophic levels were used for the biological assessment of the wastewater, namely, *Pseudokirchneriella subcapitata*, *Daphnia magna*, *Artemia salina* and *Vibrio fischeri.* The physicochemical assessment of wastewater based on "traditional" chemical parameters indicated that the quality of the wastewater complies with the limits set by the relevant national guidelines for disposal. The ecotoxicological assessment, however, indicated the presence of toxicity throughout the sampling periods and most importantly an increase of the toxicity of the treated wastewater during summer compared to winter. The resulting poor correlation between the physicochemical and biological assessments demonstrates that the two assessments are necessary and should be performed in parallel in order to be able to obtain concrete results on the overall quality of the treated effluent. Moreover, a hazard classification scheme for wastewater is proposed, which can enable the comparison of the data sets of the various parameters deriving from the biological assessment in a comprehensive way.

Keywords Whole effluent toxicity · WET · Cluster analysis · Ecotoxicity of wastewater · Hazard classification

Introduction

Wastewater reuse is extensively implemented in countries facing water scarcity (Angelakis and Durham 2008, Bixio et al. 2006, Friedler et al. 2006, Grant et al. 2012). The main applications of wastewater reuse include agricultural and landscape irrigation, surface and groundwater recharge, and industrial reuse schemes. However, in some extreme cases, like the drought in the Barcelona region during 2008, treated wastewater was used to increase drinking water resources as well (López-Serna et al. 2012).

The European Council Directive 91/271/EEC concerning urban wastewater treatment states that "treated wastewater shall be reused whenever appropriate". The term appropriate is still legally undefined. The ecotoxicological evaluation of wastewater in Europe has been indirectly recognized nowadays through the implementation of the Water Framework Directive (EU 2000). Most of the countries in which organized wastewater reuse schemes are implemented have developed national or regional regulations/guidelines (Brissaud 2008). However, the standards differ significantly, having the WHO guidelines (1989) or the California's Water Recycling Criteria (1975) as the main pillars of the regulations/guidelines. The need for uniform guidelines for wastewater reuse based on risk assessment, in order to ensure maximum safety levels, has been frequently reported (Angelakis et al. 1999, Brissaud 2008, Huertas et al. 2008).

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The biological assessment of wastewater using standard ecotoxicological bioassays can be considered as complementary to the physicochemical assessment by examining the "traditional" parameters (e.g. pH, BOD, COD and conductivity). The importance of using both chemical analyses and toxicity tests for the quality characterization and control of sewage treatment plant (STP) effluents in the framework of water quality programs is widely accepted nowadays (Martin and Voulvoulis 2009, Power and Boumphrey 2004, Teodorović et al. 2009). The whole effluent toxicity (WET) approach entails various bioassays for acute and chronic toxicity determination, and it was formalized by USEPA (1991).

The basic step is to test the effluents in their initial conditions without any treatment and dilution. Compared to chemical analysis alone, the WET programs have advantages in that they assess the potential biological effects of the chemicals present in wastewater. The WET approach has led to the identification of detrimental effects of xenobiotics such as insecticides, surfactants and treatment polymers in the environment (Grothe et al. 1996, Mount 1998). The WET approach has been included in the legislation of various countries as a tool for assessing the effects of real matrices and environmental protection (Costan et al. 1993, Yi et al. 2009).

In the framework of the present study, wastewater from two STPs in Cyprus was evaluated. Cyprus is a country that faces water scarcity and where wastewater reuse is extensively applied. The objective of this study was to monitor the physicochemical parameters and, in parallel, to perform various ecotoxicological bioassays so as to be able to correlate if possible the findings between the physicochemical and biological assessments. The objective was to identify the most significant parameters influencing the toxicity of treated wastewater. A battery assay of freshwater and saltwater microorganisms was applied. The trophic levels evaluated included a producer (*Pseudokirchneriella subcapitata*), two consumers (*Daphnia magna* and *Artemia salina*) and a decomposer (*Vibrio fischeri*). The innovation of this study lies in the development of a hazard classification approach of wastewater that can be used to understand the trends of toxicity during the course of time.

Methods

Physicochemical assessment

Two sewage treatment plants (STP A and B) with conventional activated sludge systems were monitored in the framework of this study. Both STPs consist of primary, secondary and tertiary treatment steps. The main differences between the treatment processes applied are the following: (1) STP A has a reservoir for storing the secondary-treated effluent before disinfection, (2) STP B has a nitrification/denitrification step, and (3) the flow of the inlet of STP A ranges from 90 to 200 L s^{-1} and that of STP B from 160 to 500 L s^{-1}. Both sewage treatment plants receive only municipal wastewater and are of similar designed capacity (60,000–90,000 p.e.). Composite sampling for 24 h was performed by taking every hour 100 mL of wastewater from three different treatment stages, i.e. raw influent (IN), effluent after the secondary treatment (STE) and effluent after the tertiary treatment (OUT). All samples were transferred immediately to the laboratory in cooling boxes. All experiments were performed in less than 72 h from sampling to avoid any changes in the quality of the sample. The sampling of wastewater for toxicity assessment was performed seasonally (4 samples per year) for four years (2007–2010). Regulated parameters such as pH, temperature, conductivity, BOD_5, COD, TSS, TP, TN and NH_4-N were monitored and determined according to Standard Methods (APHA et al. 1998) at the frequency set by the legislation (Table S1, Supplementary data file).

Ecotoxicological and estrogenic assessment

P. subcapitata The tests were performed using the Algaltoxkit F™ with *P. subcapitata* microalgae according the OECD guidelines (2011). In brief, 1 mL of the initial algal inoculum of 1×10^6 cells mL^{-1} was transferred to 100-mL test sample. Twenty-five mL of each test sample was then transferred to 10-cm long cells and put in a holding tray randomly. A plastic strip was slid in order to leave some opening near the middle of the long cells for gas exchange. The holding tray was incubated with constant uniform sideway illumination of 10,000 lux supplied by a cool white fluorescent lamp. The temperature was kept at 25 °C. Inhibition of the algal growth relative to the control was determined by daily measurement of the OD (optical density) at 670 nm of the algal suspensions in the long cells during the 3 days of exposure to the testing sample. The number of cells was calculated using a reference chart provided by the kit manufacturer.

D. magna The tests were performed using the *Daphtoxkit* F^{TH} *magna* according to the OECD protocol (2004). Ephippia were activated by rinsing with tap water and then were hatched for 72–90 h before testing at 20–22 °C under continuous illumination of 6,000 lux. Two hours before testing, the neonates were fed using a dilution of *Spirulina* microalgae in order to avoid mortality caused by starvation, which could bias the test results. During the subsequent 48 h of test exposure, the organisms were not fed. One hundred twenty neonates were used to perform each independent test. Five daphnids were tested in quadruplicate for each

dilution in specific test plates. The multi-well plates were covered and incubated at 20 °C in darkness. After 24 and 48 h of incubation, the number of dead and/or immobilized neonates was calculated.

A. salina The cysts were collected from a local salt lake (Larnaka) to perform acute toxicity assays. The cysts were collected using sandboxes of different sizes. The cysts were then cleaned and washed using salt water (37 ‰) and left to dry in the sun. Before testing, cysts were hatched in synthetic salt water (37 ‰), and nauplii of less than 24 h were used to perform the tests. The procedure followed (USEPA 2002) is similar to the procedure for testing *Daphnia* nauplii with the following differences: all dilutions were prepared using synthetic salt water, the salinity of the samples was adjusted to 37‰, and the *Artemia* nauplii were not fed before testing. Immobilization of *Artemia* nauplii was calculated at 24 and 48 h.

V. fischeri The bacteria (NRRL B-11177) were tested to obtain percentile bioluminescence inhibition during 5- and 15-min exposures. The Microtox® assay was performed in accordance with the operational procedures from Azur Environmental Ltd. Lyophilized bacteria (approx. one million in one preparation) were reconstituted by adding a reconstitution solution, and then, the suspensions were sequentially diluted and tested at 15 °C. The light transmissions were recorded by a luminometer (Microtox® Model 500 Analyzer, UK). All samples were adjusted at pH 8 and 2 % salinity.

Yeast estrogen assay The recombinant yeast was kindly provided by J.P. Sumpter (Brunel University, Uxbridge, UK). The protocol used for the implementation of the yeast estrogen assay is described in detail elsewhere (Routledge and Sumpter 1996). For the preconcentration of samples, solid phase extraction (SPE) was carried out. SPE cartridges (C18) were conditioned by passing 2×5 mL of deionized H_2O, 2×5 mL of methanol and 2×5 mL deionized H_2O, at a flow of 1 mL min^{-1} using a slight vacuum (J.P. Selecta, Spain). Depending on the sample type, 100 mL of untreated or 1 L treated wastewater was used. The cartridge was then rinsed with 5 mL deionized H_2O and dried to remove excess water. Elution was performed with 2×5 mL of methanol at 1 mL min^{-1}. The extract was evaporated under a gentle nitrogen stream and reconstituted in ethanol. A standard curve was calculated for 17β-estradiol for the range of 1.33–340 ng L^{-1}, and the LOD (1.33 ng L^{-1}) and LOQ (2.65 ng L^{-1}) were calculated.

Quality control and statistical analysis

Positive tests for the aforementioned bioassays were run in parallel using $K_2Cr_2O_7$ (*P. subcapitata*, *D. magna* and *A. salina*), phenol (*V. fischeri*) and 17β-estradiol (YES assay). Each independent experiment was repeated in triplicate. Negative control experiments using only the culture media were also performed. All the quality criteria set by the OECD guidelines concerning the procedure for each bioassay were met.

Data obtained from the physicochemical assessment were analysed using descriptive statistics. The Kolmogorov–Smirnov test (IBM SPSS, v. 19) was applied to recognize deviation from normal distribution. Values that failed to conform to the normal distribution were log-transformed. A K-means cluster analysis was then applied due to the large number of the data set (parameters and cases). By this analysis, the data were assigned into groups (clusters) of similar physicochemical quality in order to further investigate each one of the groups. ANOVA was used for each cluster in order to compare the mean values of each parameter among clusters and to identify which parameters influenced the most the clustering (IBM SPSS v.19).

The ecotoxicological data were evaluated using different approaches. The data obtained from *P. subcapitata* tests were analysed using a point estimate method applying linear interpolation as proposed elsewhere (Norberg-King 1993). The results referring to the *D. magna* and *A. salina* tests were analysed by Probit analysis (IBM SPSS v.19) and those referring to *V. fischeri* by the software provided by the luminometer manufacturer (MicrotoxOmni software). The YES assay results were analysed using descriptive statistics and ANOVA analysis (IBM SPSS v.19). The EC_{50} values calculated for each species were transformed to toxic units (TU), permitting a comparison among species by dividing 100 by the EC_{50} values.

It is important to note that during the presentation of results, abbreviations are used for simplicity as follows: STP A-IN denotes the composite samples taken from the inlet of STP A, STP A-STE refers to the composite samples taken after the secondary treatment at STP A, and STP A-OUT is used to indicate the composite samples taken from the outlet of STP A. The same notation is used for STP B.

Results

Examination of the physicochemical characteristics of the wastewater

A descriptive statistics analysis was applied to organize and classify the data. The range and mean values for the physicochemical parameters are shown in Table 1. The values of COD and BOD_5 parameters decrease gradually during the treatment process, and the final effluents' characteristics generally comply with the quality limits (Table S1). However, the quality limit of the final effluent for conductivity is not met at

Table 1 Characteristics of the samples collected from sewage treatment plants A and B during the various sampling periods (years 2007–2010)

Parameters	Frequency	STP A						STP B					
		Range			Medium value (SD)			Range			Medium value (SD)		
		IN	STE	OUT	IN	STE	OUT	IN	STE	OUT	IN	STE	OUT
pH	d	7.2–7.6	7.2–7.6	7.2–7.8	7.5 (0.1)	7.5 (0.1)	7.5 (0.1)	6.8–8.0	6.8–7.4	6.9–8.0	7.4 (0.2)	7.0 (0.1)	7.4 (0.3)
Temperature (°C)	d	16.4–29.9	13.9–29.5	12.1–29.5	23.3 (3.9)	22.3 (4.8)	22.0 (5.2)	14.0–-27.5	13.7–27.6	2.8–25.4	20.2 (2.8)	21.1 (3.0)	15.4 (5.2)
Conductivity (μS cm^{-1})	d	2,800–5,670	3,150–4,650	3,230–4,520	3,580 (600)	3,800 (400)	3,800 (400)	685–2,240	–	1,037–1,558	1,685 (163)	–	1,358 (66)
BOD_5 (mg L^{-1})	1/w	86–460	13–42	3–27	411 (53)	29 (8)	12 (7)	390–1,120	–	5–17	564 (167)	–	9 (3)
COD (mg L^{-1})	3/w	140–912	11–146	33–118	798 (100)	94 (24)	70 (20)	151–3198	20–273	6–65	660 (299)	49 (28)	32 (11)
TSS (mg L^{-1})	3/w	68–199	11–69	3–69	175 (20)	41 (10)	19 (13)	76–3,240	4–175	1–13	272 (282)	23 (25)	3 (2)
TDS (mg L^{-1})	3/w	–	–	–	–	–	–	800–3,500	–	100–1,900	1,381 (473)	–	518 (262)
TP (mg L^{-1})	2/w	8.3–26.0	7.1–11.1	5.0–11.0	18.9 (2.5)	8.7 (0.8)	8.4 (1.2)	8.9–26.0	–	0.8–19.6	16.9 (4.4)	–	5.2 (3.4)
PO_4-P (mg L^{-1})	2/w	–	–	–	–	–	–	0.3–25.0	0.5–20.9	0.2–16.7	12.0 (4.6)	5.8 (4.2)	4.7 (3.3)
TN (mg L^{-1})	2/w	22–84	22–60	3–25	76 (8)	44 (9)	14 (6)	8–125	–	3–23	74 (18)	–	7 (5)
NH_4-N (mg L^{-1})	d	16–69	14–65	3–19	63 (7)	35 (8)	11 (4)	15–101	0–18	0–16	54 (14)	3 (4)	3 (4)
HS^- (mg L^{-1})	1/m	–	–	–	–	–	–	0.10–15.00	–	–	1.90 (1.96)	–	–
Total Hardness as mg L^{-1} $CaCO_3$	d	–	–	–	–	–	–	–	–	218–393	–	–	295 (39)

d: daily, *w*: week, *m*: month

STP A-OUT. Furthermore, COD, total phosphorous and total nitrogen parameters slightly exceed the limits in STP A-OUT for the specific sampling period. The ammonia concentration was found to be high in both STP A- and STP B-OUT samples, whereas the total nitrogen concentration was found to be in compliance with the limits set for both plants. Ammonia concentration was generally higher in STP A than STP B, as expected, as a nitrification/denitrification process was not applied at STP A. The metal concentrations for STE samples were in the range of 0.065–0.03 for Cd, 0.035–0.229 for Ni, 0.789–1.569 for Zn and 0.06–0.166 for Pb.

The parameters used for the statistical analysis of the STP A- and STP B-IN samples were the temperature (°C), pH, TP (mg L^{-1}), TN (mg L^{-1}), BOD_5 (mg L^{-1}), COD (mg L^{-1}), TSS (mg L^{-1}) and conductivity (mS cm^{-1}). The original data demonstrated values of skewness from (−0.1400) to (+2.424) and kurtosis from (−1.2) to (+6.956), indicating that some parameters were not close to the normal distribution. The parameters were log-transformed, reducing the skewness to the range of (−1.585) to (+1.818) and the kurtosis to the range of (−1.484) to (+4.004), indicating that the data followed normal or close to normal distribution. The data set for ammonia concentration (mg L^{-1}) was not included in the cluster analysis because its kurtosis was still high even after the log-transformation, indicating deviation from the normal distribution.

The visualization of the data through boxplots indicates that the quality of STP A- and STP B-IN samples were of different quality (data not shown). In order to examine this hypothesis, K-means cluster analysis was applied due to the large number of cases. The number of clusters was set to two to four. The maximum iterations were set to 20, and the missing values were excluded pairwise. The data set was best split into two different clusters. When a greater number of clusters were investigated, the STP B cases were divided among the clusters, whereas the cases from STP A remained in one cluster. The final cluster centres are presented in Table 2. One hundred forty two (142) cases were grouped in cluster 1 and 243 cases in cluster 2. Of the cases of STP B, 100 % (142) were grouped in cluster 1, and 97.93 % (241) of the cases of STP A were grouped in cluster 2. From the *F*-values, it can be concluded that the parameters which contribute the most to the formation of the clusters are conductivity, COD and pH of the IN samples (Table S2, Supplementary data file).

The descriptive statistics of the STP A- and STP B-STE samples revealed a normal distribution of the data since the skewness ranged from (−0.881) to (+1.349) and the kurtosis from (−1.121) to (+0.803). The temperature, pH, TP, COD, TSS and nitrate concentrations were evaluated since these were the parameters monitored at both plants. The data sets were split into two clusters; 98.33 % (235) cases of STP A were grouped in cluster 1, and 100 % (98) of STP B were grouped in cluster 2, indicating that the samples from the two STE sample groups could not be considered of the same quality (Table 3). The values of all the parameters for the STP B were lower than the ones of STP A. The ANOVA table helped identify the fact that the pH was the most critical parameter in splitting the clusters, followed by the TSS, the nitrate and the COD concentrations (Table S3, Supplementary data file).

The cluster analysis split the data sets into two clusters. Cluster 1 included all the cases of STP B-OUT (146) and cluster 2 all the cases of STP A-OUT (240) (Table 4). The most influential parameters were found to be the conductivity and the nitrate concentration, followed by the COD and the TSS. The values of all the parameters of the STP B-OUT samples were found to be lower than those of the STP A-OUT samples. Regarding STP B, the conductivity and the nitrate concentration were less than 1 standard deviation from the distribution mean, followed by the COD and the TSS that were close to 1 standard deviation from the distribution mean. Regarding STP A, the conductivity, nitrate, total nitrogen, and total phosphorus concentrations deviated 0.5–1 standard deviation from the mean (Table S4, Supplementary data file).

Table 2 Final cluster centers of the *Z*-values of the log-transformed values for the inlet of the sewage treatment plants A and B (STP A and B-IN samples)

	Cluster	
	1	2
Zscore: log[Temperature]	−0.54133	0.31093
Zscore: log[pH]	−0.73929	0.42996
Zscore: log[TP]	−0.59677	0.28199
Zscore: log[TN]	−0.41560	0.17936
Zscore: log[BOD]	1.05078	−0.42889
Zscore: log[COD]	−1.01096	0.52322
Zscore: log[TSS]	0.43301	−0.25561
Zscore: log[Conductivity]	−1.22873	0.70068

Table 3 Final cluster centers of the *Z*-values for the secondary -treated effluent of the sewage treatment plants I and III (STP A and B-STE samples)

	Cluster	
	1	2
Zscore[Temperature]	0.07902	−0.18206
Zscore[pH]	0.60289	−1.42725
Zscore[TP]	0.60823	−0.33558
Zscore[COD]	0.44747	−1.14571
Zscore[TSS]	0.50529	−1.19673
Zscore[NO_3]	1.18703	−0.65353

Table 4 Final cluster centers of the *Z*-values for the outlet of the sewage treatment plants I and III (STP A and B-OUT samples)

	Cluster	
	1	2
Zscore[Temperature]	−0.66837	0.40659
Zscore[pH]	−0.31169	0.19785
Zscore[TP]	−0.54106	0.72705
Zscore[TN]	−0.53015	0.69958
Zscore[NO_3]	−1.24165	0.74053
Zscore[BOD_5]	−0.39607	0.16635
Zscore[COD]	−0.98810	0.53557
Zscore[TSS]	−0.86706	0.52409
Zscore[Conductivity]	−1.25730	0.74390

To summarize, it may be suggested that the cluster analysis revealed a significant differentiation of the quality of the samples between the IN, STE and OUT samples for STP A and B. Regarding the STP A- and B-STE and -OUT samples, the values referring to STP B were found to deviate with negative standard deviations and those referring to STP A with positive standard deviations, for all the parameters evaluated. This trend was not so obvious for the STP A- and B-IN samples, where BOD_5 and the TSS concentrations were higher in STP B than in STP A.

The clustering in different groups indicated that wastewater of different quality enters and leaves each STP, a fact that was not considered initially (since both plants serve similar population equivalents characterized also by similar habits and practices). The results from the effects' assessment of the wastewater differed for each STP. No uniform behaviour was observed concerning the quality of the flows at the various treatment stages (e.g. STP A-STE and STP B-STE) even though the treatment process applied at each plan is quite the same.

Hazard classification of wastewaters using a battery assay

As already mentioned, the EC_{50} and the TU values were calculated for each sampling period for each of the bioassays applied. In order to compare these values, a hazard classification was developed. In the context of this study, "hazard" is defined as a source of potential danger to the environment and hazard assessment as the evaluation of inherent properties of a sample to cause harm (Chapman 2000). This should be differentiated from risk assessment, which encloses the probability that a hazard will occur. The WET tests contribute to the identification of the hazard, an action that constitutes the first step in ecological risk assessment. The toxicity was ranked as suggested by Persoone et al. (2003). Samples are categorized in five classes (I–V) according to the highest toxic response shown by at least one of the tests applied. A weight score from 0 to 4 was then calculated for each hazard class to indicate the quantitative importance of the toxicity in the class.

All IN samples were found to be severely toxic (100 % effect) to the freshwater microorganisms even when only a 5 % concentration of sample was evaluated, and consequently were ranked as class V (TU > 100), as presented in Fig. 1. The calculation of an EC_{50} value was possible for *A. salina* and *V. fischeri* through sample dilution. The IN samples had TU values for *A. salina* after 24-h exposure

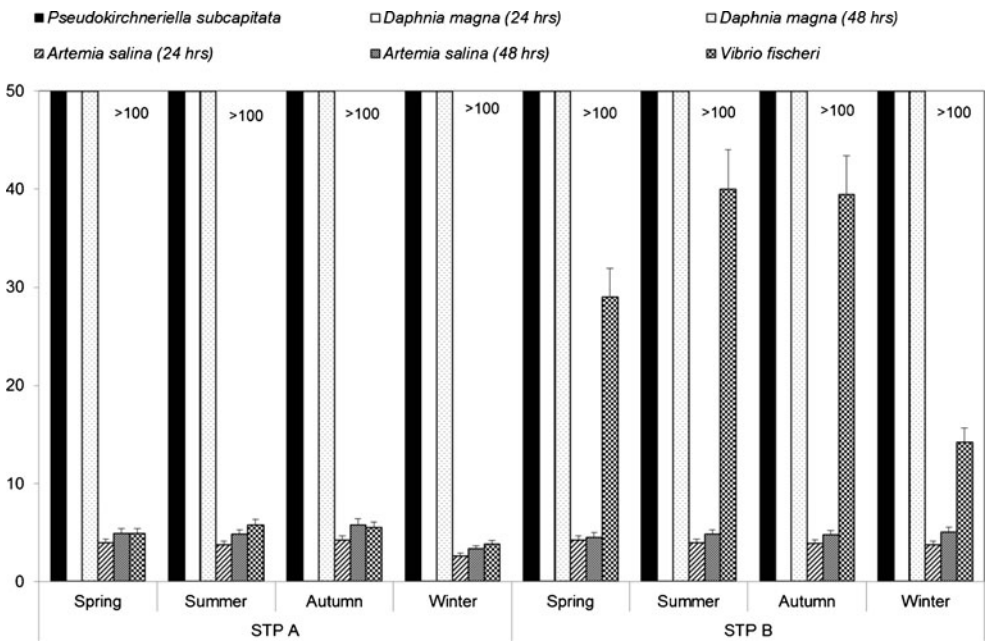

Fig. 1 Toxic units (TUs) for the inlet (IN) of sewage treatment plants A (STP A) and B (STP B). Average values plus standard deviation for a total of nine samplings per season are presented

time ranging from 2.6 to 4.2 and from 3.7 to 4.2 for STP A and B, respectively. The TU values for the 48-h exposure were ranging from 3.3 to 5.8 and from 4.5 to 5.0 in STP A and B, respectively. The TU values for *V. fischeri* were 3.8–5.8 and 14.2–40.0 for STP A and B, respectively. The ranking for the marine microorganisms was III–IV.

The STE samples of STP A showed a seasonal behaviour, with the toxicity of freshwater microorganisms during autumn and winter being lower than the one during spring and summer (the latter were found to be severely high, TU > 100), as shown in Fig. 2. The toxicity pattern was similar for the freshwater species, whereas *A. salina* was not affected by the STP A-STE samples at any time when exposed both for 24 and 48 h. *V. fischeri* was affected in a seasonal way, with summer and autumn samples being more toxic to the species. The STP A-STE samples were considered toxic for *P. subcapitata* in all tests performed, but the toxicity intensity followed a seasonal behaviour (i.e. spring and summer, > 100 TU; autumn and winter, 1.3–3.2 TU). The toxicity of the STP B-STE samples demonstrated a clear trend. The toxicity decreased from summer to winter samplings (spring and summer, >100 TU; autumn and winter, 2.0–2.1 TU). *D. magna* was affected in a similar way, with samples of spring and summer (STP A and STP B, TU > 100) being more toxic than the ones of autumn and winter (STP A, TU of 0.4–1.0; STP B, TU of 1–6.2) for both STPs. *A. salina* was not affected by the STE samples at any time. *V. fischeri* was affected in a lesser extent by the spring and summer samples demonstrating some toxicity (STP A TU of 1.4–1.5 and STP B TU of 1.4–1.8), whereas autumn and winter samples were found to be non-toxic (STP A and STP B, TU < 0.1). In summary, the STE samples were considered toxic to *P. subcapitata* and *D. magna* and to a lesser extent to *V. fischeri*, whereas *A. salina* was not affected at all. The toxicity intensity showed a variation throughout the year and between the STPs. The samples taken from both secondary treatment stages during summer were found to be severely toxic.

The toxicity evaluation of the STP-OUT samples is presented in Fig. 3. *P. subcapitata* was again the most affected species by the samples of both STPs. STP A-OUT was more toxic during spring and summer, with TU values ranging from 4.9 to >100, whereas during autumn and winter, TU values dropped to the range of 0.4–0.8. STP B-OUT samples were more toxic during the summer and autumn period, with TU values being greater than 100; during spring, the toxicity was in the range of 5 TU, and only during winter, the toxicity was less than 0.4. *D. magna* showed an increased toxicity towards summer samples of STP A and summer and autumn samples of STP B (TU > 100). Autumn and winter samples of STP A had a lower toxicity ranging between 0.3 and 1.0, whereas spring samples were found to be non-toxic (TU < 0.05). Spring and winter samples of STP B were found to be toxic, with TU values of 1.8–2.3. *A. salina* was not affected by OUT samples of either STP (TU < 0.1). *V. fischeri* demonstrated toxicity during summer samplings of STP A (TU values of 0.9) and spring to autumn samplings of STP B (TU values of 0.4–1.8). As a summary, the freshwater microorganisms were negatively affected by all samples tested. The toxicity to *P. subcapitata* was found to be of the same level as the toxicity to *D. magna* (48 h). On the contrary, the marine microorganisms were affected to a lesser extent since *A. salina* was not affected at all and *V. fischeri* demonstrated lower toxicity than the freshwater microorganisms.

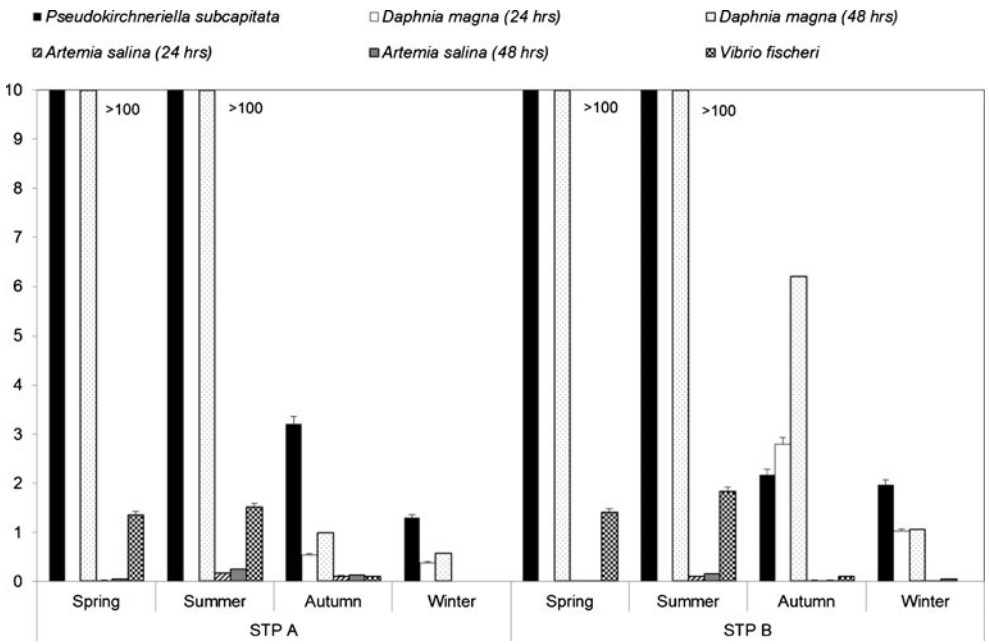

Fig. 2 Toxic units (TUs) for the secondary-treated effluent (STE) of sewage treatment plants A (STP A) and B (STP B). Average values plus standard deviation for a total of nine samplings per season are presented

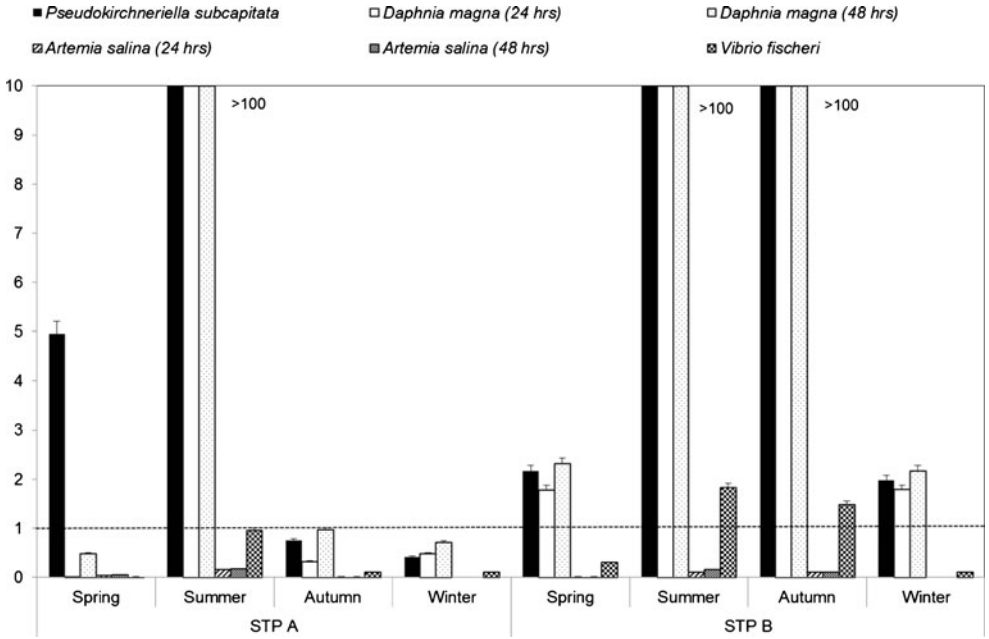

Fig. 3 Toxic units (TUs) for the tertiary-treated effluent (OUT) of sewage treatment plants A (STP A) and B (STP B). Average values plus standard deviation for a total of nine samplings per season are presented

The estrogenicity tests with regard to all STP-OUT samples had a corrected absorbance lower than 1 corresponding to 17β-estradiol equivalent concentration lower than 2.65 ng L^{-1}. However, a colour response was observed in all the samples tested, indicating that estrogenic compounds were present in the range of the LOD–LOQ (1.33–2.56 ng L^{-1}). The STP-IN samples had a maximum 17β-estradiol equivalent of 25.6 ng L^{-1}, demonstrating that the IN samples contained estrogenic equivalent compounds that were removed during treatment.

In order to compare the toxicity assessed among the IN, STE and OUT samples, the hazard classification scheme previously described was applied. As a result, Table 5 was developed to summarize the results for the assessment of effects. As the sensitivity to freshwater and marine microorganisms was quite different, a separate hazard classification for the freshwater (F) and the marine (M) microorganisms was developed.

The STP samples receive severely toxic IN samples to both freshwater and marine microorganisms. During secondary and tertiary treatments, this toxicity is reduced. Based on the methodology applied though, the reduction is not found to be substantial as hazard percentages of 50–55 % for the STE samples and 30–38 % for the OUT samples were calculated when all bioassays were taken into account simultaneously. The separate hazard scoring revealed a greater hazard for freshwater species, ranging between 68 and 71 % in STE samples and 57 and 71 % in OUT samples. On the other hand, the hazard for the marine species was very low: 0 % for the STE samples and 16 % for the OUT samples.

Correlation of physicochemical and biological parameters

During the samplings for the toxicity evaluation, the physicochemical parameters of the IN, STE and OUT samples were monitored. The values of the physicochemical parameters were compared to the mean values (Table 1) to investigate whether extreme conditions were present during the samplings for the toxicity evaluation. All the values were in the range of the mean values ± one standard deviation for both STPs investigated, indicating no statistically significant deviation from the mean values (data not shown).

Pearson's correlation coefficients were calculated for the samples of low toxicity, in which a normal distribution was observed ($p<0.05$). The toxicity to *D. magna* at 24 h could be correlated negatively to the conductivity (−0.752, $p<0.05$) and to the nitrate (−0.652, $p<0.05$) and phosphorus (−0.863,

Table 5 Hazard scoring of sewage treatment plants wastewater

	Inlet (%)			Secondary-treated wastewater (%)			Tertiary-treated wastewater (%)		
	T	F	M	T	F	M	T	F	M
STP A	100	100	100	50	71	0	30	71	16
STP B	100	100	100	55	68	0	38	57	16

T: total, *F*: freshwater organisms, *M*: marine organisms

$p<0.01$) concentrations. The same trend was observed when the toxicity to *D. magna* at 48 h was examined. Furthermore, a negative correlation to the COD was calculated (−0.536, $p<0.05$).

The Spearman rank-order correlation, a more conservative approach, was also applied to the data sets in order to overcome the *non-normality* of some parameters (e.g. *D. magna* toxicity, ammonia). The toxicity to *D. magna* at 24 h was negatively correlated with the nitrate (−0.583) and phosphorus (−0.633) concentrations ($p<0.05$). When the toxicity to *D. magna* at 48 h was examined, only a negative correlation to phosphorus (−0.686) was present ($p<0.01$). The toxicity to *A. salina* at 24 h was positively correlated to the conductivity (0.9, $p<0.05$). The toxicity to *A. salina* at 48 h was positively correlated to the conductivity (1, $p<0.01$) and to the ammonia concentration (0.812, $p<0.05$). The toxicity to *P. subcapitata* was positively correlated to the nitrate concentration (0.824, $p<0.05$).

Discussion

The main findings of this work concerning the quality of the wastewater examined can be summarized as follows: (1) the concentrations of the conventional pollution parameters regulated by the legislation were not always found to meet the limits in the OUT samples, (2) between STP A and B, the quality of the IN, STE and OUT samples was found to be dissimilar, and (3) severe toxicity was present in some of the OUT samples.

The WET approach was implemented for identifying and delineating treated effluents of concern by hazard ranking. This approach is in accordance with the EU Water Framework Directive, which refers to the use of algae, *D. magna* and fish toxicity as food chain indicators for the monitoring of surface water quality. *A. salina* and *V. fischeri* were also evaluated as indicators of marine environments as suggested by Rizzo et al. (2009a). Seasonal and between species toxicity variations were observed, suggesting that the frequency of their evaluation according to the discharge permitting law is insufficient. Regarding the seasonal variation, the findings are in accordance with previous studies performed elsewhere, in which higher toxicity was observed during the summer period (dry seasons), potentially due to less dilution and/or different composition of the wastewater. The variation of toxicity among species may imply a complex composition of the matrix tested, in which some pollutants are more toxic to one species than to another. For instance, in another study, algae were found to be more sensitive to herbicides and fungicides, whereas daphnids to pesticides (Ra et al. 2007).

Although it has been clearly proved that *P. subcapitata* is more sensitive than *D. magna* to chemically treated wastewater samples (Naddeo et al. 2009, Rizzo et al. 2009a, Rizzo et al. 2009b), the discrepancy observed between the results of *D. magna* and *P. subcapitata* tests exposed to the STP samples can be attributed to the wastewater characteristics obviously and in particular to the presence of nutrients which can increase after nitrification in the effluent (Mendonça et al. 2009). *V. fischeri* and *A. salina* were found to be less sensitive. Their use though should not be excluded since *V. fischeri* bioassay is considered to be a fast method providing information for changes in wastewater quality characteristics (Farré et al. 2002, Farré et al. 2006, Hernando et al. 2006), and *A. salina* species could be used for assessing the quality of IN samples.

Physicochemical parameters are, of course, the fundamental criteria to define the use of effluent after treatment. However, a recent study of Mendonça et al. (2009) concluded that the chemical evaluation of samples does not always correspond to the effects observed towards the tested organisms. The reverse situation can also be the case, in which some samples induce effects towards the tested organisms but no indication of potential hazards can be derived from the chemical evaluation. This underlines the significance of complementing the chemical evaluation with a biological one to maximize environmental protection. To some extent, these findings were observed in the present study, in which the correlations found between some species and chemical parameters were not able to explain the toxicity present, implying a more complex interaction scheme. For example, the toxicity to *D. magna* was negatively correlated with COD, conductivity, nitrate and phosphorus concentrations, probably indicating a nutrient deficiency. In another study, the toxicity was correlated with increased hardness, salinity and total dissolved solids, related to adverse effects on daphnids (Chapman et al. 2000, Grothe et al. 1996). Ammonia and conductivity are considered as "confounding factors" that may interfere with the biological effects of micropollutants according to Postma et al. (2002). In that study, the highest range of concentrations for which no significant effects were observed was 13–60 mg L^{-1} for ammonia and with values lower than 650 μS cm^{-1} for conductivity. In the present study, conductivity for both STPs and ammonia for STP B can be considered as confounding factors as well. The EC_{20} of ammonia reported for *D. magna* ranges from 7.37 to 21.7 mg L^{-1} (USEPA 1999a). In the study by Boillot et al. (2008), free chlorine, 2-propanol, copper and ammonia were found to be among the most important causes of toxicity of hospital wastewater in France. Conductivity greater than 1,000 μS cm^{-1} may be an indication of higher total dissolved solids (TDS) (Torres-Guzmán et al. 2010). The relationship between conductivity, TDS and metallic ions, and their contribution to the overall toxicity is the main reason for the limit of 3,000 μS cm^{-1} set by the USEPA (1999b) for the conductivity.

Exceedances on conductivity of wastewater have also been reported in previous studies (Bakopoulou et al. 2011), which were not correlated, however, to toxicity increases. Moreover, when industrial wastewater was evaluated, a direct relationship between the COD of the industrial wastewater and toxicity was shown (Cooman et al. 2003). Hence, a more complex situation seems to prevail that is not easily to be understood by monitoring the "traditional" chemical parameters alone.

Much attention has been given to natural and synthetic steroidal hormones, which are shown to induce biological effects on some organisms at part-per-trillion concentration (Parrott and Blunt 2005). In the present study, low estrogenicity was detected at STP IN and OUT samples. The OUT samples' estrogenic compound concentrations were not quantified due to their low concentrations, which is similar, however, with the ones found in other studies (Schilirò et al. 2009). This is in accordance with an earlier study by Holbrook et al. (2002) in which 51–67 % of estrogenic compounds were removed during sewage treatment. Chlorination, as a final step at the treatment plants, has been found to reduce the amount of estrogenic compounds. However, in some cases, this was coupled with an increase of the toxicity of effluents (Schilirò et al. 2009). It is not therefore a win-win situation, and as recently documented, the decrease of estrogenicity can cause an increase of the antiestrogenicity during chlorination processes (Wu et al. 2009). The assay applied could not identify the specific compounds responsible for the estrogenicity of the samples; however, most likely, the majority of activity is caused by the presence of 17β-estradiol, the synthetic 17α-ethinylestradiol and its metabolites estrone and estriol (Johnson and Williams 2004).

For future studies, innovative approaches that are a combination of in vitro bioassays for the determination of cytotoxic and genotoxic potential of wastewater could be implemented in order to determine the presence and potential impacts of pollutants in wastewater (Žegura et al. 2009). Moreover, methodologies for assessing chronic toxicity, persistence and bioaccumulation need further development (ECETOC 2004). These are mandatory in cases of countries, like Cyprus, facing water scarcity, demanding alternative and safe water resources.

The application of multivariate techniques, such as the cluster analysis, facilitated the interpretation of complicated multi-parametric data with seasonal and spatial variations. The most critical parameters affecting the quality of the wastewater were identified and correlated to other parameters to understand their effects. A substantial variability of the quality of the effluents was observed for the chemical parameters regulated. The presence of various micropollutants and other non-regulated parameters may also vary on a daily basis especially during the touristic season, which is quite extended in Cyprus. The performance of the STPs was also found to vary during the sampling periods, adding to the necessity of carrying out systematic toxicity assays.

Seasonal or monthly monitoring of chemical and toxicological assessment should be considered for all STPs in which reuse practices are in place. Furthermore, stricter and more integrated reuse and recharge guidelines should be considered, including a greater range of parameters. Furthermore, the treated wastewater quantities that will be either disposed of or reused should be also considered. Since the chemical parameters' data sets demonstrated a different quality of wastewater for the two STPs, the requirements and limits should be differentiated, as well as the reuse practices implemented. For instance, the volume to be used for recharge purposes may be set according to the environmental conditions of the water body to be affected by this action. An example of such an approach is provided by Asano and Cotruvo (2004), presenting the criteria for groundwater recharge in California. Among others, they state that wastewater used for recharge should meet all drinking water maximum concentration levels and the maximum volume of the wastewater should not exceed 50 % of the total volume of the receiving water body. In fact, the trend to overcome variability and type II errors (false negative) is to increase the number of tests and reduce the number of concentrations evaluated in each test (i.e. the influent or the whole effluent sample with no dilution). The initial required monitoring frequency is by quarters for USA and Canada. However, most water specialists acknowledge that more frequent monitoring would improve effluent representativeness (Chapman 2000).

In order to fully comprehend the ecological effects of releasing treated wastewater to the environment though, further stages are needed such as an exposure and effect characterization and assessment, and risk characterization. Tools such as long-term laboratory or field bioassays and toxicity identification evaluation should be considered for further studies. For instance, in quantitative and probabilistic studies, the duration and magnitude of actual exposures of resident communities to effluent could be implemented (Chapman 2000). Food chain effects may also need to be accounted since they represent a separate exposure route. Irrigation should be included as an additional exposure route for chemicals in terrestrial ecosystems, in order to assess the potential risks derived. The behaviour of wastewater should be studied since many regulators and scientists recognize that concentration–response patterns will not always follow the traditional pattern, especially when complex mixtures are examined. A hormetic curve or inverted U-shaped curves are some examples of these deviations. In some cases, the release of treated effluent may not be detrimental if a hormetic curve can best fit the dose–response behaviour of the treated effluents, which means that low concentrations may be beneficial. An inverted U shape, in which toxicity is observed at both ends of the U,

ascribed to endocrine disrupting chemicals, may also be a non-traditional dose–response behaviour (Chapman 2000) that requires more investigation. Chemical analyses, biological assessments of receiving waters and toxicity identification evaluations would certainly complement the weight-of-evidence approach for decision making.

Countries facing water scarcity problems are already considering implementing quaternary treatment by the end of the decade in order to improve wastewater quality and ensure its safe reuse (Brenner 2012). The need to develop advanced treatment processes, such as ozonation (Muñoz et al. 2009), ultrasonic irradiation (Naddeo et al. 2010) and photocatalytic oxidation (Rizzo et al. 2009a) just to name a few, in order to hinder the release of micropollutants included in the effluent organic matter is apparent. Their evaluation, however, via toxicity bioassays is crucial since the transformation products generated during the processes may also exhibit toxicity (Schilirò et al. 2009).

Conclusions

This work was designed to test a number of bioassays, the objective being the application of the whole effluent toxicity approach to sewage treatment plant effluents reused for irrigation and water bodies' replenishment. Four species from different trophic levels (decomposer, producers and consumers) and environmental habitats (fresh and marine water) were exposed to the samples collected from different process steps at various dilutions. Estrogenicity screening was also performed. Variation regarding seasons and species was observed. Increased toxicity especially during the summer season was identified, whereas in general, low estrogenicity was observed. A toxicity hazard classification procedure led to the identification of higher hazard to freshwater species than to marine species. The reuse of treated effluents is mainly performed during the summer period when the demand for reclaimed wastewater is higher due to the absence of rainfall. The reuse of the treated wastewater may therefore enclose unknown risks due its higher toxicity, and this is something that should be further investigated.

Variations in chemical parameter data sets and in the sewage treatment plant process efficiency were also observed. However, the toxicity could not be adequately correlated to the traditional parameters evaluated, highlighting the complexity of real matrices. However, even if the toxicity findings could not be completely explained, some chemical parameters such as conductivity and ammonia concentrations were found to be of concern in relation to the toxicity, and they should be evaluated in future relevant studies. Moreover, the toxicity should be further investigated by implementing a methodology aiming at the toxicity identification evaluation and monitoring of non-regulated parameters, such as contaminants of emerging concern.

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