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Review

Opinion paper about organic trace pollutants in wastewater: Toxicity assessment in a European perspective

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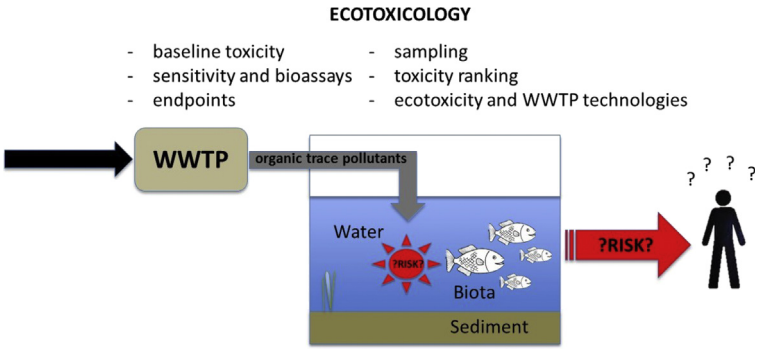
HIGHLIGHTS

- Bioassays must be chosen by taking into account the meaning of biological responses.
- Lab and in situ bioassays must be integrated, based on reliability and applicability.
- Trace pollutants can cause unpredictable and non-linear biological responses.
- Wastewater composition and flowrate variability affects any toxicity assessment.
- Environmental and socio-economic aspects underpin sewage treatment scheme choice.

GRAPHICAL ABSTRACT



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ABSTRACT

This opinion paper focuses on the role of eco-toxicological tools in the assessment of possible impacts of emerging contaminants on the aquatic ecosystem, hence, on human health. Indeed, organic trace pollutants present in raw and treated wastewater are the pivot targets: a multidisciplinary approach allows defining the basic principles for managing this issue, from setting a proper monitoring campaign up to evaluating the optimal process treatment. Giving hints on trace pollutants fate and behaviour, attention is focused on the choice of the bioassay(s), by analysing the meaning of possible biological answers. Data interpretation and exploitation are detailed with the final goal of providing criteria in order to be able to select the best targeted treatment options.
The manuscript deals with conventional and innovative analytical approaches for assessing toxicity, by reviewing laboratory and field assays; illustrative real scale and laboratory applications integrate and exemplify the proposed approach.

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Contents

1. Introduction

Ecological risk assessment is the scientific decision supporting process for gauging risks based on the occurrence of a physical or biological agent or the amount of a chemical/mixture of chemicals/emission discharged into a given environment, on the exposure of an ecological receptor (*e.g.* plant, fish, or bird) and on the inherent toxicity of the agent itself. The awareness of investigating the effects of an exposure to pollutants throughout the whole lifespan of an organism (or during specific phases of its development) quests for new approaches. This comes up beside and integrates conventional tests, issued according to established guidelines and performed on specific laboratory organisms, generally aimed to assess short to mid-term effects.

Together with the monitoring of various effects on a single biological model (i.e. early life stages), nowadays it is clear that cross-generational, ecological and ethological aspects should be investigated (Gelbke et al., 2004; Xia et al., 2013; Sunanda et al., 2016). Ecotoxicity testing strategies are developed worldwide and supported by international organizations. Risk characterization/assessment schemes are tiered, enabling a progressive refinement of exposure/effect ratios. Nevertheless, it is not possible to specify the number of tiers generally required, since they depend on each specific situation, due to the complexity of community structures and relationships among different populations.

This opinion paper aims to gather the main findings obtained by different research groups participating to ES1202 COST Action “Conceiving Wastewater Treatment in 2020 – Energetic, environmental and economic challenges” (Water_2020). The final goal is to present the strength of a multi-tiered method within the risk assessment of whole effluent approach detailing the potentialities of toxicity as a parameter for treated wastewater quality evaluation in the perspective of its reuse. Pros and cons of conventional and innovative bioassays have been investigated, including the socio-economic aspects; some case studies are showed as well.

2. Main knowledge and open issues

As far as chemical pollution is concerned, substances are prioritized based on the risk to or via the aquatic environment, according to the Water Framework Directive (European Community, 2000), and included in article 16 "Strategies against pollution of water". Since the Seventies, the progressive awareness of hazards linked with specific chemicals has been increasingly consolidated by the findings in epidemiology, the long term follow up of environmental disasters and the availability of new technological tools enabling the identification and quantification of a huge range of analytes from complex matrices also at risible concentrations (Petrović et al., 2014). For instance, it has been possible to carry out investigations on metal speciation. In addition, almost every class of organic compounds has been taken into account, starting from reactions by-products (*e.g.*, among the firstly studied, the disinfection by-products, such as the trihalomethanes), to persistent organic pollutants (POPs), and, finally, to the thousands of substances derived from the everyday use, such as PPCPs (pharmaceuticals and personal care products). Furthermore, research has focused on pollutants released into the environment, by considering, *inter alia*, micro-plastics and nanomaterials. It is now well known, that the size of the chemical agents strongly affects both the bioavailability and the effects on the organisms. So far, the scientific literature numbers lots of remarkable works focusing on the detection of (trace) pollutants, both organic and inorganic, the study of their fate and behavior into the environment, their toxicity and the feasibility of their replacement and removal from the contaminated areas (Auffan et al., 2010; Zhuang and Gao, 2014; Shyamasundar et al., 2015; Anderson et al., 2016; Sendra et al., 2017). The present knowledge indicates that thousands of organics in trace quantities are widespread in ecosystems, aquatic organisms being important targets, as they are exposed to wastewater residues over their entire life.

The Water Framework Directive defines "hazardous substances" substances or groups of substances that are toxic, persistent and liable to bio-accumulate, and other substances or groups of substances, which give rise to an equivalent level of concern. Hence, in the risk assessment process, the initial step would be hazard identification. Further, the primary investigation should already concern the possible health problems caused by the pollutants. This process uses the intrinsic properties of a chemical (persistence, solubility, K_{ow}, volatilization, etc.) to determine expected adverse effects, and on the other hand, to estimate the probability of adverse effects to occur. In addition, the physical-chemical data provide information about the relevance of some exposure paths. As the next step, and already partly depending on the nature of the substance(s) under scrutiny, proper analytical tools capable of providing deeper information on exposure and effects are required: the most commonly applied are acute toxicity, subchronic and chronic toxicity, abiotic and biotic degradability, bioaccumulation and biomagnification.

During an exposure assessment, the following questions must be answered: 1) To which pollutant doses are humans and ecosystems exposed, throughout a given lapse of time? and 2) How many individuals, species or populations are exposed? In case of dose-response assessments, quantitative data regarding biological effects under different situations and types of exposure must be supplied. Either finally, risk assessment can be carried out, comparing exposure and effects, quantitatively or qualitatively, thus determining the probability of effects occurrence. Both hazard and risk assessments are mandatory to guarantee scientific support for regulations (Tarazona and Vega, 2002).

Ecotoxicity tests can be classified based on design (field, laboratory, computer), level of biological organization (population, assemblage/community, ecosystem), exposure period (acute, sub-chronic, chronic) and endpoint (lethal, sub-lethal). Short-term ("acute") tests are generally used preliminarily, being the survival the most common endpoint. Long term ("chronic") tests (involving the observation of sub-lethal effects on organism growth or reproduction) are used afterwards, if results from short term tests combined with large safety factors indicate possible risks to the environment.

The use of acute and chronic tests in ecotoxicology has been proposed in reports from EU's REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) with aims to improve the protection of human health and the environment through the better and earlier identification of the inherent properties of chemical substances. However, despite the presence of mixtures of multiple compounds in environmental media and samples, theoretical considerations and experimental findings suggest that the overall risk may be driven by only a few components in these mixtures (Hashmi et al., 2018; Altenburger et al., 2015; Backhaus and Karlsson, 2014; Price et al., 2012). Furthermore, routinely detected chemicals often cannot explain the observed biological responses (*e.g.*, Escher et al., 2013) confirming the need to integrate biological and chemical results.

Wastewaters, due to their nature and origin (municipal, industrial, runoff, grey), evidently collect and concentrate a multitude of chemicals, that form complex mixtures including microbial consortia.

Therefore, when assessing the overall impact of any given wastewater (either raw or treated), it is essential to take into account both: i) the removal/discharge of specific micropollutants and ii) the toxicity of parent compounds, metabolites, treatment by-products and, in any case, of the whole stream. For this reason, in order to gain insight not only into the impact of individual micropollutants, but also into the effects exerted by wastewater, as a whole, bio-analytical tools are necessary. The removal of a target xenobiotic compound, indeed, does not necessarily mean that the treatment process is detoxifying, because adverse effects may be a result of the conversion of chemicals into metabolites or breakdown products more toxic than the parent compounds.

3. Which responses should be measured? A focus on "real life"

3.1. Principles

Two basic questions originated from the debates held within the present COST Action: what is the true essence of toxicology? Which role it can (and has to) play in the environmental protection. The first query implies a reflection on its genesis; therefore, it stirs to ask ourselves what we can really measure and which meaning might have our measurements. A host of scientists have debated about this issue, since the birth of the modern discipline, whose founder is considered Mathieu Orfila, author of a treatise on poisons and their effects on "physiology", published in 1815 (Hodgson, 2004). The second question concerns the true applicability of toxicological tools for the protection of the aquatic ecosystem, and, consequently, the preservation of the quality of water for human consumption. The first aspect will be treated in Sections 3 and 4, while the second subject will be broached in Section 5.

From a "real life" perspective, a range of possible responses can be expected and observed, from the molecular to the ecosystem level, in the living beings exposed to the mixture of pollutants present in raw and treated wastewaters. According to Newman and Clements (2008), ecotoxicology is the science devoted to the study of the contaminants and their effects induced on all parts of the biosphere.

Many well-known molecular and biochemical mechanisms enable to explain the toxic action of contaminants and their subsequent effects. Enzyme dysfunctions (inhibition, activation or induction), DNA alterations, oxidative stress and generation of reactive oxygen species (ROS), oxidative phosphorylation inhibition, heme biosynthesis inhibition, are typical mechanisms associated to toxicants (Newman and Clements, 2008; Carvan and Di Giulio, 2015; Barron et al., 2015). In general, the biochemical and molecular responses express morphological (at microscopic and macroscopic scale) modifications on cells and tissues, and/or functional failures of organs and systems (this topic will be detailed in Section 3.2). Frequently, the biochemical mechanisms of toxic actions are unknown; thus, the effects on cells, tissues, organs

and systems can be identified as the only target in the risk assessment. Different morphological findings linked to the exposure to organic pollutants have been described in cells and tissues, such as inflammation, necrosis, apoptosis, and sometimes hypertrophy or hyperplasia (Chen et al., 2016; Cheng et al., 2015; Ray et al., 2015; Kumar et al., 2017; Santana et al., 2018, Vicario-Parés et al., 2018).

Tissues, organs and systems are involved in vertebrate toxicokinetics: integument, respiratory and digestive organs are firstly subjected to pollutant exposure, due to their direct interface with the outside (Fatima et al., 2014; Alves et al., 2016; Salamat and Zarie, 2016; Strzyzewska et al., 2016); besides, nervous, immune, and endocrine systems are mostly studied (Kumari and Khare, 2018; Lonappan et al., 2016; Vogt et al., 2018; Xu et al., 2018; Gambardella et al., 2016; Adeogun et al., 2016; Hicks et al., 2017; Tulloch et al., 2016a).

At the organismal level, different single-species bioassays (of which some are standardized) can be applied to identify hazards through all relevant exposure routes: soil, water, air and food. The results can be applied in ecological risk assessments (ERA), to yield Species Sensitivity Distributions (SSDs), which model species sensitivity to chemicals or other stressors (Posthuma et al., 2002; Tulloch et al., 2016b; Liu et al., 2014). At the border between organismal and population levels, alterations on growth and development, reproduction and behavior, are issues of concern: tests on fish, birds, terrestrial and aquatic invertebrates, terrestrial and aquatic macrophytes, and microscopic plants are commonly part of monitoring campaigns (Kapustka, 2003; Moro et al., 2014; Gauthier et al., 2016; Schwindt, 2015; Lu et al., 2017; Díaz-Gil et al., 2017; Gür et al., 2016; Gopalapillai et al., 2014; van Wijngaarden and Arts, 2018).

Although long-term single-species tests and laboratory multispecies tests can be performed to predict or evaluate population dynamics, increasing attention has been paid to *in situ* toxicological studies. In particular, laboratory scale assays (*in vitro* and *in vivo*) may not allow a relevant simulation of real cases, due to the presence of other stressing conditions that influence the biological response. Thus, laboratory scale experiments might dramatically stray from real situation. Mesocosms and field assays provide information about real ecological effects (Tarazona and Vega, 2002; Szöcs et al., 2015; Hasenbein et al., 2017; Lemm and Feld, 2017). A critical aspect may consist in the multi-faceted scenario of responses, which can present non-monotonic trends and can be affected by hormesis and adaptation phenomena (Calabrese and Blain, 2011).

3.2. Exploiting the biological responses

As far as chemical substances are concerned, every mechanism of toxicity is initiated by the interaction between the chemical(s) and the organism (through a MIE, Molecular Initiating Event) and can be described according to the following sequence: exposure, bioavailability and formation of a bond with the ligand. Consequently, two opposite scenarios can reveal either alteration or adaptation, both driven by complex pathways. Sub-lethal responses can be evaluated by quantifying proper physiological condition indices, linked to morphometry, biochemistry and growth. Considering the toxicity pathways, a MIE is the starting point of xenobiotic metabolism pathways; afterwards, cells answer via specific and reactive modes of action (MOAs). Bioassays may reveal xenobiotic metabolism pathways, MOAs, as well as the induction of adaptive stress response, by capturing specific signals. Cell viability remains a major phenomenon to take into account. System responses, as abovementioned, regard the whole apparatuses (Escher and Leusch, 2012; Escher et al., 2014).

The effects occurring after an exposure to chemicals at molecular and cellular level can be linked with those exhibited at system and organism levels, thanks to the model of the Adverse Outcome Pathways. It represents a tool for predicting adverse effects, based on mechanistic evidence (specific key events, KEs, can be measured), without exploring chemical reactions and biotransformation; it connects the responses of in vitro, in chemico and in silico experiments with the toxicity shown in vivo and is applicable to a variety of living organisms, from invertebrates to mammals (Ankley et al., 2016; Escher et al., 2017). Recently, a possible keystone for assessing the risk connected to the exposure to chemicals and mixtures has been theorized, starting from the concept of exposome (Wild, 2012), which represents an index of the cumulative risk (Smith et al., 2015). In effect, this concept, used in epidemiology, holds the action of exposure to external stressors (throughout the entire life of an organism) and the internal events taking place in response to the exposure. The causal link between exposure and adverse effects may be investigated by merging the principles of AOPS and exposome, which complement each other; moreover, the overall external exposure, via environment and dietary intake (Aggregate Exposure Pathway) can be considered by this approach, since the AEP accounts for the key events taking place from the external exposure to the internal target. The challenge will consist mainly in the capability of measuring exogenous and endogenous chemicals (Escher et al., 2017).

Cell-based tests, however, cannot be translated directly into a toxicological effect, which is directly exploitable in water supply quality management, as pointed out by Escher et al. (2015). Moreover, regarding assays, whose answers are ascribable to multiple effects (being for instance non-specific and reactive), it is almost impossible to find a strict correlation between a positive result and the presence of a specific chemical. Apart from the receptor-mediated effects, as in the case of hormones and hormone-like substances, which can often be clearly linked with the presence of particular chemicals in the sample, in most cases the effects appear to be caused by substances that remain unidentified. Consequently, the complementarity of biological assays with respect to chemical analyses fails in a sense, within the quality assessment of a natural waterbody (Escher et al., 2011; Escher et al., 2015; Leusch et al., 2014a, b; Escher et al., 2013; Tang et al., 2014; Denslow et al., 2016; Neale et al., 2017d). Given the context, the proposal of the derivation of effect-based trigger values bioanalytical equivalent concentrations (EBT-BEQ) appears to be quite promising (Tang et al., 2013; Escher et al., 2018; Escher et al., 2015). In particular, the conflation of two concepts, namely the use of results, which might be based on effect triggers, in order to attribute the response of a non-specific bioassay to chemicals contained in a sample, and the use of a reference chemical to express the toxicity exhibited by a sample, can offer a way of predicting the actual hazard for the aquatic environment (see also Section 5.3).

Finally, it is worth underlining that the concurrence of the concentration of a substance freely dissolved in the bioassay medium, in the cell and also in the cellular membrane cannot be taken for granted. Consequently, the interpretation of the assay results can be twisted, since the response of the biological system is usually correlated to the nominal concentration, which can be overestimated, due, for instance, to partial adsorption of lipids and proteins dispersed in the medium. Therefore, the actual bioavailability is a function of chemical partitioning in vitro (Fischer et al., 2017).

3.2.1. Xenobiotic metabolism pathways

The induction of these pathways indicates the presence of pollutants, although it may not get to cytotoxicity. Among the measured endpoints it is worth citing the induction of cytochrome P 450 1 A2, the activation of aryl hydrocarbon (AhR) and pregnane X (PXR) receptors, the bond with peroxisome proliferator-activated receptor gamma (PPARγ) (Escher and Leusch, 2012); (Leusch et al., 2014a, b).

3.2.2. Specific receptor-mediated modes of toxic action

They include endocrine disruption, reproduction and development impairment, and acetylcholinesterase inhibition (Escher et al., 2014) (Escher et al., 2015).

As far as endocrine disruption is concerned, it is mandatory to select the mechanisms to investigate, by taking into consideration the biological complexity of the target organism and its physiology;

developmental and reproductive toxicity, however, are unpredictable through the execution of in vitro tests, since they are meta-cellular events (Leusch et al., 2014a, b). Several mechanisms account for endocrine impairment: the most commonly studied are the bonds with nuclear receptors (this super family includes 48 types, in case of humans), and the interactions with membrane receptors, cytosolic receptors, orphan nuclear receptors. Moreover, epigenetic changes as well as regulation cascade processes, effects on hormones and oxidative metabolism can be numbered among the modes of action of endocrine disrupting compounds. The modes of action concern all the biological levels, from single cells to the whole organisms, both with acute and chronic effects, including reproductive, immunological and neurological disorders, cancer, diabetes, obesity.

Endocrine disrupting compounds exhibit multiple modes of actions, resulting in dose-effect relationships not always following a monotone trend and changing entirely as a function of concentrations and depending on the final target. The case of bisphenol A (BPA) is emblematic: it behaves as a relatively weak estrogen towards ERα in comparison with the natural hormone estradiol, while it is equipotent towards membrane receptors (Welshons et al., 2006); (Quesada et al., 2002). Furthermore, the effects of EDCs can differ based on the developmental stage of the organisms (*e.g.*, pre-natal, post-natal and adult forms) as pointed out by Beronius and Vandenberg (2016) and UNEP (2012).

Many pathways are based on nuclear receptors that migrate into the nucleus and regulate gene transcription after hormone binding, despite their location. The main pathways are the following: thyroid signaling, estrogen signaling, glucocorticoid pathway, renin-angiotensin-aldosterone system, leptin and insulin signaling (NIEHS, 2002; Escher and Leusch, 2012; Leusch et al., 2010; Leusch et al., 2017a).

A possible side effect of endocrine disruption might be the acquisition of antibiotic resistance. Large environmental releases are caused by their intensive use and, often, overuse or misuse. Furthermore, it is worth noting that the majority of antibiotics can be only partially metabolized after medication, and, thus, are excreted directly into the wastewater. Main hotspots are soils fertilized with manure runoff water from farms (Sarmah et al., 2006), effluents of drug production units (Larsson et al., 2007; Li et al., 2010), WWTP effluents and sludge and, consequently, the receiving waterbodies (Kümmerer, 2009a; Michael et al., 2013; Lofrano et al., 2017). Antibiotic resistance is mechanistically based on inactivation or modification of the antibiotic, an alteration in the target site of the antibiotic that reduces its binding capacity, a modification of the metabolic pathways to circumvent the antibiotic effect or a reduction in the intracellular antibiotic accumulation by decreasing the permeability and/or increasing the active efflux of the antibiotic (Schmieder and Edwards, 2012). Acquisition of antibiotic resistance may occur by mutation of its own genes (vertical evolution) or by acquiring new genes from other strains or species (horizontal gene transfer) (Blair et al., 2015). The latter is mediated by the so-called mobile genetic elements (MGE) such as phages, plasmids, integrons and transposons. The pool of genetic material maintained by the environmental bacterial communities, named the resistome, provides the molecular functions for protecting bacteria against the majority of clinically important classes of antibiotics and constitutes a reservoir of ARGs that can be mobilized into human pathogenic bacteria (Cantón, 2009; Allen et al., 2010). ARGs have gained increasing attention in recent years (Zhang et al., 2011; Kristiansson et al., 2011; Schmieder and Edwards, 2012; Yang et al., 2013); there is still a critical lack of knowledge about the diversity, distribution and origin of resistance genes (Kümmerer, 2009b), especially for the unculturable majority of environmental bacteria, of which less than 1% are estimated to be culturable (Hugenholtz et al., 1998).

Recent developments in genomics, together with the decrease of equipment prices and the wide availability of sophisticated tools (such as DNA micro-arrays) have contributed to a tremendous exploitation of molecular techniques. Starting from genome sequencing, this led to the study of expression profiling (m-RNA transcripts, miRNA, ncRNA), the so-called transcriptomics, until the characterization of protein (proteomics), peptide (peptidomics) and metabolic profiles (metabolomics).

The application of these analyses to toxicology (toxicogenomics) has rapidly spread to the impact assessment of chemicals, mixtures and effluents towards the whole ecosystems (with regard to water matrices), thus turning the research field into ecotoxicogenomics. This novel discipline, by investigating transcripts, proteins and metabolites, overcomes several gaps inherent to the traditional approach, such as long response time and relationships between exposure duration and possible adverse effects. Meanwhile, it is possible to gain information on basic biology of organisms, also highlighting common patterns of modes of action (Snape et al., 2004; Miracle and Ankley, 2005).

The identification of gene expression mechanisms due to stimulation of natural hormones and xenobiotics has been studied by means of DNA microarrays. Research has been focused mainly on the estrogen nuclear receptors, which behave as transcription factors, *i.e.*, they interfere with the DNA transcription process. On the contrary, knowledge of the response elements in gene promoter regions is still lacking (Iguchi et al., 2006), (Iguchi et al., 2007). Transcriptome differs from proteome, due to post- translational modifications; each environmental stimulus affects these mechanisms, as well as gene expression. The challenge is, thus, to find the link between the "protein expression signatures", which are constituted by biomarker patterns and the modes of actions of chemicals. At the same time, however, the physiological levels, from the sub-cellular up to the organism, must be scrutinized to investigation, to avoid collecting a huge amount of protein sequences without getting any relative response (Lemos et al., 2010), (Shepard et al., 2000).

3.2.3. Reactive modes of action

They cover crucial effects, such as mutagenicity, genotoxicity and reactive oxygen species (ROS) formation.

The toxicity towards the DNA and the genetic processes exhibits a wide spectrum of effects, and, therefore, can be investigated by means of several complementary tests. The observed phenomena include genotoxicity (not directly transmissible), mutagenicity (heritable change in a genotype), mechanisms of DNA repair, carcinogenesis, and genetic-related developmental toxicity.

Briefly, damage to DNA involves alkylation (which mainly induces H bonds alteration, errors in base-pairing); hydroxylation (hence, errors in base-pairing), deamination (bringing on changes from cytosine to uracil, then errors in base-pairing and base substitution) formation of base analogues (for instance by replacement of H atoms with halogens) leading to errors in base-pairing and base substitutions, strand breaks, intra/interstrand cross links. Large planar molecules can intercalate within the double helix, without reacting but disrupting replication, recombination and repair. The mutations consist in point mutations (referred to nucleotide substitutions), yielding to errors in amino acids coding, and chromosomal mutations (consisting in deletion or insertion of several contiguous genes, inversion of genes on a chromosome, exchange of large segments of DNA between non-homologous chromosomes) which lead to several mistakes in amino acids coding and, thus, to major phenotypic consequences. Bioassays often exploit mechanisms of DNA repair, which aim, for instance, at restoring its pristine function or destroying the damaged cell by means of apoptosis. They are based on the action of multiple enzymatic reactions, which, allow repairing base excision, nucleotide excision, double strand breaks, and base mispairing. (Chatterjee and Walker, 2017; Croom, 2016; Stalter et al., 2016; Claxton et al., 2010; Dearfield et al., 2002; Turkez et al., 2017; Verheyen, 2017; Cartus and Schrenk, 2017; Basu, 2018).

Production of reactive oxygen species (ROS) and free radicals can be associated with carcinogenesis, immunotoxicity, teratogenesis and genotoxicity.

Although oxidative processes and the subsequent generation of free radicals are normal in the cellular metabolism of organisms (Finkel and Holbrook, 2000), oxidative stress is a condition of imbalance between

the antioxidant defense and the production of ROS, so that the defense is overcome by the formation of radicals (Halliwell and Gutteridge, 2015). This process may cause oxidative damage to membrane lipids, DNA and proteins, and lead to cellular dysfunction and tissue injury (Schieber and Chandel, 2014; Valavanidis et al., 2006; Sies, 2015; Neale et al., 2017a; Sies et al., 2017).

Oxidative stress can be induced through different mechanisms. They may affect the redox cycle by donating electrons to or withdrawing electrons from cell components. During metabolism, they may deplete glutathione (endogenous antioxidant) or even inactivate other endogenous antioxidants (Lushchak, 2011). In short, oxidative stress can act either through overproduction of free radicals or alteration of the antioxidant homeostasis (Abdollahi et al., 2004). Indeed, a close relationship was described between metal cytotoxicity, the total GSH content and the dissociation energy of the sulphur-metal bonds, confirming the involvement of antioxidant defense mechanisms in the toxic action of these ions (García-Fernández et al., 2002). Oxidative stress is also due to the alteration of antioxidant enzymes as glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferase (GST), catalase (CAT) and superoxide dismutase (SOD), which may lead to elevated lipid peroxidation (Bayoumi et al., 2001); (Garcia-Fernandez et al., 2002); (Koivula and Eeva, 2010). Increased plasma and erythrocytes concentration of thiobarbituric acid reactive substances (TBARs), changes in the antioxidant status, and impaired activities of cellular enzymes like superoxide dismutase (SOD) and catalase (CAT) indicate higher oxidative stress in pesticides sprayers. Hence, many researchers have associated exposure to pesticides with oxidative stress (Wafa et al., 2013), although concentrations occurring in water are usually too low to elicit this effect (Neale et al., 2017b).

Biomarkers can be chosen based on the biological damage they are linked to. For instance, membrane disruption might be associated with malondialdehyde, ethylene and ethane, isoprostanes concentration; ROS production affects glutathione, photosynthetic pigments, total phenols content. Other biomarkers may indicate more general phenomena, such ageing, decay and cell integrity (putrescine, spermidine, spermine) and undetermined stressors (proline) (Rhee et al., 2007; Pisoschi and Pop, 2015).

For such a complex scenario, it is essential to use an array of biomarkers to detect oxidative stress. Different antioxidants are involved in the protection against ROS through a close cooperation between them, and antioxidant defense may respond differently depending on the species used (Costantini and Verhulst, 2009). Hence, at least one marker of oxidative damage should be measured in order to draw inferences about oxidative stress (Costantini and Verhulst, 2009). Previous studies have shown that antioxidant enzymes, particularly GPx, CAT and SOD, and lipid peroxidation may function as useful biomarkers of metal induced effects on the antioxidant system in different bird species (Espín et al., 2014a; Espín et al., 2014b; Espín et al., 2016). Further studies on other taxa will yield a better understanding of the toxicity mechanisms induced by metal in wild birds and the definition of concentrations prone to cause effects on the antioxidant system.

3.2.4. Adaptive cellular stress response pathways

They allow the preservation of cell homeostasis after an exposure to external stressors (physical and chemical agents) and are measurable at low concentrations almost immediately after the external induction. The stressing agents include hypoxia, heat shock, exposure to chemicals and radiations; the defense mechanisms are mediated by specific transcription factors, being the endpoints, for instance, the modulation of cytokine production, the activation of Nrf2-antioxidant response element (ARE) pathway (Escher and Leusch, 2012; Leusch et al., 2014a, b; Escher et al., 2014; Neale et al., 2017b).

3.2.5. Baseline toxicity

Also termed "non-specific toxicity", it starts from the interaction between the substances and the cell membrane; hydrophobicity affects the capacity of the molecules to react and pass through these barriers, whose fluidity can thus be deeply modified. As a consequence, several biochemical pathways can be impaired, such as the electron transfer chain in photosynthesis, in case of vegetal cells, or specific enzymatic activities, linked for instance with the electrical signal transmission and the transport mechanism in case of animal cells. Baseline toxicity is quantifiable only at higher chemicals concentrations with respect to those triggering the adaptive stress response pathways. In several cases depending on the features of cell lines, bioassays can provide information which are transferable to the whole living system (Escher and Leusch, 2012; Escher et al., 2014). Among the most commonly used organisms (also with regard to laws in force and current regulations) there are the luminescent marine bacterium *Vibrio fischeri*, the cladoceran *Daphnia magna* and the green alga *Raphidocelis subcapitata*. The employment of whole organisms can highlight apical effects, which can derive from multiple toxicity pathways (Neale et al., 2017c).

4. Definition of assays for testing ecotoxicity: a focus on "lab-life"

In this section, attention will be focused on how the different biological responses or stresses potentially caused by micropollutants present in wastewater can be assessed in lab-scale tests. It is crucial that such assays can simulate the actual conditions occurring in case of receiving waterbodies and reused waters. Due to the challenges in collecting representative samples without losses and the inherent high costs for conducting proper toxicity assessments, a well thought-through sampling strategy and sample collection and preparation are of major importance.

4.1. Sampling strategy, sample collection and preparation

Wastewater concentrations of a wide range of compounds exerting plenty of unwanted biological responses vary considerably as a function of time, agglomerate type and treatment plant performance. All these factors can be dramatically different from site to site. For instance, the hydraulic retention time (HRT) and sludge retention time (SRT) are highly dependent on plant design (*e.g.* type and size of treatment units and internal flow patterns, including sludge treatment and reject water) and changes in volumetric loading (*e.g.*, due to storm water intrusion). When assessing acute toxicity, the worst-case scenario would usually be appropriate. However, chronic effects would better require average or median exposure conditions. Ort et al. (2010) detailed and explained all these aspects, highlighting the importance of the sampled volumes, collection duration, storage conditions and data elaboration.

If possible, (considering the storage time constrains) composite samples are preferred, taken by means of automatic samplers, usually collecting a volume aliquot every 10 min over a certain period, typically 24 hours. This would normally cover at least 1 HRT at most WWTPs, and it would be within the maximum recommended storage time (if stored refrigerated) for the most relevant compounds (ISO, 2012; McCall et al., 2016). In case of a longer sampling period, more 24-hours composite samples can be summed, possibly after a pre-treatment (see below). When planning the monitoring campaign, depending on the final goals, the expected weekly and seasonal variations may be taken into account (Sui et al., 2011), as well as the conditions of receiving waterbodies (see Section 5). For instance, the concentrations of some illegal drugs have been found to increase towards the weekends or in relation to popular events which draw the crowds (EMCDDA, 2016).

Passive samplers, able to discriminate hydrophilic and hydrophobic substances, represent an irreplaceable tool for monitoring the quality of the receiving bodies, thus, the impact of a wastewater treatment plant effluent (Li et al., 2013; Novák et al., 2018; Liscio et al., 2014; van der Oost et al., 2017).

Several factors can undesirably influence the composition of wastewater from collection to analysis: i) Compounds may be adsorbed to or

diffuse from the sampler tubing and container. Most of the larger WWTPs have an automatic sampling equipment installed, which should preferably be used to minimize these effects. ii) Depending on the WWTP scheme, the compounds of interest in effluent water samples will usually be in the range of μg/L to pg/L together with suspended solids and microorganisms (mg/L). Biodegradation preferably occurs in raw sewage and in the effluents, with respect to the receiving waterbodies. Hence, it is important to limit both biotic and abiotic processes after sampling. Sterile filtration (<0.2 μm) is an efficient way to stop biotransformation, though enzymes may still be present. It is also necessary prior to solid-phase extraction (SPE) to prevent clogging. 0.45 μm filters are more commonly used since they are less prone to blocking. Anyway, the choice of filter type is also crucial: polycarbonate or (low static charge) cellulose acetate filters may be preferred as *e.g.* nitrocellulose filters tend to bind proteins while nylon filters tend to bind proteins, DNA and RNA. Acidification with HCl or HNO_3 is often adopted, alone or after filtration, to preserve the sample. However, this may alter the speciation and stability of the compounds and, therefore, should be applied with care (Comerton et al., 2009). iii) Unaltered sample should be used in toxicity tests, but for sensitivity reasons (i.e. frequently, toxicity tests are short-term based assays) micropollutants in the filtered sample can be cleaned up and concentrated prior to application in bioassays by stepwise SPE and elution. The composition and concentration of the eluate depend on parameters such as physico-chemical properties of the compounds themselves, type of SPE, sample volume and percolation rate, sorbent cartridge volume, type of elution solvent and elution volume (see (Comerton et al., 2009) for a more detailed discussion). Anyway, solid phase extraction cannot retain most metals and salts; likewise, the majority of volatile organic compounds escape, thus modifying the final composition of mixture in the assay medium (Shane and Leusch, 2018). For this reason, recently it has been proposed to test the raw sample just immediately after a pre-filtration for sterilizing it prior addition to concentrated cells suspension (Niss et al., 2018)

The specificity of sorbent materials, by selecting the chemicals, reduces the range of substances subsequently exposed to the biological systems in the bioassays. The combination of different sorbent materials into a single cartridge during the solid phase extraction increases the recovery capacity, thus widening the number of chemicals possibly linkable with the toxicity registered during the execution of a bioassay (Neale et al., 2017d; Osorio et al., 2018; Neale et al., 2018).

Effect-directed analysis (EDA) is revealing a promising tool to find a causal link between chemicals and the induced adverse effects (in particular, in case of estrogenicity and androgenicity), by fractioning the sample by means of RP-HPLC (reversed phase-high performance liquid chromatography); single sample fractions can be characterised by lower cytotoxicity and masking effects (Hashmi et al., 2018).

Interestingly, the adoption of the high-resolution mass spectrometry (*e.g.*, Orbitrap and time-of-flight instruments) as a quantification technique, is appearing extremely advantageous, because both targeted and non-targeted analytes can be detected (Osorio et al., 2018). Non-targeted-analyses (NTA) are undoubtedly one of the most promising research perspectives (Shane and Leusch, 2018).

4.2. From research to standards: multifaceted approach in bioassays

Several national and international authorities and scientific and technical organizations are instrumental in compiling and evaluating toxicity tests such as the Organisation for Economic Cooperation and Development (OECD), World Health Organization (WHO), Food and Agriculture Organization (FAO), International Organization for Standardization (ISO), American Society for Testing and Materials (ASTM), United States Environmental Protection Agency (USEPA), United States Army Corps of Engineers (USACE), American Public Health Association (APHA), Association Française de Normalisation (AFNOR), Deutsches Institut für Normung (DIN), Italian Association for Standardisation in the Chemical Sector (UNICHIM). The level of worldwide methods harmonization is sometimes limited, thus a plethora of standard protocols exist with overlapping normalisation actions that sometimes can be conflicting in terms of sensitivity, meaning that each protocol has its own feasibility. Time-by-time authors must clearly declare which method they follow, to assure data reproducibility and correct interpretation.

Table S1 (Supplementary material) reports a list (necessarily not exhaustive) of the most commonly adopted toxicity tests, by pointing out the issuing agency and the measured response at the cell, tissue, organ, organism and ecosystem level. The principle of adverse outcome pathways, AOP (Perkins et al., 2015) allows to start from the initiating event, which possibly causes an adverse effect and to explore the whole biological pathway, up to the ecosystem level, by following a mechanistic approach. Endocrine activity testing is an example of such an application. The available assays can highlight both the interference with the hormone receptors, by means of agonistic/anti-agonistic activities, and, more generally, the interference with hormone synthesis and release.

The architecture of an assay involves simple cases, such as the mere formation of a bond between a ligand (either radio-labeled or bound with a fluorochrome) and an isolated receptor (thus gaining only analytical information). Options that are more complex rely on specific endpoints, such as protein activity (both in terms of protein synthesis and protein interactions with co-factors), cell proliferation and direct receptor activation linked with a gene reporter. The aforementioned tests employ several different techniques for the quantification of the biological activity. They range from basic approaches with UV-VIS spectroscopy, to the most exploited tools like ELISA, radio-immunology, and fluorometry (including flow cytometry) (NIEHS, 2002; Escher and Leusch, 2012; Scognamiglio et al., 2016).

Reporter gene-assays involve the use of cells (deriving from bacteria, yeasts, fish, humans and other mammals) to assess gene expression mediated by chemicals. The endpoints consist in cell proliferation in case of E-SCREEN (Scognamiglio et al., 2016; Bicchi et al., 2009; Schenk et al., 2010; Selma, Atsushi, Junkyu, Jamila, and Hiroko, 2014), while, most assays are based on gene expression, often after specific transfection. The main testing tools are the following: CALUX, CAFLUX, PALM, MELN, MVLN, T47D-kBluc, HELN, HGELN, MDA-kb2, PR reporter gene assay, YES, YAS, BLYES, BLYAS, BLYR (luciferase/fluorescent protein gene expression, β-galactosidase synthesis induction); they measure the binding with estrogen, androgen, progesterone, glucocorticoid, peroxisome proliferator activator receptors (Scognamiglio et al., 2016; Bertanza et al., 2010; Di Dea Bergamasco et al., 2011; Bertanza et al., 2011; Metcalfe et al., 2013; Bain et al., 2014; Wang et al., 2015; Conley et al., 2015; Bazin et al., 2016). Other tests are focused on the quantification of the production of proteins, such as vitellogenin, choriogenin, *zona radiata* protein after estrogenic stimulation (Ihara et al., 2015; Xuereb et al., 2011; Adeogun et al., 2016; Cavallin et al., 2016). Steroidogenesis based tests look promising in providing additional information on disruption mechanisms ((Cavallin et al., 2016; (Garcia-Reyero et al., 2011)) although *in vivo* compensation of the effects which occur during *in vitro* assays is far from being defined. Among the *in vivo* assays aimed to evidence the gene expression induced by pollutants, there is the application of the genetically modified *Danio rerio* (green fluorescent protein expression, controlled by a thyroid hormone response promoter of *Xenopus laevis*) already applied to environmental samples (Terrien et al., 2011; Scholz et al., 2013). An *in vitro* reporter gene assay (ER_α-luc assay) can be used for estrogen receptor activation to quantify the total estrogenic activity in liquid samples. Extracts from environmental samples (*e.g.* in petroleum ether) can be used to measure the estrogenic activity with a reporter gene assay (ER_α-luc assay) based on U2OS-ER_αcells, with luciferase as reporter (Quaedackers et al., 2001). The method to culture and expose the cells and to assay luciferase activity has previously been described in de Weert et al. (2008). Measurement of the estrogenic activity of nonylphenol during biological degradation showed a decrease of the estrogenic activity during microbial

degradation, and, consequently, can be used to determine the ecotoxicological risk of an environmental sample. An overview of pros and cons of the main assays applied for ecotoxicological purposes, in case of water ecosystems, together with basic technical information is reported in the paper recently published by Brack et al. (2016).

Besides, the study of the effects of EDs on populations and communities requires the setting up of mesocosm assays or the direct observation of real scenarios. It is worth noting that the pollutants (and mixtures) are effective, in parallel, at increasing levels, up to the ecological aspects, hence yielding to significant changes on the trophic web. This is the case, for instance, for *R. rutilus*, a planktivorous fish, whose grazing capacity is deeply reduced by the exposure to EE2; the population of plankton, as a result, can undergo a development (Hallgren et al., 2014).

Among the agencies and organizations which are facing the issue of endocrine disruption, the OECD approach can be cited, since it prescribes further subsidiary levels of investigations, in order to draw a complete profile of endocrine disruption (OECD, 2012). The five levels consist of: 1) acquirement of existing data about chemical, physical and toxicological properties, 2) execution of *in vitro* assays aimed to highlight endocrine pathways, 3) execution of *in vivo* assays aimed to highlight endocrine pathways, 4) execution of *in vivo* assays aimed to highlight adverse effects on endocrine endpoints, 5) execution of *in vivo* assays aimed to highlight adverse effects on endocrine endpoints throughout the whole life of an organism and across generations.

As far as genetic toxicity is concerned, the assays proposed in the scientific literature, the international standards (in particular, issued by OECD and ISO) and available on the market (automatized, in most cases) allow highlighting and quantifying multiple effects, from early, hence reversible modifications of genetic material, up to irreversible damages, which can evolve to either apoptosis or neoplastic formations. Therefore, the assays can be usefully integrated in a multi-layer frame, also due to the option of testing organisms of growing biological complexity (prokaryotes and eukaryotes) and situated at different levels of the trophic web (producers, consumers and decomposers). The detection of genetic damage induced by various mechanisms is made possible by performing *in vitro* and *in vivo* tests. The endpoints can involve a) gene mutations; b) chromosomal damage (to parts of the chromosomes); c) genomic damage (loss/gain of entire chromosomes) c) epigenetics.

Among the large number of tests either standardized or just proposed for the evaluation of water matrices, it is worth mentioning: a) the Ames test, for detecting point mutations in *S. typhimurium* bacterial strains; it is based on the growth of histidine revertant bacteria over specific culture media, with or without the addition of rat liver microsomal fractions. It is the most applied in case of environmental evaluations (Bertanza et al., 2013; Gonzalez-Gil et al., 2016; Magdeburg et al., 2014; Masood and Malik, 2013; Papa et al., 2016; Sharif et al., 2016). It takes 48 h to obtain a result. b) The micronuclei test, for detecting chromosomal mutations (generally performed on root cells of *A. cepa*, throughout 72 h) (Bertanza et al., 2013; Masood and Malik, 2013; Papa et al., 2016); it is a biomarker of chromosomic damage and genome instability. Its exposure depends on the employed organism. c) The Comet assay (also called SCGE, Single Cell Gel Electrophoresis), for quantifying the primary DNA damage; it is typically carried out on eukaryotic cells (Bertanza et al., 2013; Gonzalez-Gil et al., 2016; Papa et al., 2016; Sharif et al., 2016; Penders et al., 2012). d) The reporter gene assays, which detect the SOS response induced by DNA damage and have a duration of several hours; often automatized, they are less sensitive and robust than the aforementioned tests (Magdeburg et al., 2014; Weltens et al., 2012). e) The GreenScreen assay (GSA) which employs cells of *S. cerevisiae*; it detects a DNA damage, based on the quantification of a green fluorescent protein linked to the promoter of the RAD54 gene (Keenan et al., 2007; Zounková et al., 2007). f) The sister chromatide exchange (SCE assay) based on mammal cells (Penders et al., 2012), (Ohe et al., 2009).

Traditionally a wide number of enzymes, known to be involved in reactions against pollutants, are employed in toxicity tests. Unfortunately, in several cases enzymes react by means of induction or inhibition mechanisms, without a direct connection to the chemistry (*e.g.* leaving groups, electrophilic or nucleophilic functions) of the specific pollutants. Moreover, it is well-known that the effects of chemicals can be disguised by the action of several environmental factors, such as the feeding regime, temperature, water chemism, matrix effect, as well as biological aspects, including population genetics, reproductive cycles (Ippolito et al., 2017; Neale and Escher, 2013). Enzymes may rarely induce general stress rather than detoxification.

Therefore, it is important to clearly denominate the purpose of the assay in the frame of the toxicity testing. Enzymes like SOD, CAT, APOX, DHAR, MDHAR, GPOX and GR are members of the Halliwell-Asada-pathway (Halliwell and Gutteridge, 2015) detoxifying radicals and toxic oxygen species that might build up under xenobiotic stress (Lyubenova et al., 2009).

Enzymes of the metabolic cascade of xenobiotics, like the P450 and POX, as examples for phase I, would, on the contrary, act on the xenobiotic directly and activate it by inserting —OH groups into the molecule. Similarly, in phase II, GST and GT would conjugate glutathione or glucose to the activated xenobiotic, thereby detoxifying it (Schröder, 2007). However, there are also examples of direct attacks towards the pollutant, as for P450 and diclofenac or acetaminophen, and GST and lamotrigine.

Despite these differences in function, the mentioned enzymes are inducible by xenobiotics, and might exhibit elevated levels of activity in the respective assays. Table S2 (Supplementary material) lists the main enzymes employed in bioassays.

Among pharmaceuticals, antibiotics give seriously cause of concern, due to their indirect adverse effect on human health linked to the phenomenon of bacterial resistance. In clinical microbiology standardized susceptibility tests they clearly dominate among the available methods, aimed to detect possible drug resistance in common pathogens and to assure susceptibility to drugs for a particular infection (Jorgensen and Ferraro, 2009). In these tests, resistance is detected by carrying out growth inhibition tests broth (*e.g.* the macrobroth dilution test and the miniaturised broth dilution test) or by agar diffusion (*e.g.* the gradient diffusion test and the disk diffusion test). In most of these tests (except the disk diffusion test) the lowest concentration of antibiotic that prevents growth, represented by the minimum inhibitory concentration (MIC), is quantified. A more detailed discussion of advantages and drawbacks of these methods is given by Jorgensen and Ferraro (2009) and Balouiri et al. (2016). Such culture-based approaches typically require 1–2 days for fast-growing bacteria like *Escherichia coli* or *Salmonella* spp., and several weeks for slow-growing bacteria, like *Mycobacterium tuberculosis*. However, the main drawback of cultural methods is that the vast majority of strains present in environmental microbial communities (<1%; (Hugenholtz et al., 1998)) still cannot grow outside their host environment. Assessment of antibiotic resistance in such communities based solely on cultivable bacteria will therefore easily generate unrepresentative and biased results (Amann et al., 1995).

For that reason, tools for molecular detection of antibiotic resistance genes (ARG) have become increasingly popular (Schmieder and Edwards, 2012; Zhang et al., 2009; Gilbride et al., 2006). Polymerase chain reaction (PCR) assays such as multiplex PCR and quantitative real-time PCR (qPCR) have frequently been applied to amplify and detect specific ARGs in environmental samples (Zhang et al., 2009). Nevertheless, they only target well-studied pathogens or resistance-causing genes (as the primers are based on known resistance genes only) and cannot easily be used for broad-spectrum screening (Schmieder and Edwards, 2012). DNA microarray is a more powerful molecular method than the PCR assays as it is able to detect the presence or absence of a large range of ARGs simultaneously in a single assay (Gilbride et al., 2006). However, its use for environmental samples has been limited

as it is hampered by low detection limits (partially overcome if coupled with PCR) and the need for complicated pre-treatment to reduce the presence of other compounds that inhibit DNA extraction and/or target gene amplification (Zhang et al., 2009). Furthermore, both microarray and PCR based technologies are not conclusive regarding the detection of resistance genes in metagenomes (Mullany, 2014).

Metagenomic analysis is one of the latest modern approaches for analysing complex microbial communities and enables to describe the genetic potential of a community and to detect the presence/absence of genes or genetic variations responsible for antibiotic resistance (Schmieder and Edwards, 2012). Metagenomic analysis usually follows two different approaches, namely sequence-based and functional. In the first case, a sample of DNA from the studied metagenome is extracted and completely, but randomly, sequenced in relatively short contiguous sequence read lengths. These sequences are then compared with known sequences that have accumulated over the years in public databanks (reference sequences; *e.g.* McArthur et al., 2013) to identify resistance genes and/or mutations that are known to cause resistance (Schmieder and Edwards, 2012). This approach has the potential to identify all known resistance genes in a given metagenome. Though, important shortcomings are that it can only identify known ARGs and that it gives no information on expression of the resistance genes (Mullany, 2014). This is, however, overcome by the second approach, functional metagenomics, in which the extracted DNA is shot-gun cloned into cloning vectors and subsequently expressed in a cultivable surrogate host (usually *E. coli*) plated onto antibiotic-containing agar. If bacterial artificial chromosomes (BACs) are used, a larger gene fragment can be inserted, potentially making it possible to trace the phylogenetic origins of the original host bacteria (Mullany, 2014). These larger gene fragments are also more likely to include antibiotic resistance that is encoded by multiple genes. Disadvantages of using BACs is the low copy number (though, they are usually more stable than higher copy vectors) and the need for the transcription and translation signals to be efficiently recognized by the host organism. If vectors that only accept small inserts are used, the copy numbers are higher, and the host's transcription and translation systems can be used, hence the drawbacks of using BACs are circumvented. However, the small size of the insert will not normally allow information about the genetic background of the resistance gene. However, if coupled with sequence-based metagenomics, this disadvantage can be overcome to some extent. See Mullany (2014) and Schmieder and Edwards (2012) for a more thorough discussion of advantages and drawbacks in metagenomics analyses.

4.3. Criteria for selecting a bioassay

Toxicity assessment can be prescribed by regulatory or voluntary requirements, generally, referring to standardized methods to acquire, analyze and interpret data. On the contrary, when the final goal is a deeper investigation of the impact of effluents (or chemicals/mixtures) on specific biological targets, at different levels (from sub-cellular components to the whole community), either within a routine monitoring or for the evaluation of a specific polluted site, several alternatives arise (Neale et al., 2017c). The criteria underpinning the selection of a bioassay (or a battery of complementary assays) should include the duration, the required volume (smaller volumes may favor the miniaturization, hence the automation of the procedure), the price (capital expenses: building with related services, such as hydraulics and electrics, instrumentations; operation expenses: consumables, personnel, license fees), the throughput, the sensitivity (by taking into consideration possible non-monotonic responses), the specificity, the requirement of trained and skillful operators, the possibility to measure acute/chronic/transgenerational effects, the capability of evidencing toxicokinetic or, more generally, specific metabolic pathways of interest (Campana and Wlodkowic, 2018; Leusch et al., 2017).

A pivotal role is played by the personnel cost, which differs highly among the countries: the European example is revealing, varying the minimum wage per month from less than 250 € for Albania, to nearly 2,000 € for Luxembourg (Eurostat, 2016). Furthermore, the same test (*e.g.*, Ames on *S. typhimurium*) can be carried out either by adopting the conventional microbiological approach and cultivated bacteria or using commercial kits, including also genetically modified microorganisms.

A scientific advisory panel of California State recommended refining the criteria for modeling and predicting the environmental concentration and possible hazards of emerging pollutants by taking into account other aspects such as land and chemicals, population density. Besides, in vitro high-throughput bioassays focusing on the same mode of action should complete the monitoring, with the final goal of finding a link with potential health implications, like cancer onset (Maruya et al., 2014).

On the other hand, in case the objective is the assessment of the health of organisms living into an aquatic ecosystem, information provided by chemical and toxicological analyses may reveal inadequate in predicting and inferring their actual conditions: only a direct in situ monitoring of biological indicators can throw light on the ecological integrity. Moreover, a wrong prediction of the actual hazard for the aquatic organisms is likely to occur, by taking into account from the thresholds defined with the common laboratory bioassays (Ode and Schiff, 2009; Windsor et al., 2018; Leusch et al., 2010; Escher et al., 2018).

Laboratory protocols of the majority of bioassays, together with relative data analysis procedures still require harmonization, standardization and the implementation of a quality system (Leusch et al., 2010; Hartung, 2010); in several cases, there is still lack of regulatory acceptance (Leusch et al., 2014a, b; Shane and Leusch, 2018). Furthermore, there is a wide gap between the "academic toxicology" and the "regulatory toxicology", due to the scarce compliance, in the first case, with quality systems, like the Good Laboratory Practice. Consequently, reproducibility and repeatability of the results can seldom fade out; likewise, effectiveness of models, which include a multitude of partial and still stand-alone proposals (for instance concerning a specific mode of action) (Hartung, 2010).

An encouraging step forward has been taken in the field of drinking water, by the German Federal Environment, with the recommendation of health-related indicator values (HRIV), which provide for thresholds, set as a function of availability and completeness of toxicological data. The key effects include genotoxicity, neurotoxicity and germ cell-damaging potential; further investigations may profitably complement this battery (Kuckelkorn et al., 2018). A similar approach should be followed in case of treated wastewater.

It is worth noting that, similarly to Green Chemistry, Green Toxicology proposes a list of principles, which should be taken into account before planning the execution of a testing session: energy and materials saving, use of harmless reagents, minimization of animal use (in accordance with the 3Rs – reduction, refinement and replacement approach) are fundamental suggestions. A cultural change is. required by companies and policy makers: computational tools might provide early information about toxicity mechanisms of substances and health and safety. In silico and fully automated in vitro testing might precede and complement a further multitiered assays battery (whose quantity and burden could then be reduced) (Crawford et al., 2017; Maertens and Hartung, 2018). Starting from the Quantitative Structure–Activity Relationships (QSARs) and the QVIVE (quantitative in vitro-to-in vivo extrapolation) (QIVIVE) approaches, by further implementations, it is possible to predict adverse outcomes based on the effect concentrations (Ankley et al., 2010; Tang et al., 2013). Nevertheless, a definitive assessment of water quality cannot be reached by performing only in vitro tests (Shane and Leusch, 2018).

5. Environmental risk assessment: challenges and limitations

5.1. Traditional environmental risk assessment

Environmental Risk Assessment (ERA) deals with the interactions of agents or hazards, humans and ecological resources. It describes human

populations, ecological resources and agents, analyzes agents and exposure potential, characterizes the potential for adverse effects, defines uncertainties, generates options to deal with the risks, and communicates information about the risks to humans and ecosystems. ERA is a process that evaluates the likelihood or probability that adverse effects may occur to environmental values, because of human activities (i.e., a formal procedure for identifying and estimating the risk of environmental damage). ERA provides information for making reasoned decisions by defining the range of risks associated with various options, but it does not dictate a specific outcome. ERA also provides a mechanism for managers to communicate forecasted risks associated with decisions, such that stakeholders and the public are informed of the implications for environmental values.

Based on the toxicological data and measured environment concentrations found in the literature, the risk for acute toxic effects is unlikely but chronic adverse effects cannot be excluded. Therefore, risk characterization is one of the important tools to estimate the environmental risk, particularly in view that co-occurrence of diverse micropollutants in environmental matrices may lead to additive, synergistic, and antagonistic toxic effects which is difficult to predict if only concentration is available.

5.2. Wastewater toxicity assessment, ranking, and reuse

The problem of wastewater toxicity data management and interpretation is still a current issue, especially when high toxicity levels are recorded and there are compulsory legislative threshold limits to comply with Libralato et al. (2010a), Libralato et al. (2010b), and Libralato et al. (2016). Around the world, countries have developed various toxicity-based methods to assess the quality of treated wastewater to increase the accessibility to water and sanitation in order to avoid human health impacts and ecosystem services impairment. Several procedures for discharge hazard estimation have been proposed generating assessment toolboxes including limit-based threshold approaches, and toxicity score and index for data integration and interpretation including expert judgment as well (Libralato et al., 2010a). The main goal of wastewater ecotoxicity assessment and ranking should be to minimize the adverse impact onto the receiving water body as well as treated wastewater recovery and reuse (Libralato et al., 2012). Apart from the possibility of using toxicity tests to estimate potential hazardous effects on the ecosystem, they can favour the protection and the optimization of wastewater treatment plant operation, by discriminating the best available technologies (Libralato, 2013); (European Comission, 2014)). Consistent wastewater toxicity assessment can increase the general level of sustainability in the management of water resources pushing ahead both “zero emission” and “zero discharge” along with the precautionary principle (OSPAR, 2005).

Toxicity is currently used to check effluent quality into various national legislation around the world to be included in water monitoring and control programs like direct toxicity assessment (van Dam and Chapman, 2001), whole effluent toxicity, integrating controlling of effluent, whole effluent environmental risk, environmental effects monitoring (Power and Boumphrey, 2004), and whole effluent assessment (OSPAR, 2005), (Protection and Assessment, 2000). Apart from any program peculiarities, the main question is still how to use or “interpret” toxicity data keeping in mind that the objective is to protect the environment and not the “white rat” testing species (Calow, 1994).

Generally, legislative requirements tend to refer to a toxicity limit based on a single test or a battery of toxicity tests considering as result the worst registered data. This method is quite simple, but not environmentally realistic, depending on the biological model-endpoint pairs considered and the weight-of-evidence score attributable to each of them. Sometimes, the classification is attributed just on a logarithmic (Bulich, 1982; Sarakinos et al., 2000) or order of magnitude basis (Costan et al., 1993; Swedish EPA, 1997; Tonkes et al., 1999; Persoone et al., 2003) or expert judgment and regression analysis pair (Vindimian et al., 1999). Some authors tried to overcome such drawbacks by identifying tools to integrate and weight toxicity data on a statistical basis also according to the ecological relevance of the considered endpoint (Libralato et al., 2010a). For example, Libralato et al. (2010a) and Libralato et al. (2010b) applied the minimum significance distance (MSD) criterion to support general decisions about the presence or absence of toxicity from wastewater samples on a database of more than 100 wastewater toxicity data including domestic, municipal and industrial discharges (Phillips et al., 2001; Thursby et al., 1997). This method enabled the consideration on a species-specific basis. Thus, the relative sensitivity of the biological model made the assessment of toxicity independent to reference wastewater as well. Moreover, expert judgement was reduced to a minimum just in relation to the choice of the number of ranking classes and their extension in case of more toxic samples. This kind of approach produced a toxicity score with classes (absent, low, medium, high and very high toxicity) composed of two sub-scores. The first series of sub-scores (absent or low toxicity) was partly based on the percentage of effect responses and partly on toxic unit values. The second series of sub-scores was entirely defined on toxic unit values including a medium, high and extremely high toxicity threshold. The main limits of this approach are related to the fact that each toxicity score is species-specific and databases including wastewater toxicity data must be developed *ad hoc* also to support the data statistical reliability.

Further efforts are necessary to identify case-specific toxicity tests (country- or discharge-based), supporting their round robin and toxicity data integration methods in the perspective of EU legislative harmonization.

5.3. How reliable is our risk assessment in the receiving water bodies?

Within the Water Framework Directive (WFD) the term “ecological status” of a water body primarily embraces the biological responses caused by other pollutants than micropollutants, but priority micropollutants are taken into account through an environmental risk assessment (ERA) scheme by implementing Environmental Quality Standards (EQS) that should not be exceeded in the environment (Directive, 2013). The EQS values are set by each member state based on the predicted no effect concentration (PNEC) for each compound in water, sediment and/or biota. However, available ecotoxicity data are often limited, especially for metabolites and transformation products. Therefore, traditional ERA, as described by the European Commission Technical Guidance Document (TGD), allows the use of assessment factors (AFs) to account for the uncertainty in deriving PNEC values based on acute toxicity data and a limited number of species (EC, 2003). The intention of the use of AFs is to predict a concentration below which an unacceptable effect will most likely not occur. Data on persistence in the environment (i.e. lack in biodegradability) and bioaccumulation should also be considered. An AF of 1000 is advised if only acute toxicity data are available for three trophic levels (algae, daphnids and fish). Only highly rarely sufficient data on long-term effects at several trophic levels and taxonomic groups exist for a given compound to be used for statistical extrapolation methods to derive a PNEC value. For biologically active compounds such as pharmaceuticals, this approach may, however, overlook sub-lethal and subtle subcellular effects that might occur in some species at much lower concentrations during chronic exposure. The complexity implied by the cocktail effects of compound mixtures and the large number of unknown transformation products during degradation in the environment warrants a switch to a more effects-oriented approach when assessing the environmental risk. Hence, the combined effects from all compounds in water or sediment samples are assessed using a set of toxicity tests targeting *e.g.* baseline toxicity, estrogenic and mutagenic activity and oxidative stress. The main drawback of this effects-oriented approach is that it is not able to identify the actual compound(s) that are asserting the observed effects. But if it is combined with the above-described MEC/PNEC (or

MEC/EQS), any major discrepancies between the observed effects and the calculated MEC/PNEC values relevant to the respective effects may be used to identify "missing" contributing compounds and warrant more detailed analyses or studies. Still, true food web effects are not covered, leaving the question open whether an ecosystem hazard may be possible. Discharges from WWTPs are only one of many possible routes for micropollutants to enter the aquatic environment, and the environmental risk assessment (ERA) of discharges to a water body should take them all into account. Similar approaches as described above for the water body may be performed. Instead of measuring the actual environmental concentration (MEC), the environmental concentration is predicted (PEC) from concentrations in the effluent from the WWTP, the total discharged volumes and the immediate local dilution in the receiving waters. For compounds that are persistent in the environment and/or bioaccumulate a more long-term and regional assessment may be needed, including the potential accumulation in sediment.

Actually, any industrial agricultural, farming, commercial and recreational activity (including boats and ships), as well as living units discharging wastewater to water bodies, standing both on freshwater and marine environments, need to know the nature and the extent of impacts associated with their liquid emissions. These issues are driving the need for a more detailed assessment of the impact of wastewater discharges to support decision-making. The integrated assessment of biological effects of discharges in the ecosystems is relevant and ecotoxicity tests are referred to as extremely useful tools for the identification of environmental impacts (Mendonça et al., 2009). The use of the ecotoxicology can provide an added value to hazard and risk assessment of discharges to the receiving water bodies. Environmental management can take advantage from safe and non-toxic treated wastewater, supporting its recovery and reuse, as in case of non-potable purposes. Ecotoxicity tests can identify the hazard and be directly used in ecological risk assessment. Within the WFD, direct toxicity assessment of WWTP discharges can contribute to attain or keep ecological quality objectives in water masses and finally provide the postulated "good" quality of all water bodies in the EU.

Besides, the assessment of traditional acute and chronic (short- and long-term) toxicity tests, treated wastewater evaluation in the perspective of its reclamation and reuse presents new potential ranking tools like effect-based trigger values (EBTs), as abovementioned in Section 3.2. EBTs can be derived from the safe levels based on average daily intakes from existing toxicity databases according to with various approaches to be explicitly declared each time (i.e. different algorithms produce different thresholds). This means that EBTs approach is a chemically oriented approach based on specific pollutants (*e.g.* androgenic (AR), estrogenic (ERα), glucocorticoid (GR), and progestagenic (PR)) rather than on effects on bioindicators. About EBTs, discussion is still open on how to include the mixtures (Escher et al., 2018), and how to cope with substantial difference between whole effluent testing (WET) and bioanalytical assessment considering that EBTs are derived only for organic micropollutants. Thus, they cannot be applied to wastewater in the case of other non-organic components (i.e. metals and other inorganics) as the main causative agent (Escher et al., 2014; Escher et al., 2018). This means that traditional toxicity tests integrating the effects of reclaimed wastewater to an exposed population cannot be entirely substituted just with EBTs, at least according to their current definition. Moreover, also traditional bioassays should be considered prevalently in their chronic exposure: quality standards must be highly demanding for both traditional bioassays and the use of EBTs because once (ground)water is contaminated the treatment/remediation could be very expensive or sometimes impossible to be carried out.

5.4. Socio-economic aspects

Monitoring and predicting trace pollutant concentrations in the aquatic environment, together with their possible subsequent toxicity, are vital in order to better assess the environmental impact as well as the risks for human health. Thus, new effective tools for estimating the occurrence of these substances are needed. A recent method is based on online search queries, though this only applies to those that are widely known by the public. For example, considering pharmaceuticals, the prescription issuing in the UK of several substances included in the EU watch list for water monitoring (2015/495, 2015) is suggested to be correlated to online search queries (Mavragani et al., 2016). As the concentrations of antibiotics in wastewater seem to follow the trend of prescriptions (Le-Minh et al., 2010), search traffic data could be proven a valuable tools in predicting the occurrence of pollutants in wastewater.

The choice of proper removal treatment as well as the overall assessment of its environmental, economic, and social impacts needs to be assessed with caution (Melvin and Leusch, 2016), and must necessarily take into account pollutants loads, which, unfortunately, can be affected by extreme variability. Therefore, all the cost items might be accurately overweighed, to avoid wastes of energy and material resources, land consumption, and to reduce pollution towards other environmental matrices. Recently, Life Cycle Assessment (LCA) has been applied to evaluate the economic and environmental viability of processes aimed to remove trace pollutants from wastewater ((Hernández-Padilla et al., 2017; Pintilie et al., 2016)); this instrument provides standardized criteria to compare alternative options by taking into account different impact categories.

In any case, the effective step towards the reduction of trace pollutants emissions and, consequently, their effects on the environment is definitely a management at the source. Green chemistry principles (Anastas and Warner, 1998) are the essential criteria for designing new production and supply chains, as well as disposal and treatment. The example of pharmaceuticals is emblematic. Medical professionals and patients should employ, if possible, products manufactured in accordance with the green pharmacy principles, *e.g.* using pharmaceuticals that are designed to be better biodegradable (Rastogi et al., 2014; Kümmerer and Clark, 2016). Disposal of unused medicines is mostly carried out through household waste (Bound et al., 2006), toilets and sinks (Kotchen et al., 2009; Straub, 2016; Tijani et al., 2013). As many do not regard this as an environmental issue (Bound et al., 2006), it is evident, that public awareness is vital, together with the need for better public information (Straub, 2016). Over the past decades, attention has also focused on return policies advertisements (Bound et al., 2006) and the importance of people information on the correct disposal (Bound et al., 2006; Straub, 2016). As a consequence, population willingness to pay for a better waste treatment system increases (Kotchen et al., 2009; Logar et al., 2014). Governments should implement the regulatory frameworks for improving the whole water cycle management (Morris et al., 2017). According to the Polluter Pays Principle, environmental damage should be decreased by introducing advanced treatment technologies, which should be paid by the final users. Therefore, conventional tariff policies aiming to charge all households as a function of wastewater production are not in accordance with the Polluter Pays Principle. It has been shown, that increased charge rates and penalties do not contribute to more environmentally friendly practices (Lu et al., 2016). Thus, in order to internalize the externalities of using products, which potentially release micropollutants, the purchase cost should be increased in order to subsidize the removal/remediation expenses. Revenues should be allocated to upgrade WWTPs, with the unfailing support of national (and, possibly, international) policies which consider the global social and environmental costs due to the use of such substances, together with the costs for water treatment (from drinking water supply, to wastewater collection and purification).

6. Interpretation of eco-toxicity data: case studies

In recent years, some authors have applied toxicity tests to diverse applications. In this section, some case studies are presented, which demonstrate the power and versatility of such investigations. For this

purpose, the examples chosen include a range of different scenarios, in terms of: employed bioassays (crustaceans, algae, bacteria, etc.); tested matrices (*e.g.*, municipal and complex wastewater); adopted treatment systems (conventional activated sludge process, membrane bioreactor, ozonation, photocatalysis, sonication, anaerobic process). Some of the aforementioned experiences have been carried out at the full scale.

The pivotal role of bioassays in the integrated assessment of the environmental impact of wastewater is clearly manifest in all the reported cases.

6.1. Ecotoxicity removal from complex wastewaters: comparison among conventional and advanced technologies

Currently, water quality standards and wastewater discharge limits in the European Union are mostly based on a limited number of chemical parameters. The aim of The European Water Framework Directive (European Parliament, 2000) is to obtain water bodies with a "good" biological quality. The biological or ecological impact of complex industrial effluent discharges however, cannot be estimated using chemical assays only, but should be measured using whole effluent toxicity (WET) tests (*e.g.* OSPAR, 2005).

A typical example of a complex industrial effluent is the water originating form tank truck cleaning (TTC) activities. The TTC process mainly involves the cleaning of tank truck interiors. The wide spectrum of transported cargo, ranging from food products to hazardous chemicals, results in wastewater with a highly variable composition. De Schepper et al. (2010) reported that a significant residual toxicity was still present in biologically treated TTC effluent. A battery of acute ecotoxicity assays, with *Raphidocelis subcapitata* (primary production), *Vibrio fischeri* (decomposition) and *Daphnia magna* (primary consumption) was applied to assess the whole effluent toxicity. It was found that the effluent of the full-scale treatment plant was extremely toxic to *R. subcapitata* with toxicity values ranging from 800 to 3260 TU (toxic units).

The aim of a subsequent study was to investigate the removal of acute toxicity from TTC wastewater by a series of key unit operations applied during the treatment of industrial wastewater, i.e. chemical coagulation, activated sludge treatment and sorption by activated carbon (Dries et al., 2013). The treatments steps were performed on a laboratory scale, in order to assess the full toxicity removal potential of these technologies. The rapid *V. fischeri* bioluminescence inhibition test (applying a 30 min contact time) was used to assess toxicity removal. Chemical pretreatment of the wastewater by coagulation with $FeCl_3$ removed approx. 38% of the influent chemical oxygen demand (COD) and reduced the bioluminescence inhibition by 8%. Biological treatment with activated sludge subsequently removed another 77% of the remaining COD. This treatment step also reduced the bioluminescence inhibition but the removal efficiency varied strongly from 5 to 92% for the different samples.

The ecotoxicity of the biotreated samples was also analyzed with the 72 h algal growth inhibition assay using *R. subcapitata*. The TU values ranged from 610 to 5470, confirming the very high algal growth inhibition reported for the same type of wastewater by De Schepper et al. (2010).

Powdered activated carbon (PAC) almost completely removed the remaining COD and inhibition in all samples. The algal growth inhibition after PAC addition ranged from 23 to 82 TU, corresponding to a reduction of more than 95%.

These results suggest that conventional technologies did not suffice for complete removal of toxicity from TTC wastewater, and that advanced wastewater treatment technologies are required for a satisfactory detoxification.

6.2. Removal of estrogenicity from municipal wastewater: comparison between MBR and CAS systems

A monitoring campaign was conducted on a full scale municipal WWTP, consisting of 2 CAS and 1 MBR (ultrafiltration) parallel lines. The design size is 250,000 p.e. and the influent load is split about 50% on the MBR train and 25% on each CAS line. The plant is operated according to the modified Ludzak-Ettinger process scheme, with chemical phosphorus removal (aluminium sulphate dosage into the biological reactors).

Both chemical and biological analyses were carried out all along a 19 days period, in order to compare the CAS and MBR processes in terms of EDCs removal. The following target substances were measured: 4-nonylphenol (NP), its parent compounds 4-nonylphenol monoethoxylate (NP1EO) and 4-nonylphenol diethoxylate (NP2EO), and bisphenol A (BPA). The same wastewater samples used for chemical analyses were submitted to the measurement of hormonal activity by means of human breast cancer MCF-7 based reporter gene assay, using 17β-estradiol (E2) as a standard.

Removal efficiency and residual effluent concentration of target compounds were quite similar for both CAS and MBR lines, ranging between 70% (BPA) and 95% (NP1EO) and from 0.3 mg/L (NP1EO) to 0.8 mg/L (NP), respectively. The CAS and MBR lines were operated at a sludge age of 9 and 15 days, respectively, the sewage temperature being around 23 °C. The reason for the different plants to have similar performances can be explained based on the well-known relevance of these operating parameters: Clara et al. (2004) and Clara et al. (2005) demonstrated that any increase of sludge age and temperature above 10 days and 10 °C does not lead to noticeable improvements, regardless of the type of process (either CAS or MBR). Moreover, several Authors (*e.g.* Koh et al., 2009; McAdam et al., 2010; Verlicchi et al., 2012; Hicks et al., 2017) evidenced the positive effect of an efficient nitrification on EDCs removal.

Nevertheless, even if no appreciable difference in the EDCs effluent concentration was detected, biological measurements showed that the MBR effluent exerted a lower estrogenic activity (estrogenicity, expressed as Relative Light Units, and normalized towards protein concentration, was up to 50% lower in MBR effluent samples, ranging from 1.0 to 3.5×10^7 RLU/$mg_{protein}$). The higher performance of the MBR system is likely attributable to the more efficient retention of suspended solid, and, consequently, of specialized slow-growing bacteria and of the organics to be degraded (in case they are adsorbed onto the suspended solids).

The findings confirm the irreplaceability of bioassays in the monitoring of any impact on the ecosystems (in this case, the biological reactor o fa WWTP). Detailed results are reported in Bertanza et al. (2011).

6.3. Removal of antibiotics and their effects of anaerobic and aerobic systems

As the working principle of antibiotics inhibits biological activities directly, their adverse/inhibitory effects on the biodegradation of organic compounds in the wastewater treatment plants are one of the main concerns. In order to evaluate the inhibitory impact of these compounds in biological systems, two different experimental approaches are commonly applied: short-term (acute) and long-term (chronic) tests. The short-term, acute tests usually involve a non-acclimated microbial population to the inhibitor. In long-term experiments with continuous feeding of the antibiotics, the test may reflect, aside from changes in substrate removal and utilization, adaptation and/or resistance of the microbial community or even shifts in microbial composition in response to continuous exposure (Pala-Ozkok et al., 2014a; Cetecioglu et al., 2016). While Kümmerer and his colleagues (Kümmerer et al., 2004) argue that short-term assays would not be sufficient to investigate the effect of antibiotics on complex microbial systems because of different mechanisms associated with acute and chronic inhibition, Alighardashi et al. (2009) propose that the microbial community becomes well adapted to a synthetic substrate, which is a significantly different scenario from biomass in a full-scale plant under long-term exposure. Despite different opinions expressed in the

literature, these two inhibition tests complement one another and reflect real-life inhibition schemes encountered in wastewater treatment.

In the light of this knowledge, acute and chronic tests were applied to aerobic and anaerobic biological treatment systems with three selected antibiotics: sulfamethoxazole (SMX), tetracycline (TET) and erythromycin (ERY).

For the aerobic acute tests; laboratory-scale fill-and-draw reactors with hydraulic retention time of one day were established and sustained at sludge ages of 10 and 2 days at steady state under aerobic conditions (Pala-Ozkok, 2012), and a series of fully aerated batch reactors for kinetic investigations of peptone-meat extract mixture biodegradation and acute/chronic inhibition of the selected antibiotics (Ozkok et al., 2011; Pala-Ozkok and Orhon, 2013; Pala-Ozkok et al., 2014b). Fill-and-draw reactors were fed with peptone-meat extract mixture at concentrations characterizing domestic wastewaters. To determine the acute and chronic inhibition effects of the selected antibiotics, batch experiments were conducted with 50 mg/L antibiotic additions (Pala-Ozkok, 2012). Respirometric tests were performed to determine the effect of antibiotics on non-acclimated (acute effect) and acclimated (chronic) biomass, which yielded oxygen uptake rate (OUR) profiles. Obtained OUR profiles were used for simulation to determine the kinetic properties of each activated sludge biomass (Pala-Ozkok and Orhon, 2013; Pala-Ozkok et al., 2014b). Reactors were monitored for COD, suspended solids (SS), volatile suspended solids (VSS) and polyhydroxyalkanoates (PHA) (Beun et al., 2000). The inhibitory impact of selected antibiotics was observed as a decrease in the amount of oxygen consumed in the OUR tests, which led to the conclusion that antibiotics have the property to block the microbial substrate consumption (Pala-Ozkok, 2012; Ozkok et al., 2011). The kinetic evaluation revealed that antibiotic substances mainly increase endogenous decay levels, the half-saturation constant of the substrate and inhibit hydrolysis of different COD fractions (Pala-Ozkok, 2012; Ozkok et al., 2011; Pala-Ozkok and Orhon, 2013; Pala-Ozkok et al., 2014b).

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For the determination of short-term inhibition effects of the selected antibiotics under anaerobic conditions, a series of batch reactors seeded with acclimated microbial culture were run and fed with volatile fatty acids (VFAs) in terms of acetate, butyrate, and propionate. Each reactor was also inoculated with a different concentration (1–1 000 mg/L) of the selected antibiotics (Cetecioglu et al., 2012; Cetecioglu et al., 2015a). The batch reactors were kept running for 6 days. Soluble COD and VFAs concentrations were monitored both at the beginning and at the end of the observation period. Total COD with soluble and particulate fractions were measured at the completion of the test in selected reactors for mass balance. Biogas production and methane generation were measured daily through- out the experiment. Organic substrate removal was monitored by both soluble COD and acetate measurements, together with daily measurements of biogas and methane generation. Sole acetate fed test showed that acetate was almost fully removed in all experiments, while methane generation exhibited a significant drop with increasing antibiotics doses (Cetecioglu et al., 2012). Almost complete methane inhibition was observed for antibiotics doses above 500 mg/L. The monitored effect was found coherent with uncompetitive inhibition, which similarly exerts a binding impact on substrate–enzyme complex. For VFA mixture (acetate, propionate, and butyrate fed system), at lower doses, the VFA mixture was completely removed but partially used, leading to reduced biogas and methane generation, suggesting the resemblance of uncompetitive inhibition (Cetecioglu et al., 2015a), (Cetecioglu, 2011).

Anaerobic chronic inhibition tests represented different results from acute tests. The experiments involved anaerobic sequencing batch reactors fed with a synthetic substrate mixture including glucose, starch, and volatile fatty acids, and operated in a sequence of different phases with gradually increasing antibiotics, for more than five months. TET exerted a terminal/lethal effect at 8.5 mg/L on the microbial community, which caused the inhibition of substrate/COD utilization and biogas production and leading to a total collapse of the reactor (Cetecioglu et al., 2013). The microbial activity could not be retrieved and re-started within a period of more than 10 days, even after stopping TET dosing. During the experiments, TET was partially removed either through biodegradation or conversion into its by-products. The adverse long-term effect was quite variable for fermenting heterotrophic and methanogenic fractions of the microbial community based on changes generating on the composition of remaining/residual organic substrate. The results revealed that anaerobic treatment was suitable for pharmaceutical industry wastewater with concentrations of up to 40 mg/L of SMX. Higher levels exserted toxic effects on the microbial community under anaerobic conditions, inducing the inhibition of substrate/COD utilization and biogas production and leading to a total collapse of the reactor. The adverse long-term impact was quite variable for fermentative bacteria and methanogenic archaeal fractions of the microbial community depend on changes inflicted on the composition of the residual organic substrate and mRNA expression of the key enzymes (Cetecioglu et al., 2015b). ERY fed reactors showed that methane production and VFA recovery are simultaneously possible up to 2 mg/L of ERY. ERY exerted a terminal effect at 3 mg/L on the biomass, and the activity could not be recovered after stopping ERY dosing (Cetecioglu, 2011).

Also, another study was performed to reveal if anaerobic-aerobic biological treatment strategy is proper for antibiotic production waste streams. Although activated sludge treatment systems are inhibited by the low concentration of antibiotic mixture, the same aerobic system can tolerate higher concentrations of the same mixtures after an anaerobic pre-treatment (Cetecioglu, 2014).

6.4. Removal of estrogenicity from textile wastewater by means of ozonation

A pilot scale ozonation plant was installed at the outlet flow of a CAS plant (design size 370,000 p.e., located in Northern Italy) treating mainly domestic wastewater. The CAS process scheme includes primary settling, pre-denitrification and oxidation-nitrification, secondary settling. Main CAS effluent characteristics are: 30 mg COD/L, 5 mg BOD_5/L, 12 mg TSS/L, 6.5 mg TKN/L; 4 mg NH_4^+-N/L, 4 mgNO_3^--N/L, <0.1 mgNO_2^--N/L, 1.3 mgP_{TOT}/L.

The O_3 pilot plant consisted of a stainless-steel tubular reactor (volume = 1460 L) equipped with a pure oxygen supply system (capacity = 400 gO/h). The reactor was fed with a flow-rate up to 6 m^3/h in a continuous mode of operation. Two different dosages were tested, namely 8 and 11 mg O_3/L, with an HRT of 20 min.

The estrogenicity of wastewater was reduced from 7.35 down to 3.25×10^7 RLU (Relative Light Units)/$mg_{protein}$ (about 55% removal efficiency) by means of ozonation, under the lower dosage conditions.

Nevertheless, while the higher O_3 dosage led to an appreciable improvement of EDCs removal (data not shown: see full data in (Bertanza et al., 2011)), only a slight additional reduction of hormonal activity was achieved (measured value = 2.90 x 10^7 RLU/$mg_{protein}$; removal efficiency = 60%). The difference between the chemical and the biological answer may be due to the formation of active by-products, metabolites and/or conjugates, able to exert an estrogenic activity comparable to those of parent compounds, and to the synergistic and additive effect among the different compounds.

In summary, the information gathered from chemical analyses was somehow misleading: the power of ozonation was overestimated; on the contrary, the bioassay gave a more realistic evaluation of the results obtainable.

6.5. Removal of emerging pollutants from municipal wastewater by means of photocatalysis and ultrasound treatments

Photocatalysis and ultrasound treatments have been widely investigated for the treatment of emerging pollutants in urban wastewaters, including EDCs, pharmaceuticals, personal care products, drugs (Belgiorno et al., 2007; Rizzo et al., 2009; Carotenuto et al., 2014; Lofrano et al., 2016). Since during the oxidation process some by-products (intermediates) are formed and the effluent may become more toxic than the untreated solutions or the parent compounds, respectively, the overall efficiency of the treatment process for this class of chemical pollutants strictly depends on the toxicity and estrogenic potency of treated effluents.

The toxicity of photocatalytic degradation of caffeine, the number one drug worldwide, has been investigated in aqueous suspensions of titanium dioxide (TiO_2) (29.3–170.7 mg/L) and initial drug concentrations (0.76–9.24 mg/L) by Carotenuto et al. (2014)). Caffeine was quickly degraded, but not mineralized as quickly, and it was found that persistent toxic organic intermediates resist further oxidation producing toxicity on *D. magna* at 24 h and 48 h. *Raphidocelis subcapitata* showed to be more sensitive to by-products than *L. sativa*.

A set of bioassays (*Daphnia magna*, *Raphidocelis subcapitata* and *Ceriodaphnia dubia*) was performed to evaluate the potential detoxification of the antibiotic vancomycin B hydrochloride (VAN-B, 50 mg/L) and its oxidation by-products under acute and chronic conditions. The toxicity of the photocatalytically treated VAN-B samples varied during the oxidation, due to the formation of some intermediate by-products that are more toxic than VAN-B. Despite almost total removal of VAN-B that was achieved within 120 min of irradiation with 0.2 g TiO_2/L, a significant increase in toxicity was observed in chronic tests proving that the chronic assays are more sensitive than acute ones to detect the impact of by-products formed during the photocatalytic degradation of antibiotics (Lofrano et al., 2014). The residual toxicity of photocatalitically treated solutions of chloramphenicol sodium succinate (CAP, 25 mg $L^{/L}$), which is a broad-spectrum antibiotic, evidenced a decreasing trend in toxicity at increasing concentrations of TiO_2 and photo-oxidation times. After 120 min of photo-oxidation the most significant effect on *Vibrio fischeri* ($p < 0.05$) was obtained at 1.6 g/Lof TiO_2 with a residual toxicity of 8 ± 6% (5 min) and 10 ± 4% (15 min). Lower TiO_2 concentrations showed toxicities ranging between 45–62% (5 min) and 53–76% (15 min) (Lofrano et al. (2016)).

The toxicity of the mixtures of three pharmaceuticals (2.5 mg/L, diclofenac, DCF, 2.5, 5 and 10 mg L^{-1}, amoxicillin, AMX, 2.5, 5 mg/L carbamazepine, CBZ) at different concentrations in contaminated urban wastewater treated by ultrasound has been evaluated by Naddeo et al. (2009). Sonication decreased toxicity of contaminated WW sample to *R. subcapitata* and no significant effect on this decrease by either the sonication time or the applied power density was observed. *R. subcapitata* was found more sensitive than *D. magna*.

Toxicity data about photocatalysis and ultrasound treatments are still in their infancy, especially for sonolysis where just few studies have been performed. From the available results it can be stated that photocatalysis can be suitable to fully remove toxicity at the discharge but focused research must be oriented specifically, not only on target compound removal but also on effluent toxicity goal. Moreover, toxicity investigation must comply with the international recognized approach, considering the integration of at least three species belonging to different phylogenetic levels [149], [150].

7. Conclusions

This paper reports the shared opinions of the participants to COST Action ES1202 Conceiving Wastewater Treatment in 2020-Energetic, environmental and economic challenges (Water_2020) about the topic of toxicity of wastewater trace organic pollutants.

Notwithstanding the valuable literature production, which, up to now, includes also hundreds of reviews, the choice to write another work about the topic of toxicity of wastewater organic trace pollutants arose from the awareness that there are still gaps between the different scientific sectors involved in this research. The debated subjects, indeed, pertain to several disciplines and have been connected based on the final goal to propose criteria for choosing the proper tools to assess and reduce the possible environmental impact of such pollutants on the human health and the aquatic ecosystems.

Keeping in mind that: 1) toxicity proceeds by following a cascade of events, after the initial molecular event, and it spreads, in principle, up to the ecosystem level; 2) it may be possible to link MIEs with KEs up to the different outcomes, by following single toxicity pathways 3) it may be possible to make the results extrapolation “in vitro to in vivo” 4) several emerging pollutants of concern (as well as unknown molecules) can be measured and linked with the toxicity exhibited by a sample (and, even from single fractions of it), a basic question still remains unanswered. Is such huge amount of information (acquired costly in terms of time and money) capable to describe the health state of an ecosystem and to assess effectively possible risks towards the organisms which live in (and get sustenance from) it? In other words, once obtained the biological responses and a chemical characterization, are we able to define the actual effects of a discharge and, consequently, to intervene in order to prevent/reduce possible damages to the aquatic ecosystem (and maybe to human health)?

Finally, which contribution might our detailed and more and more accurate monitoring give to policy makers in terms of threshold values and quality goals definition? How can we transfer the composite and complex knowledge, acquired in most cases by following unique, even if rigorous protocols?

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