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Use of ecotoxicology tools within the environmental footprint evaluation protocols: the case of wastewater treatment plants

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ABSTRACT

This work focuses on the application of the protocol for the assessment of the environmental footprint of products and organisations, performed accordingly with the prescriptions of the European Commission Recommendation 2013/179/EU. Although scientific and technical literature already reports some example cases carried out in several industrial fields, this methodology has not been run yet for the evaluation of the environmental performances of a wastewater treatment plant. The research has been carried out on a real scale plant, by acquiring, in parallel, operation data, chemical, physical and biological parameters throughout a 2-week campaign. Effluent toxicity towards the aquatic ecosystem was measured, by adopting a multi-tiered approach, the test organisms being crustaceans, bacteria and algae, respectively. The protocol for the evaluation of the environmental footprint was followed by considering as input data either the results of the chemical, physical and biological analyses or those deriving from the bioassays execution. The findings clearly illustrate the invaluable importance of bioassays in the process of evaluation of the environmental impact of any work, together with the need of combining different tests based on specific endpoints and involving organisms playing different trophic roles.

Keywords: Bioassays; *Daphnia magna*; Freshwater; PEF/OEF; *Raphidocelis subcapitata*; *Vibrio fischeri*

1. Introduction

Life cycle assessment (LCA) is a standardised and sophisticated tool that allows to quantify and compare the potential impacts, associated to the consumption of resources and emissions of pollutants in the environment, occurring along the life cycle of products, services or processes (from the extraction of raw materials to final disposal). Since the nineties, several LCA studies in the field of wastewater treatment have been conducted, for example, in order to assess the environmental impacts of WWTPs [1,2], compare the environmental performance of different treatment options [3], conventional and non-conventional technologies [4–7], control strategies [8,9] or water and sewage management options [10–15]. The application of LCA to WWTPs has been widely described in several literature reviews [8,15,16].

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Although the LCA protocol was defined by ISO 14040 and 14044 standards [17,18], a range of several methodological approaches to develop a life cycle impact assessment [19], has been explored yielding quite different conclusions [20,21]. The Recommendation 2013/179/EU [22] establishes a new protocol for an overall assessment of the environmental footprint of products (PEF) and organisations (OEF), by harmonizing and overcoming the previous conventional tools. Even though several PEF/OEF studies focus on products and organisations in different industrial sectors [20,23], there is still lack of knowledge and experience regarding the application to WWTPs.

In life-cycle-based methodologies, the assessment of the effects on the different impact categories is based on mass flows of pollutant discharged in the environment or resources used. Yet, the evaluation of the impact of the WWTP effluents cannot be solely based on the quantification of emission loads with a "compounds-based" methodology. Indeed, the identification and quantification of all those micropollutants and their transformation products, which may be of environmental concern is quite difficult [24,25]. In addition, the analytical methods might not be sufficiently sensitive to measure all the components [26]. Moreover, the chemical characterisation alone cannot take into account possible antagonistic, subtractive, additive or synergistic effects of a mixture of different pollutants [27]. Several studies suggest that the limitations of the chemical "single substances" approach can be overcome by carrying out multi-tiered biological assays, which can directly and more accurately measure the impact of a stream [28–31].

In this paper, the results of a study basing on a previous work by Pedrazzani et al. [32] are presented. In particular, the environmental footprint of a real WWTP has been evaluated by applying the PEF/OEF protocols. Furthermore, an innovative procedure for using the results of ecotoxicity tests for the evaluation of the "freshwater ecotoxicity" impact category is proposed and discussed.

2. Materials and methods

In this section, the following methodology and the data used within the environmental footprint assessment are described, as well as the WWTP selected as case study. Then, the innovative procedure for the evaluation of the freshwater ecotoxicity based on results of bioassays is explained.

2.1. Case study

The studied WWTP is a conventional activated sludge plant (design size 370,000 p.e.) treating mainly domestic wastewater and located in Northern Italy. The water line includes preliminary treatment (fine screening, grit and oil removal), primary settling (10,400 m^3, three parallel lines), pre-denitrification (7,200 m^3, five parallel lines) and oxidation–nitrification (16,600 m^3, five parallel lines), final sedimentation (26,100 m^3, six parallel tanks). The sludge treatment line consists of dynamic thickening, anaerobic digestion, biogas production used to produce on site electricity, and mechanical dewatering.

The main operational data (typical values referred to the year 2016–2017) are the following: wastewater flow rate = 75,874 m^3/d; influent characteristics: 545 mg COD/L, 297 mg BOD_5/L, 374 mg TSS/L, 49 mg TKN/L, 7.5 mg P_{TOT}/L. Effluent characteristics: 24 mg COD/L, 5.3 mg BOD_5/L, 8.0 mg TSS/L, 3.9 mg TKN/L, 1.5 mg NH_4^+–N/L, 11.2 mg NO_3^-–N/L, 0.2 mg NO_2^-–N/L, 1.6 mg P_{TOT}/L. total suspended solids concentration in the biological reactors = 3.9 g TSS/L.

2.2. OEF/PEF protocols

The environmental footprint (EF) assessment of the real WWTP, was performed using the software SimaPro (version 8.5.2), in which the impact categories and impact assessment models were selected in order to ensure the compliance with the Recommendation 2013/179/EU. An additional impact category, which is not included in the default list, provided in the PEF guide, has been selected: the quantification of life cycle energy consumption along the supply chain (GER). Impact categories, as well as impact assessment models adopted in this study are reported in Table 1.

The system boundaries (Fig. 1) include all the up-stream and core processes involved from wastewater collection to the treatment and the discharge of the effluent into the environment together with the management of waste generated throughout the operation of the plant.

Raw materials (chemicals and other auxiliaries) production and consumption, energy on-site and off-site generation and use, direct emission in air, water and soil, waste production and treatment have been modelled.

Regarding the sludge, only the impacts related to the transportation to composting facility were considered, while the composting process and the application of the compost to agricultural soil were excluded due to insufficient data pertaining the characteristic of the compost produced and the impact related to the emissions to soil. The environmental impacts of construction (both civil works and equipment), dismantling and end-of-life options (e.g., total recycling for steel and iron parts, plastics and rubber incineration and landfill disposal without concrete recycling) of all the components of the sewer system and the WWTP were also accounted in the analysis. The sewer system lifetime (661 km of pipes) and the treatment plant were set equal to 100 and 30 years, respectively. The selected functional unit (FU) is 1 m^3 of treated wastewater.

In order to compare the magnitude of the contributions of the different EF impact categories with the pressure exerted by an average European citizen, a normalisation was carried out: the normalisation factors, defined by Benini et al. [33] and reported in Table 2, were compared with the annual impact generated by the treatment of 73 m^3 (corresponding to a daily average consumption of 200 L of water per person) of wastewater by WWTP selected as case study.

2.3. Data set for the application of the standard protocols

The calculation of the 16 environmental footprint indicators, defined by the PEF/OEF protocol, was based on mass flows of pollutants discharged into the environment. Primary data (measured directly in the field) consists of effluent characteristics (in terms of flow rate, organic and inorganic compounds concentration), amounts of chemicals (sodium hypochlorite, sulfuric acid, sodium hydroxide,

Table 1
Details of the environmental footprint impact categories considered in this study: units of measurement (impact category indicator) and impact assessment models

EF impact category	Impact category indicator	EF impact assessment model
Gross energy requirement (GER)	MJ	Cumulative energy demand
Global warming potential (GWP)	$kgCO_{2,eq}$	Bern model – global warming potentials (GWP) over a 100-year time horizon
Ozone depletion (OD)	$kgCFC\text{-}11_{eq}$	EDIP model based on the ODPs of the World Meteorological Organization (WMO) over an infinite time horizon
Photochemical ozone formation (POF)	kgNMVOC	LOTOS-EUROS model
Acidification (A)	$molH^{+}_{eq}$	Accumulated exceedance model
Terrestrial eutrophication (TE)	$molN_{eq}$	Accumulated exceedance model
Marine eutrophication (ME)	kgN_{eq}	EUTREND model
Freshwater eutrophication (FE)	kgP_{eq}	EUTREND model
Human toxicity – cancer effects (HT-CE)	CTUh	USEtox model
Human toxicity – non cancer effects (HT-NCE)	CTUh	USEtox model
Freshwater ecotoxicity (FET)	CTU_{e}	USEtox model
Particulate matter (PM)	$kgPM2.5_{eq}$	RiskPoll model
Ionizing radiation (IR)	$kg\ U^{235}_{eq}$	Human health effect model
Land use (LU)	$kgC_{deficit}$	Soil organic matter (SOM) model
Water resource depletion (WD)	$m^{3}_{water,eq}$	Swiss ecoscarcity model
Mineral, fossil and renewable resource depletion (MD)	$kgSB_{eq}$	CML2002 model

Source: Adapted from the study by European Commission [22].

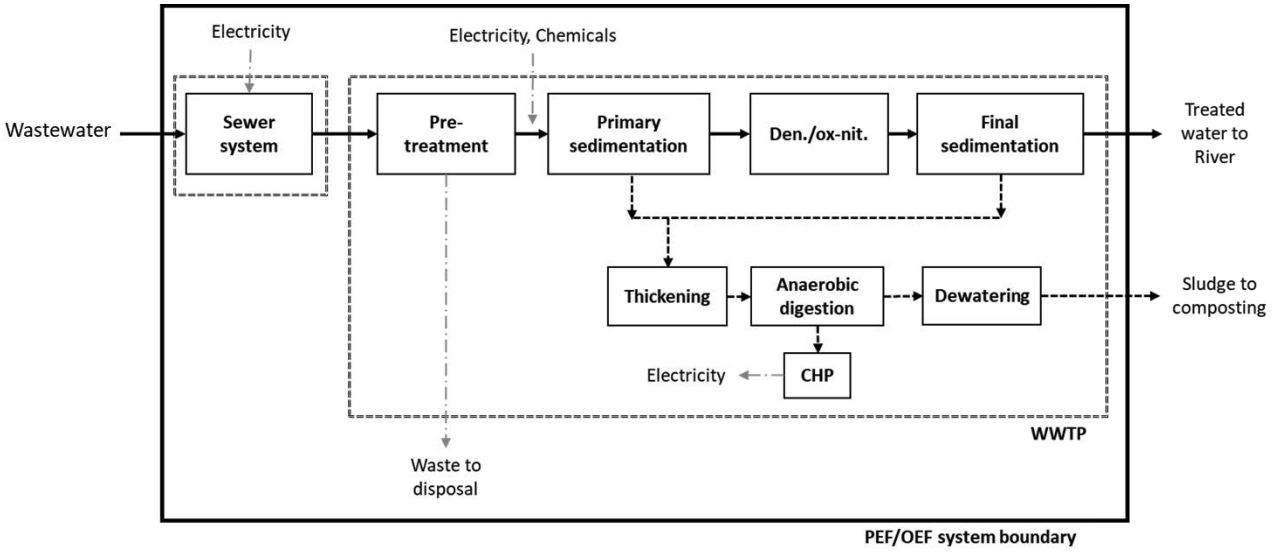

Fig. 1. System boundaries considered in this study for the PEF/OEF analysis.

polyelectrolytes) and energy used (purchased and produced from the CHP unit), as well as produced waste (solid residues, sand, greases, sewage sludge, household-like municipal solid waste). These data derived from plant routine monitoring of the year 2017 and average values were taken for modelling. Table 3 reports the parameters used for the characterisation of the effluent, while Table 4 shows the results of the chemical analyses of metals and semimetals, carried out on a composite sample of 15 d (each daily sample being flow-dependent and covering 24 h). It is worth noting that all the measured polynuclear aromatic hydrocarbons, chlorinated insecticides and herbicides resulted below their limits of detection, a complete list of the results of the chemical analyses is reported in supplementary material,

Table 2
Normalisation factors for EU-27 [33]

Impact category	Normalisation factor per person
Global warming potential (GWP), $kgCO_{2,eq}$	9,22E+03
Ozone depletion (OD), $kgCFC\text{-}11_{eq}$	2,16E-02
Human toxicity – cancer effects (HT-CE), CTUh	3,69E-05
Human toxicity – non cancer effects (HT-NCE), CTUh	5,33E-04
Particulate matter (PM), $kgPM2.5_{eq}$	3,80E+00
Ionizing radiation (IR), $kg\ U^{235}{}_{eq}$	1,13E+03
Photochemical ozone formation (POF), kgNMVOC	3,17E+01
Acidification (A), $molH^{+}_{eq}$	4,73E+01
Terrestrial eutrophication (TE), $molN_{eq}$	1,76E+02
Freshwater eutrophication (ME), kgP_{eq}	1,48E+00
Marine eutrophication (ME), kgN_{eq}	1,69E+01
Freshwater ecotoxicity (FET), CTU_e	8,74E+03
Land use (LU), $kgC_{deficit}$	7,48E+04
Water resource depletion (WD), $m^3_{water,eq}$	8,14E+01
Mineral, fossil and renewable resource depletion (MD), $kgSB_{eq}$	1,01E-01

together with the followed analytical methods. In addition, N_2O direct air emission from biological nitrogen removal processes was estimated as 1% of inlet nitrogen load [34–36].

2.4. Ecotoxicity tests

The wastewater treatment plant effluent (deriving from the same composite samples submitted also to chemical analyses) was tested for ecotoxicity by applying specific bioassays with the aim of proposing an innovative procedure for including the results of the different tests in the OEF/PEF protocols. Attention was focused on the assessment of the baseline toxicity exhibited by aquatic organisms, playing a different role in the trophic web chain, and reasonably linkable with freshwater ecotoxicity. *Daphnia magna* and *Vibrio fischeri* acute test, as well as *Raphidocelis subcapitata* test were carried out according with ISO standard procedures [37–39].

2.5. Integration of ecotoxicological tools within PEF/OEF protocols

An innovative procedure for assessing the impact on the category "freshwater ecotoxicity" is proposed: bioassays results are converted into biological equivalent concentrations of reference substances. Then, the equivalent loads of reference substances can be employed as input data instead of single pollutant loads derived from the routine chemical monitoring.

Consequently, four reference substances (inorganic: zinc and cadmium ions; organic: 3,5-dichlorophenol and dodecylbenzene sulphonic acid) included in the list provided by the International Reference Life Cycle Data System (ILCD) for the midpoint category "freshwater ecotoxicity", were selected. The reference substances were submitted to bioassays in order to obtain dose–response curves. Thus, the results of the bioassay could be converted into equivalent concentrations of reference substances, corresponding to the same value of the toxicity exhibited towards each organism.

With the aim of testing the suitability of the proposed approach, the impact related to freshwater ecotoxicity of 16 different scenarios was calculated. Each scenario was characterised by a specific concentration of a reference substance, based on the results of the bioassays carried out on each organism. For each scenario, the reference substances equivalent concentrations were used as input data; the USEtox model characterisation factors for freshwater ecotoxicity [40] are reported in Table 5.

3. Results and discussion

This section reports the upshot of the application of PEF/OEF protocol. In particular, the outcomes of using either chemical analyses or bioassays for data production are discussed. The results of the proposed procedure applied to 16 different scenarios (Case #1–Case #16) are described and compared with the results obtained with the traditional approach, based on chemical data only (Case #0).

Table 3
Average, minimum and maximum effluent concentration (referred to the year 2017) used for assessing the WWTP impact

Parameter	Average concentration	Minimum concentration	Maximum concentration
COD, mg/L	29	20	127
BOD_5, mg/L	6	5	7
Nitrogen, mg/L	15.9	0.5	37.0
Phosphorus, mg/L	1.5	0.5	12.0
TSS, mg/L	8.5	2.0	158.0
Surfactants, mg/L	0.47	0.10	0.90
Sulfate, mg/L	84	72	101
Chlorides, mg/L	109	99	120

Table 4
Values of metals, and semimetals effluent concentration used for assessing the WWTP impact. It is worth noting that all the measured polynuclear aromatic hydrocarbons, chlorinated insecticides and herbicides resulted below their limits of detection

Parameter	Composite sample concentration
Boron, μg/L	119
Vanadium, μg/L	1.5
Chromium III, μg/L	13
Manganese, μg/L	23
Iron, μg/L	249
Nickel, μg/L	36
Copper, μg/L	8.1
Selenium, μg/L	0.37
Arsenic, μg/L	0.85
Cadmium, μg/L	0.07
Antimony, μg/L	1
Aluminium, μg/L	175
Mercury, μg/L	0.30
Lead, μg/L	6.1
Zinc, μg/L	75

3.1. Life cycle impact assessment results

In Table 6, the results for each impact category, per cubic meter of treated water, are reported. A hotspot analysis of the potential environmental impacts of the WWTP has been developed, in order to highlight the most relevant phases. As reported in Fig. 2, direct emissions are particularly relevant on the impact categories freshwater eutrophication, marine eutrophication, human toxicity cancer effects and freshwater ecotoxicity.

Thus, the analysis was focused on those impacts, being those on which the management can exert the greatest degree of control, hence, having the most significant opportunities to improve the environmental performance [41]. Furthermore, the relevance of the abovementioned categories becomes clearer, after normalising the impact on different categories with respect to the total environmental burden generated by an average European citizen throughout a year (Table 7).

It is worth underlining, that, in accordance with the most recent guidelines [42], the three toxicity-related impact categories (human toxicity-cancer effects, human toxicity-not cancer effects and freshwater ecotoxicity) cannot not be used for communication purpose and must be excluded from the identification procedure of the most relevant impact categories, due to the uncertainty related to the USEtox model CFs. Nonetheless, freshwater ecotoxicity has been included in this study in order to allow the comparison between the results derived from the traditional model and those derived from the approach described in Section 2.5. An analysis of the most relevant impact categories is presented in the following paragraphs.

3.1.1. Eutrophication

The impact on freshwater eutrophication is 0.0016 kgP_{eq} per FU, while the impact on marine eutrophication is equal to 0.016 kgN_{eq} per FU. They are caused, respectively, by the direct emissions of P and N in the water effluent, which account for 94.3% and 97.1% of the total impact.

Table 5
Selected reference substances, characterisation factors for the evaluation of freshwater ecotoxicity and equivalent concentration (expressed as mg/L) obtained by performing toxicological assays

Case #	Reference substances	Organism used in the biological tests	Characterisation factors (CF) for freshwater ecotoxicity (CTU_e/kg)	Equivalent concentration (mg/L)
1	Zinc	*D. magna* (24 h)	38,600	3.134
2	Zinc	*D. magna* (48 h)	38,600	3.127
3	Zinc	*V. fischeri*	38,600	1.262
4	Zinc	*R. subcapitata*	38,600	0.041
5	Cadmium	*D. magna* (24 h)	9,710	0.117
6	Cadmium	*D. magna* (48 h)	9,710	0.030
7	Cadmium	*V. fischeri*	9,710	5.532
8	Cadmium	*R. subcapitata*	9,710	0.028
9	3,5-Dichlorophenol	*D. magna* (24 h)	6,910	2.236
10	3,5-Dichlorophenol	*D. magna* (48 h)	6,910	1.250
11	3,5-Dichlorophenol	*V. fischeri*	6,910	4.585
12	3,5-Dichlorophenol	*R. subcapitata*	6,910	1.850
13	Dodecylbenzenesulfonic acid	*D. magna* (24 h)	3,110	3.579
14	Dodecylbenzenesulfonic acid	*D. magna* (48 h)	3,110	2.854
15	Dodecylbenzenesulfonic acid	*V. fischeri*	3,110	31.395
16	Dodecylbenzenesulfonic acid	*R. subcapitata*	3,110	33.996

3.1.2. Toxicity towards human health and freshwater ecosystem

CFs for the three toxicity-related impact categories have been currently under revision by the European Commission and the ECHA (European Chemicals Agency). Thus, as abovementioned, these categories cannot be included in the definition of the relevant life cycle stages [42]. The aim of this paper, therefore, is to underline the crucial role of bioassays within the assessment of freshwater ecosystem toxicity: a comparison between the traditional approach, based on chemical analysis, and our innovative approach (based on ecotoxicological tools) is presented.

The human toxicity-cancer/non cancer effects categories reach a percentage of 3.53 and 12.55, respectively (Table 7) being their absolute values 2.6×10^{-7} and 6.3×10^{-8} CTU h. The carcinogenic effect is mainly attributable to the construction phases of the work (84.6% of the total impact); non-carcinogenic effects, however, appear to be linked with the plant operation (63.4% of the total impact). In particular, the contribution of direct emissions to the HT-NCE accounts for the 48.5% of the impact and is related principally to the presence of zinc in the effluent. The impact on freshwater ecotoxicity, estimated based on chemical analyses, is equal to 10.01 CTU_e per m^3 of treated water. This value is due to the emission of metals (Zn, Cu, Ni, Sn, Pb) in the effluent, accounting for the 43% of the total impact. It is important to note that not all the substances measured in the composite sample collected during the monitoring campaign are included in the USEtox model. For this reason, the impact related to these substances cannot be considered. A significant contribution to the freshwater ecotoxicity derives also from the construction of sewer system and WWTP, accounting, respectively, for the 21.7% and 11.5% of the total impact, and from the management of the waste generated during the process (13.6% of the total impact).

Table 6
Absolute environmental footprint calculated for each impact category

EF impact category	Value
Gross energy requirement (GER), MJ	7.258
Global warming potential (GWP), $kgCO_{2,eq}$	0.750
Ozone depletion (OD), $kgCFC\text{-}11_{eq}$	3.1E-08
Photochemical ozone formation (POF), kgNMVOC	1.4E-03
Acidification (A), $molH^+_{eq}$	1.9E-03
Terrestrial eutrophication (TE), $molN_{eq}$	5.1E-03
Freshwater eutrophication (ME), kgP_{eq}	1.6E-03
Marine eutrophication (ME), kgN_{eq}	1.6E-02
Human toxicity – cancer effects (HT-CE), CTUh	2.6E-07
Human toxicity – non cancer effects (HT-NCE), CTUh	6.3E-08
Freshwater ecotoxicity (FET), CTU_e	10.008
Particulate matter (PM), $kgPM2.5_{eq}$	2.2E-04
Ionizing radiation (IR), kg U^{235}_{eq}	2.9E-02
Land use (LU), $kgC_{deficit}$	1.018
Water resource depletion (WD), $m^3_{water,eq}$	2.3E-02
Mineral, fossil and renewable resource depletion (MD), $kgSB_{eq}$	1.9E-05

All values are referred to the FU (1 m^3 of treated wastewater).

3.1.3. Global warming potential

Although the normalisation of the results highlights that global warming potential (GWP) is not a relevant impact category for the selected WWTP, it is commonly judged as a key environmental problem from a political and social perspective. In this category, the main impact is usually ascribed to the consumption of the purchased electricity (especially from fossil fuel combustion) for WWTPs operation [43–45] and the emissions of N_2O [46,47]. The relative impact of energy use is reduced by the production of energy (electricity and heat) from biogas obtained by sludge anaerobic digestion: while the characterisation factor for the electricity mix is equal to 0.548 $kgCO_{2,eq}$/kWh, the characterisation factor for the electricity produced in the plant is equal to 0.494 $kgCO_{2,eq}$/kWh.

It is worth mentioning that literature reviews report a large variation of coefficients used for the estimation of the emissions of N_2O and methane leakage from biogas storage [48,49]. Several studies report that methane leakage may achieve up to 5% of gross methane production [7,50]. A sensitivity analysis was thus conducted to evaluate the effect of different assumptions related to the N_2O and CH_4 direct emissions on the GWP.

3.2. Innovative procedure results

The assessment of toxic impacts on the environment and human health, by means of LCA-based procedures handles separately the contribution of each substance. Zang et al. [58] by reviewing the literature state of art about the application of LCA on WWTPs, highlight some technical gaps, such as the need of broadening the range of organic chemicals (e.g., introducing trace pollutants) included in databases and using spatial differentiated CFs, in order to consider the interaction effluent/receiving body. Nevertheless, ecotoxicology studies clearly point out the inadequacy of a mere chemical monitoring for the assessment of any impact on living organisms and, consequently, on a whole ecosystem [51–55]. The main reason lies in the inherent toxicity pathways, which may involve additive, subtractive and synergistic effects. Furthermore, the actual bioavailability of a substance, in terms of speciation, cytoplasmic and lipidic concentration plays a pivotal role in triggering the biological reactions [56]. Recent findings, in addition, explore the significance of internal and external exposure to chemicals, that is, both endogenous, which have been produced by the living systems as a response to an event, and exogenous [57].

Therefore, an innovative procedure for the evaluation of freshwater ecotoxicity, based on the integration of biological assays in the OEF/PEF procedure described in Section 3.1, was proposed, with the final aim of overcoming such limitations. 16 different scenarios (case #1–case #16) were obtained by inserting, instead of the loads derived from the routine monitoring (case #0 reported in Table 6), the equivalent

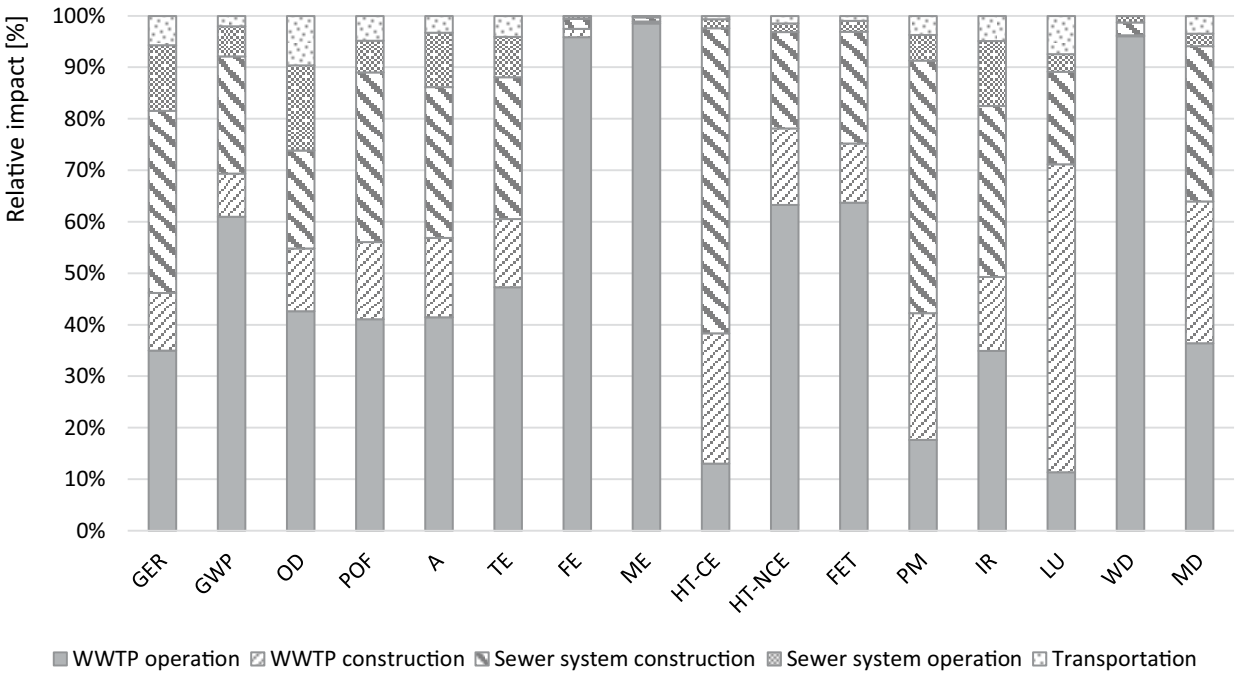

Fig. 2. Relative impact of plant operation, plant construction, sewer system construction, sewer system operation, transportation, on each category, respectively.

Table 7
Normalised results of the different impacts caused by the studied WWTP, with respect to the burden generated by an average European citizen

EF impact category	Normalised value (%)
Global warming potential (GWP)	0.59
Ozone depletion (OD)	0.01
Photochemical ozone formation (POF)	0.31
Acidification (A)	0.30
Terrestrial eutrophication (TE)	0.21
Freshwater eutrophication (FE)	7.95
Marine eutrophication (ME)	7.05
Human toxicity – cancer effects (HT-CE)	3.53
Human toxicity – non cancer effects (HT-NCE)	12.55
Freshwater ecotoxicity (FET)	8.36
Particulate matter (PM)	0.43
Ionizing radiation (IR)	0.19
Land use (LU)	0.10
Water resource depletion (WD)	2.06
Mineral, fossil and renewable resource depletion (MD)	1.38

concentration of a reference substance derived from the results of the ecotoxicity tests.

In Table 8, the results of freshwater ecotoxicity assessment, expressed as CTU_e per m^3 of treated water, for each of the 16 different scenarios are reported. The freshwater ecotoxicity potential, calculated with the proposed procedure, is mostly higher than the impact calculated based on chemical analyses. As expected, the choice of the reference substance deeply affects the biological responses. No significant correlation was found between the impacts calculated on the results of the tests with the same organism for the different substances.

For better emphasizing the variation of results depending on the bioassay and substance considered, calculations were referred only to the direct emissions, which are strictly correlated with the quality of the effluent. For examples, as shown in Fig. 3, the impacts, due to the direct emissions, derived from the test with *D. magna* varied in a range between 2,806% and 258% of the case #0, for the zinc (case #1) and the dodecylbenzenesulfonic acid (case #13), respectively; while the impacts based on the test with *R. subcapitata* are 37% (case #4) and 1,303% (case #16) of the case #0, respectively.

Furthermore, the freshwater ecotoxicity assessment, based on the results of bioassays, is clearly affected by the organism used for the biological assays and the relative end-points. In particular, considering the estimated impact related to the equivalent concentration of cadmium and 3,5-dichlorophenol, the calculation of the freshwater ecotoxicity potential based on the tests with *D. magna* and *R. subcapitata* yield to results of the same magnitude order, while the evaluation based on the test with *V. fischeri* shows a higher impact. For the other reference substances, however, significant differences appear in case of *D. magna*, *V. fischeri* and *R. subcapitata*.

These differences can be attributed to the calculation method of the freshwater ecotoxicity impact. Indeed, the target organisms of the ecotoxicological tests provide a different response to each selected substance, yield to different

Table 8
Results of the impact on the freshwater ecotoxicity of the WWTP, expressed as CTU_e per m^3 of treated water, calculated for the different scenarios (case #1–case #16) with the proposed procedure (all emissions in all the life cycle included)

Case #	Reference substance	Organism used in the biological tests	CTU_e/FU
0	–	–	10.01
1	Zinc	*D. magna* (24 h)	126.69
2	Zinc	*D. magna* (48 h)	126.41
3	Zinc	*V. fischeri*	54.41
4	Zinc	*R. subcapitata*	7.28
5	Cadmium	*D. magna* (24 h)	6.84
6	Cadmium	*D. magna* (48 h)	5.99
7	Cadmium	*V. fischeri*	59.41
8	Cadmium	*R. subcapitata*	5.97
9	3,5-Dichlorophenol	*D. magna* (24 h)	21.15
10	3,5-Dichlorophenol	*D. magna* (48 h)	14.34
11	3,5-Dichlorophenol	*V. fischeri*	37.38
12	3,5-Dichlorophenol	*R. subcapitata*	18.48
13	Dodecylbenzenesulfonic acid	*D. magna* (24 h)	16.83
14	Dodecylbenzenesulfonic acid	*D. magna* (48 h)	14.57
15	Dodecylbenzenesulfonic acid	*V. fischeri*	103.34
16	Dodecylbenzenesulfonic acid	*R. subcapitata*	61.90

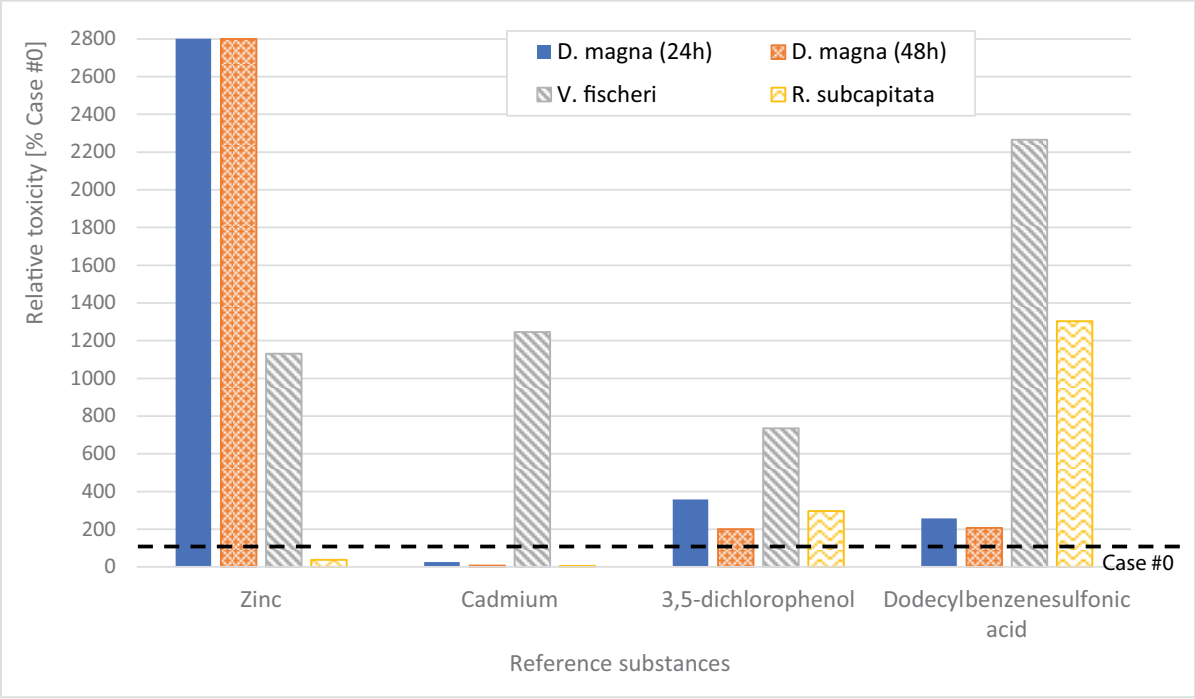

Fig. 3. Impacts on freshwater ecotoxicity due to only the direct emissions, calculated with the proposed procedure. Values have been normalised with respect to the result obtained with the chemical analyses (case #0) from the OEF/PEF calculation.

dose–response curves. Consequently, different equivalent concentrations were obtained for the three tests performed. However, the freshwater ecotoxicity evaluation procedure, based on the USEtox model, involve one CF for each chemical substance present in the database of the model. The CF is an indicator of the ecotoxicity potential that considers the effect of a substance, emitted in a specific part of environment, on the whole ecosystem and do not refer to the impact on a single organism. This results in different impacts for the same reference substance as a function of

Table 9
Equivalent concentration (expressed as mg/L) obtained by considering the results of all ecotoxicological tests performed and related impact on the freshwater ecotoxicity calculated with the proposed procedure (all emissions in all the life cycle included)

Case	Reference substance	Equivalent concentration (mg/L)	CTU_e/FU
#0	–	–	10.01
A	Zinc	1.48	62.83
B	Cadmium	1.87	23.86
C	3,5-Dichlorophenol	2.70	24.35
D	Dodecylbenzenesulfonic acid	17.76	60.93

the selected target organism. Moreover, any toxicological approach should be based on multi-tiered battery of tests, which explore as many trophic levels (or taxa) as possible. For these reasons, a more suitable approach for the calculation of the equivalent concentration should include the results of different bioassays covering, at least, the main trophic categories (producers, primary consumers and decomposers) as in the proposed case.

Therefore, a further step was taken: the equivalent concentration was calculated using the results of the assays on the three target organisms (*D. magna*, *V. fischeri*, *R. subcapitata*) instead of the results of only one ecotoxicological test. In particular, the equivalent concentration of each substance was calculated as the average of the geometric mean of the equivalent concentrations derived from the results of the assays on every individual species. In Table 9 the reference substances and the equivalent concentration used for the evaluation of the freshwater ecotoxicity impact for the four scenarios (case A–case D) as well as the results, expressed as CTU_e per m^3 of treated water are reported. The impact on freshwater ecosystem due to the direct emissions (Fig. 4), based on the biological assays, assumes a value ranging between 196% (case B) and 785% (case A) with respect to case #0. Also, this approach highlights the impact underestimation in case of running the model with chemical data only. No significant difference was observed between the freshwater ecotoxicity assessment based on the concentration of cadmium and 3,5-dichlorophenol while the results based on the equivalent concentration of zinc and dodecylbenzenesulfonic acid are much higher.

Nevertheless, it is worth noting that several studies highlight that the development of a better tools for the evaluation of the toxic impacts of metals is needed [58,59]. Indeed, the CFs for the heavy metals and other substances are still associated with a high degree of uncertainty [60,61] and, for this reason, classified as "interim" in the USEtox

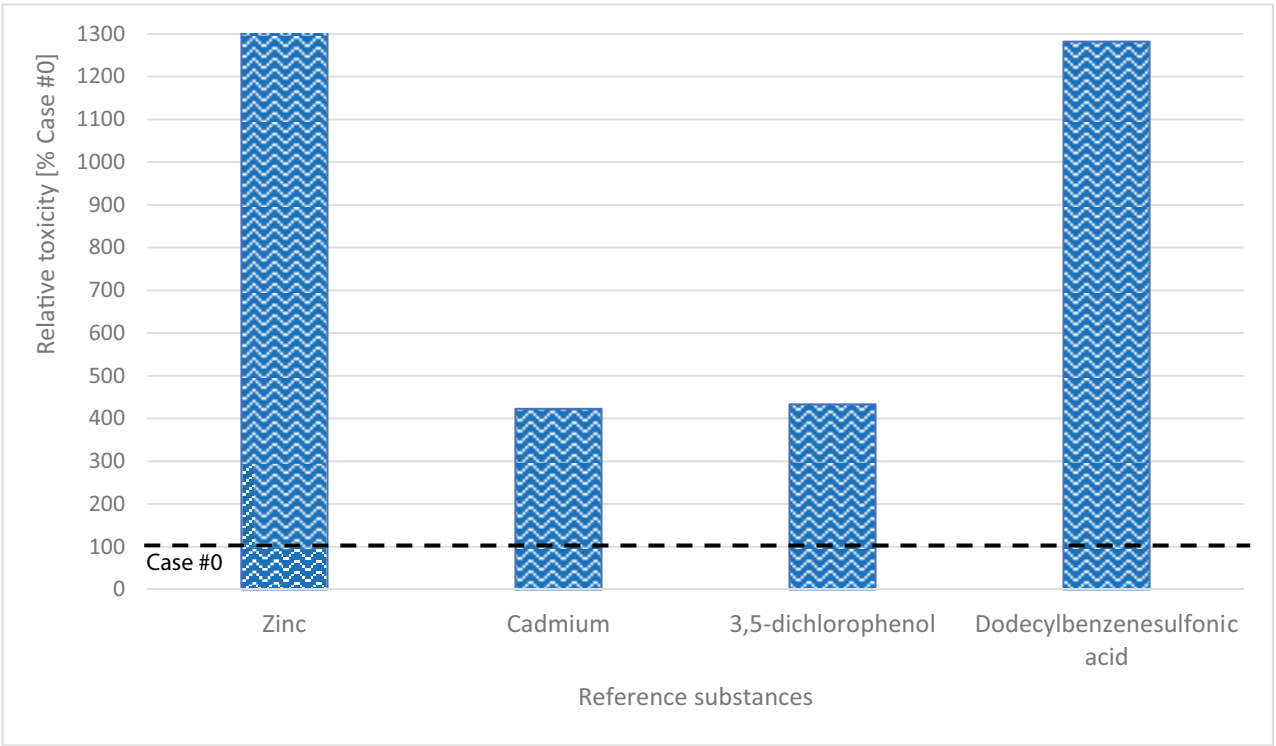

Fig. 4. Impacts on freshwater ecotoxicity due to only the direct emissions, calculated with the proposed procedure. Values have been normalised with respect to the result obtained with the chemical analyses (case #0) from the OEF/PEF calculation.

model [40]. In order to avoid the uncertainty in the characterisation factors estimation, the choice of the reference substances must be provided between the substances with a characterisation factors classified as "recommended".

4. Conclusions

The environmental footprint of a real WWTP was assessed, based on the OEF/PEF protocols. For almost the majority of impact categories, the operation phase contribution (direct emission, energy consumption, waste production and chemicals addiction) was higher than 40%. Interestingly, the values normalised with respect to the total environmental burden generated by an average European citizen, made freshwater eutrophication, marine eutrophication, human toxicity-not cancer effect and freshwater ecotoxicity the most impacting categories.

In order to overcome the intrinsic limitations of the traditional approach, we proposed an innovative procedure, which includes the bioassays in the freshwater ecotoxicity assessment. It is this approach the researchers believe that offers some advantages, compared with the procedures based on chemical analyses only:

- The whole effluent impact is considered (instead of taking into account each measured/estimated pollutant, which represent the model input data)
- Possible additive/subtractive/synergistic/antagonistic effects among the different substances in the effluent can be taken into consideration.

By following the proposed procedure, it appears that the impact due to the direct emission calculated with the traditional approach is significantly lower than the impact calculated based on bioassays in most of cases. Moreover, within bioassays execution, the choice of reference substances and target organisms plays a crucial role in freshwater ecotoxicity evaluation. For this reason, it might be necessary to plan a multi-tiered assays battery, in order to include at least three trophic levels and to develop further evaluation in order to determine which reference substance is the most representative to assess the eco-toxicity impact. Finally, the reference substance should be chosen among the substances classified as "recommended" in the USEtox model, to limit the high uncertainty related to the CFs evaluation.

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Supplementary Information

Table S1
Quantification of metal, semimetals, herbicides, insecticides and polynuclear aromatic hydrocarbons

Parameter	Method	Measure unit	Value
B	EPA 6020B	mg/L	0.1193
V	EPA 6020B	µg/L	1.50
Cr	EPA 6020B	µg/L	13.15
Mn	EPA 6020B	µg/L	23.26
Fe	EPA 6020B	µg/L	249.44
Ni	EPA 6020B	µg/L	36.26
Cu	EPA 6020B	mg/L	0.0081
As	EPA 6020B	µg/L	0.85
Se	EPA 6020B	µg/L	0.37
Cd	EPA 6020B	µg/L	0.07
Sb	EPA 6020B	µg/L	1.02
Al	EPA 6020B	µg/L	175.35
Hg	EPA 6020B	µg/L	0.30
Pb	EPA 6020B	µg/L	6.12
Herbicides (sum)	Calculation	µg/L	<0.01
Alachlor	EPA 536	µg/L	<0.01
Ametrin	EPA 536	µg/L	<0.01
Atrazine	EPA 536	µg/L	<0.01
Cyanazine	EPA 536	µg/L	<0.01
Desethyl atrazine	EPA 536	µg/L	<0.01
Desethyl terbuthylazin	EPA 536	µg/L	<0.01
Atrazine-desisopropyl	EPA 536	µg/L	<0.01
Desethyldesisopropylatrazine (DACT)	EPA 536	µg/L	<0.01
Flufenacet	EPA 536	µg/L	<0.01
Isoxaflutole	EPA 536	µg/L	<0.01
Metolachlor	EPA 536	µg/L	<0.01

(Table S1 Continued)

Molinate	EPA 536	µg/L	<0.01
Oxadiazon	EPA 536	µg/L	<0.01
Pendimethalin	EPA 536	µg/L	<0.01
Prometrine	EPA 536	µg/L	<0.01
Simazine	EPA 536	µg/L	<0.01
Terbuthylazine	EPA 536	µg/L	<0.01
Terbutryn	EPA 536	µg/L	<0.01
Alpha-BHC	EPA 3510 C 1996 + EPA 8270 D 2007	mg/L	<0.01
Beta-BHC	EPA 3510 C 1996 + EPA 8270 D 2007	mg/L	<0.01
Delta-BHC	EPA 3510 C 1996 + EPA 8270 D 2007	mg/L	<0.01
Gamma-BHC	EPA 3510 C 1996 + EPA 8270 D 2007	mg/L	<0.01
Heptachlor	EPA 3510 C 1996 + EPA 8270 D 2007	mg/L	<0.01
Aldrin	EPA 3510 C 1996 + EPA 8270 D 2007	mg/L	<0.01
Heptachlor epoxide Isomer B	EPA 3510 C 1996 + EPA 8270 D 2007	mg/L	<0.01
Endosulfan I (alpha)	EPA 3510 C 1996 + EPA 8270 D 2007	mg/L	<0.01
4,4′-DDE	EPA 3510 C 1996 + EPA 8270 D 2007	mg/L	<0.01
Dieldrin	EPA 3510 C 1996 + EPA 8270 D 2007	mg/L	<0.01
Endrin	EPA 3510 C 1996 + EPA 8270 D 2007	mg/L	<0.01
Endosulfan II (beta)	EPA 3510 C 1996 + EPA 8270 D 2007	mg/L	<0.01
4,4′-DDD	EPA 3510 C 1996 + EPA 8270 D 2007	mg/L	<0.01
Endrin aldehyde	EPA 3510 C 1996 + EPA 8270 D 2007	mg/L	<0.01
Endosulfan II (beta)	EPA 3510 C 1996 + EPA 8270 D 2007	mg/L	<0.01
4,4′-DDT	EPA 3510 C 1996 + EPA 8270 D 2007	mg/L	<0.01
Methoxychlor	EPA 3510 C 1996 + EPA 270 D 2007	mg/L	<0.01
0,0,0-Triethylphosphorothioate	EPA 3510 C 1996 + EPA 8270 D 2007	mg/L	<0.01
Thionazin	EPA 3510 C 1996 + EPA 8270 D 2007	mg/L	<0.01
Sulphotep	EPA 3510 C 1996 + EPA 8270 D 2007	mg/L	<0.01
Forate	EPA 3510 C 1996 + EPA 8270 D 2007	mg/L	<0.01
Dimethoate	EPA 3510 C 1996 + EPA 8270 D 2007	mg/L	<0.01
Disulfoton	EPA 3510 C 1996 + EPA 8270 D 2007	mg/L	<0.01
Methyl parathion	EPA 3510 C 1996 + EPA 8270 D 2007	mg/L	<0.01
Parathion	EPA 3510 C 1996 + EPA 8270 D 2007	mg/L	<0.01
Famphur	EPA 3510 C 1996 + EPA 8270 D 2007	mg/L	<0.01
Anthracene	EPA 3510 C 1996 + EPA 8270 D 2007	µg/L	0
Pyrene	EPA 3510 C 1996 + EPA 8270 D 2007	µg/L	0
Benzo[a]anthracene	EPA 3510 C 1996 + EPA 8270 D 2007	µg/L	0
Benzo[a]pyrene	EPA 3510 C 1996 + EPA 8270 D 2007	µg/L	<0.005
Benzo[b]fluoranthene	EPA 3510 C 1996 + EPA 8270 D 2007	µg/L	0
Benzo[g,h,i]perylene	EPA 3510 C 1996 + EPA 8270 D 2007	µg/L	0
Benzo[k]fluoranthene	EPA 3510 C 1996 + EPA 8270 D 2007	µg/L	0
Chrysene	EPA 3510 C 1996 + EPA 8270 D 2007	µg/L	0
Dibenz[a,h]anthracene	EPA 3510 C 1996 + EPA 8270 D 2007	µg/L	0
Indeno[1,2,3-cd]pyrene	EPA 3510 C 1996 + EPA 8270 D 2007	µg/L	0
PAHs (sum)	Calculation	µg/L	0

Method 6020B (2014) Inductively Coupled Plasma – Mass Spectrometry, Part of Test Methods for Evaluating Solid Waste, Physical/Chemical Methods.

Method 536 (2007) Determination of Triazine Pesticides and Their Degradates in Drinking Water by Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry (lC/ES-MS/MS).

Method 3510C (1996) Separatory Funnel Liquid–Liquid Extraction, part of Test Methods for Evaluating Solid Waste, Physical/Chemical Methods.

Method 8270D (20007). Semivolatile Organic Compounds by GC/MS.