Opinion paper on strategies for toxicity assessment of organic trace pollutants in wastewater

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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 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 has been focused on the choice of bioassay(s), by analysing the meaning of biological answers. Data interpretation and exploitation are detailed with the final goal of providing criteria 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 integrated and exemplified the proposed approach.

Keywords

Aquatic ecosystem; bioassays; ecotoxicity; micro-pollutants; risk assessment; wastewater treatment

Highlights

- Bioassays-derived information in assessing organic trace pollutants toxicity is explored
- Meaning, role and choice of proper bioassays are discussed
- Hints on sampling raw and treated wastewater, which are a complex matrix, are given
- Interpretation and use of results of bioassays are suggested
- Real and laboratory scale experiences are presented as case studies

1. Introduction

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

Together with the monitoring of different effects on a single organism (from the first life periods), nowadays it is clear that also cross-generational, ecological and ethological aspects must be taken into account [1], [2], [3]. 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, as the complexity of community structures and relationships among different populations should be considered.

This opinion paper aims to gather the main findings obtained by various research groups involved in the COST Action ES1202 Conceiving Wastewater Treatment in 2020-Energetic, environmental and economic challenges (Water_2020). The final goal is to present the strength of a multi-tiered approach within the risk assessment of pollutants/whole effluents detailing potentials and weighing pros and cons of conventional and innovative bioassays. The work considers some exemplifying case studies of wastewater treatment and, finally, examines the socio-economic aspects of the whole procedure of ecotoxicity evaluation.

2. Principal well-established knowledge and open issues

As far as chemical pollution is concerned, substances are prioritized for action based on the risk to or via the aquatic environment, according to the Water Framework Directive (2000/60/EC; WFD) [4], and included in article 16 for the "Strategies against pollution of water". Compounds must be identified by a targeted risk-based assessment focusing solely on aquatic ecotoxicity and human toxicity via the aquatic environment. According to WFD, "hazardous substances" are defined as 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. In the Risk Assessment Process, the initial step would be the hazard identification. Further, the primary investigation should already include the possible health problems caused by the pollutants. This process uses the intrinsic properties of chemicals (i.e. persistence, solubility, K_{ow}, volatilisation, ...) 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, sub-chronic and chronic toxicity, abiotic and biotic degradability, and bioconcentration or bioaccumulation. Consequently, these tests can be used for toxicants and hydrophobic chemicals with low solubility in water that may accumulate and concentrate in lipids of aquatic organisms. The storage of these toxicants within the organism demonstrates cumulative toxicity to exposed organisms. These chemicals are usually considered in bio-concentration tests, which use bio-concentration factors (BCF) to predict the accumulation of hydrophobic contaminants. BCFs may be obtained either from calculations (log K_{ow}, (Q)SAR, or other computational-based model), or from experiments. This parameter represents the partitioning of the chemicals between an organism and the surrounding environment [5] based on the observation of the hydrophobic character. Thus, values of BCF > 1 indicate for hydrophobic character,

not bioaccumulative if the BCF in the range 1000–5000. Further, in case BCF is > 5000, the substance is very bioaccumulative [6].

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 each effect occurring. Both hazard and risk assessments are mandatory to guarantee scientific support for regulations [7]. In the last decade there has been a growing interest about the impact on the whole ecosystem exerted by chemicals, on aquatic and terrestrial organisms and wildlife [8], [9], [10], [11], [12].

Ecotoxicity tests can be classified with regards to design (e.g. field, laboratory, computational), level of biological organization (population, assemblage/community, ecosystem), exposure period (acute, sub-chronic, chronic) and endpoint (lethal, sublethal). Short-term ("acute") tests are generally used first, with survival as the most common endpoint. Long term ("chronic") tests (with the observation of sublethal effects on organism growth or reproduction) are then used when results from short-term tests combined with large safety factors suggest that there may be risks to the environment.

The use of acute and chronic tests in ecotoxicology has been proposed in reports from EU´s Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) aiming to improve the protection of human health and the environment through better and earlier identification of the inherent properties of chemical substances. Moreover, in order to reduce the use of animals in laboratory experiments, REACH suggests a number of possibilities to adapt the testing requirements and to use existing data, as well as alternative assessment approaches [13].

Since the Seventies, the progressive awareness of hazards linked with specific chemicals has been increasingly consolidated by epidemiology findings, the long-term follow up of environmental disasters and the availability of new technological tools to isolate and quantify a huge range of analytes from complex matrices also at risible concentrations [14]. For instance, it has been possible to carry out investigations on metal speciation. In addition, almost every class of organic compounds has been considered, starting from reaction 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) and their metabolites and transformation products. Furthermore, research has focused on the size of pollutants released into the environment, by considering, *inter alia*, micro-plastics and nanomaterials. It is now well known that the size of chemical agents strongly affects both the bioavailability and the effects on the organisms. So far, the scientific literature numbers lot 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 [15], [16], [17], [18], [19].

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.

Concentrations of pharmaceuticals in the range pg/L - μg/L have been reported worldwide in effluents from sewage treatment plants, surface, ground and even drinking water [20]. Since now, over 3,000 different agents in human pharmaceuticals are used and continuously released into the environment, it is urgent to increase our knowledge about the potential combined effects of these substances on the biota. Most research regarding the ecotoxicological effects of pharmaceuticals is generally focused on aquatic organisms and their food webs in standardized tests. 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 few components in these mixtures [21], [22], [23]. Furthermore, routinely detected chemicals often cannot explain observed biological responses [24], which point to a mismatch in the generally applied assessment approaches.

Wastewaters, due to their nature and origin (i.e. municipal, industrial, runoff, grey), evidently collect and concentrate a multitude of chemicals, that make up complex mixtures including microbial consortia. Therefore, when assessing the overall impact of any given wastewater (either raw or treated), it is essential to consider both: i) the removal/discharge of specific micropollutants and ii) the residual toxicity of parent compounds, metabolites, transformation products and by-products after treatment or, in any case, the discharge. For this reason, 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 compulsory. The removal of a target xenobiotic compound 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. In Figure 1, the temporal and logical structure of the above-mentioned process has been depicted: after identifying and characterizing the chemicals, it is necessary to consider their integrated effects towards both humans and all levels of food webs.

Figure 1. Towards a global risk assessment of aquatic pollution: present and future scenarios.

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

3.1 Principles

From a “real life” perspective, a lot of possible responses can be expected and observed in the organisms exposed to the mixture of pollutants present in raw and treated wastewaters.

According to [25], ecotoxicology is the science devoted to the study of the contaminants and their effects induced on all parts of the biosphere. Thus, all species, including human beings, must be considered as potential targets of the toxic action of the pollutants. Therefore, upon exposure to chemicals, different responses can be observed, ranging from the molecular to the ecosystem level.

The tools available to assess effects on ecosystems [7] can be related to the different steps of the hierarchical ecotoxicology described by [25]: organismal, population, community, and ecosystem. None of them must be considered the most or the least relevant in the evaluation process, as each of them represents a specific target.

At the organismal level, different single-species bioassays (of which some are standardized) can be applied to identify hazards through all relevant routes of exposure: soil, water, air and food. Several of these tests can be run using diverse species providing data on chemically induced effects, which can be applied in ecological risk assessments (ERA) to yield Species Sensitivity Distributions (SSDs). SSDs are models of the variation in sensitivity of species to chemicals or other stressors [26]. In following steps, long-term single-species tests and laboratory multi-species tests can be used to predict or evaluate population dynamics. Finally, mesocosm and field assays can offer information about real ecological effects [7].

Lots of well-known biochemical and molecular 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 [25]. It is also relevant that some products of detoxification reactions may be more toxic or reactive than the parent compound and, therefore, specific tests on drug metabolism have been developed. In general,

the biochemical and molecular responses express morphological (microscopic and macroscopic scale) modifications on cells and tissues, and/or functional failures of organs and systems.

Frequently, the biochemical mechanisms of toxic action are unknown, and therefore the effects on cells, tissues, organs and systems can be identified as the only target in the risk assessment. Different morphological findings related to the exposure to organic pollutants have been described in cells and tissues, such as inflammation, necrosis, apoptosis, and sometimes hypertrophy or hyperplasia. Genetic alterations can explain pathologic cell proliferation and cancer.

Tissues, organs and systems involved in vertebrate toxicokinetic, and the effects displayed on respective targets are important subjects in both toxicology and ecotoxicology. Integument, respiratory and digestive organs are first subjected to organic pollutant exposure, due to their direct interface with the outside. The first route of exposure to organic pollutants regards undoubtedly the derma, which is also the target of their toxicant action. In addition, dermal structures play a crucial role also in case of excretion processes (e.g., by means of fish and reptile scales, bird feathers and hair of mammals) and bioaccumulation phenomena. Secondly, respiratory exposure is included, while organs associated with digestion represent the third major exposure route, *i.e*., ingestion. In addition, liver/hepatopancreas and kidney, and other analogous organs of invertebrates can be relevant as target of the toxic action, influencing the kinetic metabolism and excretion of the chemicals.

Nervous, immune, and endocrine are mostly studied; a wide range of toxicants affects adversely nervous system cells and tissues, through both structural and functional alterations, expressing also a wide range of manifestations at the organismal level [25]. Concerning the immune system, research focuses mainly on either cellular or humoral immune dysfunction in adult and developing vertebrates. The relevance of these immune alterations is related to the ability to induce infection, infestation, or other diseases like cancer; with consequences, not only at organismal level, but also at population, and community levels.

Endocrine disruption was historically investigated especially in terms of estrogenic/androgenic activities, but, so far, plenty of hormone-mediated mechanisms have been explored.

At the border between organismal and population levels, alterations on growth and development, reproduction, and behavior, are issues of concern to public and environmental health that justify the implementation and development of specific tests to evaluate these alterations induced by the exposure to chemicals. The studies of pollutant effects on growth are common because the collection of data is easy, and they have been implemented on amphibians, fish, birds, terrestrial and aquatic invertebrates, terrestrial and aquatic macrophytes, and microscopic plants. An exhaustive list of plants for toxicity tests has been compiled by [27] and [28]. In relation to reproductive effects, there are standardized tests on aquatic and terrestrial invertebrates' species, such as *Daphnia magna* or collembolans, as well as on vertebrates such as birds. Finally, modifications and alterations of the behavior are also commonly selected endpoints to evaluate the risks associated to environmental polluted scenarios. These endpoints range from individual alterations, such as movement or foraging behavior, to social interactions, such as social structuring or mating behaviors.

Figure 2 illustrates the core message of this paragraph: there exists an indissoluble connection between each target phenomenon and the biological hierarchy. It must be underlined that the investigator must be aware of the inevitable partiality of any obtained information, which just accounts for an element of a complex framework.

Figure 2. Approaching the bioassays: the conceptual framework.

3.2 Biological responses

Among the variety of biological effects currently studied in environmental research, this section focuses on the responses that can be used to assess the activity of wastewater.

A basic principle in toxicology research is the study of a toxicant fate in an exposed organism. To assess this, the Absorption, Distribution, Metabolism and Excretion (ADME) concept has been developed decades ago. Each of the mentioned stages of pollutant impact on an organism is characterized by specific and quantitative measures, like distribution coefficients that assess uptake potential into certain organs or cell types. Metabolism is characterized by the enzymatic activation, cleavage or conjugation of parent compounds, and excretion by the velocity of transport from the target back into the environment. For compounds or conditions where clearance from the body cannot be reached, toxicology tries to assess the biological activity by describing the mode of action (see 3.2.1 Baseline toxicity). However, reliable information on mode of action is still lacking for many chemicals. Currently, there is neither an agreed inventory of modes of action, nor a defined set of criteria on how to characterize or predict a mode of action for data-poor chemicals or how to group chemicals into assessment groups [29].

Two approaches can generally be followed to assess the toxicity of pollutants and effluents, based on different methodological criteria: Toxicity Identification Evaluation (TIE), which focuses on the whole organisms (therefore, bioavailability and baseline toxicity are the core issues) and Effects-Directed Analysis (EDA), which relies on *in vitro* assays, by considering specific endpoints, *i.e.*, specific modes of action [10].

3.2.1 Baseline toxicity

As far as chemical substances are concerned, every mechanism of toxicity is initiated by the interaction between the chemical(s) and the organism and can be described accordingly 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.

Baseline toxicity involves the interaction between the substances and the cell membrane; the hydrophobicity affects the capacity of the molecules to react and pass through these barriers, whose fluidity can thus be deeply modified. Therefore, 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.

Regarding the field of environmental research, increasing attention has been paid to *in situ* toxicological studies. Laboratory scale assays (*in vitro* and *in vivo*) may not allow a relevant simulation of real cases, due to the presence of other stressing factors in environmental conditions that influence the biological response. Thus, laboratory scale experiments might dramatically stray from real situation. In terms of baseline toxicity, it is even more difficult to assess the impact of pollutants on populations and communities, represented by for example biomass decrease or change in the total numbers of individuals can be a possible outcome.

Finally, another critical aspect inherent to toxicity assessment is the multi-faceted scenario of responses, which can present non-monotonic trends and can be affected by hormesis and adaptation phenomena [30].

3.2.2 Endocrine disruption

As pointed out by [31], different organizations have issued their own definition of EDCs, taking into account growing levels of biological effects. The well-known and often cited definition of United States Environmentla Protection Agency (USEPA). considers the effects on "*maintenance of the homeostasis and the regulation of developmental processes*" [32], while World Health Organization (WHO) refers to

"*adverse effects in an intact organism, or its progeny, or (sub-)population*" [33]. The Endocrine Society [34] more strictly states that EDCs "*interfere with any aspect of hormone action*". Consequently, the evaluation of possible endocrine activity caused by a substance, a mixture or any other complex matrix (such as sewage, sludge, etc.) must follow the precise definition of the final goal.

Secondly, it is mandatory to select the mechanisms to investigate, by taking into consideration the biological complexity of the target organism and its physiology. 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 EDCs. 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, and obesity.

EDCs 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 estrogen receptor alpha (ERα) in comparison with the natural hormone estradiol, while it is equipotent towards membrane receptors [35], [36]. 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 [31] and [37].

The most studied mechanisms are related to the interference with sexual hormones (androgens and estrogens), thyroid and adrenal disorders and glucose metabolism impairment [38]; therefore, assays have been designed basically aiming at their assessment.

Many biological pathways are based on nuclear receptors that migrate into the nucleus and regulate gene transcription after hormone binding, despite their location. The main pathways include: thyroid signaling,

estrogen signaling, glucocorticoid pathway, renin-angiotensin-aldosterone system, leptin and insulin signaling. The main elicitors of estrogenic activity are micropollutants such as: the natural (estrone, estradiol, estriol) and synthetic (ethynil-estradiol) estrogens, alkylphenols (octyl- and nonyl-phenol) and the bisphenol A. The main adverse impact related to this kind of biological activity is represented by impairment of reproductive performance in wildlife. Routledge et al. [39] documented that steroidal hormones can induce feminization in fish and other aquatic organisms at concentrations as low as 1 ng/L or less estradiol equivalents. Witters et al. [40] reported that significant reproduction effects in male and female fish might appear at levels above 10 ng/L of estradiol equivalents. Moreover, any interference in their homeostasis is related to several human diseases (breast, ovary and prostate tumours, cardiovascular diseases, infertility, etc.), due to the physiological role of estrogens.

3.2.3 Genetic toxicity

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 developmental toxicity. Beside chemical agents, which are the core object of this paper, also biological and physical stressors can cause DNA damages (e.g., viruses, ionizing radiations, UV radiations); furthermore, the overall conditions of the organism, in terms of feeding regime, disruption of regulatory pathways and cellular checkpoints dramatically favors adverse events to DNA. Consequently, the assessment of possible genetic toxicity caused by trace pollutants contained in wastewater should be considered the coexistence of the aforementioned factors, together with the fact that wastewater are mixtures of chemicals, whose concentrations are extremely variable (as explained in paragraphs 4.1 and 4.2). DNA damage can be related to alkylation (i.e 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, and 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. Mechanisms of DNA repair are often exploited in bioassays; they are aimed to restore its pristine function or to destroy the damaged cell by means of apoptosis. They are based on the action of multiple enzymatic reactions, which, for instance, allow repairing base excision, nucleotide excision, double strand breaks and mismatches.

Decades of research have shown that mutagenesis is a critical component and that there is a strong correlation between carcinogenicity and mutagenicity [41].

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 particular 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 [42], [43].

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 [44], [45]. Transcriptome differs from proteome, due to post- translational modifications; each environmental stimulus affects these mechanisms, as well as gene expression. Thus, the challenge is finding 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 [9], [46].

3.2.4 Oxidative stress

A detrimental response generated by pollutants on a cellular level includes the production of reactive oxygen species (ROS) and free radicals. These "basic" processes 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 [47], 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 [48]. This process may cause oxidative damage to membrane lipids, DNA and proteins, and lead to cellular dysfunction and tissue injury [49], [50].

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 [51]. In short, oxidative stress can act either through overproduction of free radicals or alteration in antioxidant homeostasis [52]. Indeed, a close relationship was described between metal cytotoxicity, 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 [53]. Oxidative stress is also due to the alteration of antioxidant enzymes such as glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferase (GST), catalase (CAT) and superoxide dismutase (SOD), which may lead to elevated lipid peroxidation [54], [55], [56]. Increased concentration of plasma and red blood cell thiobarbituric acid reactive substances (TBARs), changes in the antioxidant status, and altered activities of cellular enzymes such as superoxide dismutase (SOD) and catalase (CAT) indicated higher oxidative stress in pesticides sprayers. Hence, many researchers have associated exposure to pesticides with oxidative stress [57].

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 phenolics content. Other biomarkers may indicate more general phenomena, such ageing, decay and cell integrity (putrescine, spermidine, spermine) and undetermined stressors (proline).

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 [58]. Hence, at least one marker of oxidative damage should be measured in order to make inferences about oxidative stress [58]. Previous studies have shown that antioxidant enzymes, particularly GPx, CAT and SOD, as well as lipid peroxidation may function as useful biomarkers of metal induced effects on the antioxidant system in different bird species [59], [60], [61]. Further studies on other taxa will help to better

understand the mechanisms of metal toxicity in wild birds, and the concentrations prone to cause effects on the antioxidant system.

3.2.5 Antibiotic resistance

Diverse spectres of antibiotics (chemotherapeutic agents) are being developed to protect humans and animal husbandry against bacterial, fungal and protozoan infections. Large environmental releases are caused by their intensive use and, often, overuse or misuse. Furthermore, it is worth noting that most antibiotics can be only partially metabolized after administration, and, thus, are excreted directly into the wastewater. Main hotspots are soils fertilized with manure runoff water from farms [62], effluents of drug production units [63] [64], WWTP effluents and sludge and, consequently, the receiving waterbodies ([65], [66], [67]). Since microorganisms also naturally produce antibiotics, they are ubiquitously and perpetually present in ecosystems. Consequently, bacterial resistance to naturally occurring antibiotics is common in nature. However, the increased presence of antibiotics in the environment increases the risk of development and spreading of antibiotic resistance [68], also to human pathogens [69], which has become of great global concern in clinical environments. WWTPs are considered as important reservoirs for antibiotic resistance genes (ARGs) since sewage receives gut bacteria previously exposed to antibiotics [70].

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 [69]. 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) [71]. The latter is mediated by 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 most classes of clinically important antibiotics and constitutes a reservoir of ARGs that can be mobilized into human pathogenic bacteria [72], [73]. ARGs have gained increasing attention in recent years [74], [68], [69], [75]; there is still a critical lack of knowledge about the diversity, distribution and origin of resistance genes [76], especially for the unculturable majority of environmental bacteria, of which less than 1% are estimated to be culturable [77].

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

Different biological responses or stresses potentially caused by micropollutants present in wastewater (see Section 2) can be assessed in lab-scale tests. Such assays have to be able to simulate the actual conditions occurring in case of receiving waterbodies and reused waters. Due to the challenges in collecting representative samples without losses and inherent high costs for conducting proper toxicity assessments, a well thought-through sampling strategy and sample collection and preparation are of major importance. Figure 3 shows the main factors, which must be considered before planning a monitoring by adopting the bioassays.

Figure 3. Factors to be accounted for when selecting the proper bioassay.

4.1. Sampling strategy

Chemicals present in wastewater can considerably vary as a function of time, urban populations 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, a worst-case scenario would usually be appropriate. However, chronic effects would better require average or median exposure conditions.

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 [78], [79]. 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 [80], as well as the conditions of receiving waterbodies (see Chapter 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 [81]. The economic aspects are crucial, and often decisive, in setting the total number of samples to acquire. Hence, for the acute toxicity assessments, uncertainty is marked, if case campaigns do not cover the actual worst case, specifically in case of the investigation of the combined effects of all substances contained in sewage. Furthermore, routinely detected chemicals often cannot explain the observed biological responses (e.g. [24]) pointing to a mismatch in the assessment approaches generally applied. Therefore, it may be difficult to pinpoint the best or most appropriate term to conduct the sampling campaign.

Ort et al. [82] detailed and explained all these aspects, focusing on sample volumes, collection duration, storage conditions and data elaboration.

4.2. Sample collection and preparation

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 µg/L - pg/L concentration range together with solids, microorganisms, beside a great number of dissolved or suspended compounds in the mg/L concentration range. 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 (see below). 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 [83]. iii) Micropollutants in the filtered sample are usually cleaned up and concentrated prior to application in the toxicity tests by step-wise 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 [83] for a more detailed discussion).

Ideally, the whole, unaltered sample should be used in toxicity tests. However, this is not possible for practical reasons for most of the tests (see Section 3.2). Some sorts of sample clean up and concentration, as briefly described above, are usually needed. If only the filtrate is submitted to toxicity tests, the effects of hydrophobic compounds and other pollutants adsorbed to the particulate matter will probably be

underestimated. Moreover, if only an extract of the filtrate is tested, even more compounds may be lost. To improve the recovery of micropollutants from the original sample, the substances retained by the filter might also be extracted using the same solvent as applied for SPE elution.

4.3. 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), WHO, Food and Agriculture Organization (FAO), International Organization for Standardization (ISO), American Society for Testing and Materials (ASTM), 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, [84] 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). More complex cases are based 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. Tests employ 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) [38], [85], [86].

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 [86], [87], [88], [89], 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 [86], [90], [91], [92], [93], [94], [95], [96], [97]. Other tests are focused on the quantification of the production of proteins, such as vitellogenin, choriogenin, *zona radiata* protein after estrogenic stimulation [98], [99], [100], [101]. Steroidogenesis based tests look promising in providing additional information on disruption mechanisms [101], [102] 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 [103], [104]. 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 [105]. The method to culture and expose the cells and to assay luciferase activity has previously been described in [106]. Measurement of the estrogenic activity of nonylphenol during biological degradation showed a decrease of the estrogenic activity during microbial degradation and can hence 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. [12].

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 [107].

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 [108]. 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 (mainly 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 are represented by 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 [109], [110], [111], [112], [113], [114]. It takes 48 hours to obtain a result. b) The micronuclei test, for detecting chromosomal mutations (generally performed on root cells of *Allium cepa*, throughout 72 h) [109], [112], [113]; 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 [109], [110], [113], [114], [115]. 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 [111], [116]. e) The GreenScreen assay (GSA) which employs cells of *Saccaromices cerevisiae;* it detects a DNA damage, based on the quantification of a green fluorescent protein linked to the promoter of the RAD54 gene [117], [118]. f) The sister chromatide exchange (SCE assay) based on mammal cells [115], [119].

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 chemistry, as well as biological aspects, including population genetics, reproductive cycles [120]. 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 [48] detoxifying radicals and toxic oxygen species that might build up under xenobiotic stress [121].

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 [122]. 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 [123]. 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 [123] and [124]. 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 %; [77]) 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 [125].

For that reason, tools for molecular detection of antibiotic resistance genes (ARG) have become increasingly popular [69], [70], [126]. 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 [70]. 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 [69]. 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 [126]. 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 [70]. Furthermore, both microarray and PCR based technologies are not conclusive regarding the detection of resistance genes in metagenomes [127].

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 [69]. 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. [128]) to identify resistance genes and/or mutations that are known to cause resistance [69]. This approach has the potential to identify all known resistance genes in a metagenome. Though, important shortcomings are that it can only identify known ARGs and that it gives no information on expression of the resistance genes [127]. 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 [127]. 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 [127] and [69] for a more thorough discussion of advantages and drawbacks in metagenomics analyses.

4.4 Criteria for selecting a bioassay

Toxicity tests can be prescribed by the law or by voluntary regulations and, consequently, be completely standardized. Also the results interpretation, in this case, is almost plane. 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) several alternative choices arise. The criteria underpinning the selection of a bioassay (or a battery of complementary assays) should include the required volume (smaller volumes may favor the miniaturization, hence the automation of the procedure), the price (consisting in capital and operation costs, as well as in the license fee), 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.

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 [129]. Furthermore, the same test (e.g., Ames on *S. typhimurium*) can be carried out either by adopting the conventional microbiological approach and cultivating bacteria or using kits available on the market. Among the options, there is also the possibility to use genetically modified microorganisms, which allows eliminating the addition of rat liver S9. Therefore, time is reduced (hence the analyst costs) but CAPEX and OPEX become significant.

Besides, also these two items can assume different commercial values: metagenomics assays are getting progressively less expensive, but the number of clones and libraries increases rapidly.

Finally, in any case, before planning a monitoring campaign focused on the assessment of toxicity of a sewage/effluent/mixture, one has to pay attention to possible species-specific responses and to the inherent criticism of *in vitro* assays, whose results cannot be translated as a whole to a real situation, due to the occurrence of compensation effects and the multifactorial nature of toxicity.

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, analyses 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 Calculation of predicted no-effect concentrations and risk assessment

Risk calculation is an essential step for quantitative evaluation of risk from chemical exposure. Risk characterization involving exposure and effects using effects-related Environmental Quality Standards

and Quality Norms is a single-substance concept for dangerous substances and priority hazardous substances. The environmental risk posed by certain trace pollutants in aquatic ecosystem is characterized and assessed through calculation of the risk quotients (RQ). Therefore, the hazard quotient method is commonly used for screening level risk characterization. Namely, under EU legislation context, guidelines have been developed for new or existing substances as well as compounds, such as biocides or hazardous substances [130], [131], [132], [133], [134], [135]. Moreover, this approach has been generally accepted and has widely been used all over the world: Taiwan [136], [137], Japan and China [64], [138], [139], [140], Germany [141], United Kingdom and the USA [142], Spain [143], [144], Korean [145] etc. for environmental risk assessment. The RQ is calculated for each micropollutant by dividing the measured environmental concentration (MAC) by the predicted no effect concentration (PNEC - the concentration at which no adverse effect is suspected to occur) using the lowest value of all endpoints, as given in Eq. 1:

$$RQ = MEC/PNEC \quad (1)$$

PNEC is calculated (Eq. 2) by dividing the lowest chronic no observed effect concentration (NOEC) by the assessment factor (AF) or uncertainty factor (UF) chosen according to European guidelines [146].

$$PNEC = NOEC/AF = EC_{50} \text{ or } LC_{50}/AF \quad (2)$$

This method recommends the use of chronic toxicity data for calculation of PNEC. However, for some compounds, the availability of chronic data referring to NOEC for some compounds is limited. In absence of NOEC values, acute toxicity data could be used and where no experimental toxicity data are provided, the toxicity values could be predicted using QSAR (Quantitative Structure Activity Relationship) models. An assessment factor (AS) is applied to the toxicological benchmark value according to [134], laying down the principles for the assessment of risks to man and the environment of existing substances in

accordance with Council Regulation [133].

AF or UF of 1000 is applied when at least one short term L(E)C_{50} from each of the three evaluated trophic levels are provided; an AF of 100 is used when one long term NOEC was available for either cladocerans or fish in case of pharmaceutically active compounds, and finally AF of 50 is used when two long term NOEC values are provided for species in two different trophic levels. When the MEC is larger than the PNEC, giving RQ larger than 1, the investigated MP is risky to human health or the environment, while a RQ value of 0.01-0.1 and 0.1-1 indicates low or medium risk, respectively. If 1.0<RQ<10, some adverse effects or moderate hazard are probable, and if RQ>10, high hazard is anticipated [146].

Although risk assessment is a useful tool, the results should be taken with attention, due to several factors. Single compound exposure scenario, indeed, is unrealistic in the real aquatic environment, since trace pollutants are present as complex mixtures. They often exhibit toxic effects at concentrations lower than the NOEC values for each compound if acting alone [147].

The overall effect is synergistic, additive or subtractive, depending on each specific mode of action; a compound might also behave completely independent from the others [148]. Also, if the exposure data used for calculation are based on single sampling events, these data do not accurately represent the long-term exposure of the organisms present in the studied aquatic matrices. This should be taken with caution when chronic exposure is estimated for the calculation. Nevertheless, the used exposure data only represent concentration in water, and, besides, factors like as bioconcentration or bioaccumulation, real uptake rates should be considered as well. Additionally, available dose-response data in the case of pharmaceutically active compounds, which are the most studied, are limited, especially regarding chronic data. Furthermore, the toxicity data provided by QSAR models for fish, algal and *Daphnia* spp. are for chemicals with non-specific mode of action and hence, should also be applied with caution. On the other hand, information on compounds for which uncertainties about potential endocrine disrupting effects exist is still needed. Jones et al. [149] when evaluating chemical mixture toxicity on sites where multiple

chemicals of potential ecological concern can simultaneously occur used HQs, which are commonly referred as Toxic Units (TUs) in order to estimate the relative toxicity of the waters from each site. Thus, the sum of TUs for each sampling point is calculated by means of the sum of chemical-specific HQ for each site. As in the case for individual HQ, the sum of TUs should be taken as preliminary for the quotient addition approach assuming that toxicities are additive or approximately additive. In the case of some pharmaceuticals similar modes of action of compounds in the same therapeutic group could account for a certain level of overestimation of the risk. However, at the same time, possible synergic interactions among compounds with different modes of action could underestimate the risk. Although the risk assessment scenario and the results obtained using this tool could be accepted with caution due to the mentioned limitations, its applicability demonstrates the need for water quality monitoring using composite and periodical samplings, since the discharge of trace pollutants and their metabolites is time dependent, i.e., fluctuating daily, monthly or seasonally. Nevertheless, the results could show the need for improving of sewage treatment technologies and could generate concern about the efficacy of the applied water treatment.

5.3 Wastewater toxicity assessment and ranking

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 [150], [151], [152]. 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 [150]. 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 [153]. 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 [154], [155]. 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 [156].

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 [157], whole effluent toxicity, integrating controlling of effluent, whole effluent environmental risk, environmental effects monitoring [158], and whole effluent assessment [156], [159]. 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 [160].

Generally, legislative requirements tend to refer to a toxicity limit based on a single test or a battery of toxicity tests considering as final 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 [161], [162] or order of magnitude basis [163], [164], [165], [166] or expert judgment and regression analysis pair [167]. 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 [150]. For example, Libralato et al. [150], [151] 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 [168], [169]. 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.

5.4 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 [170]. 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 [171]. 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.

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 [172]. 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.

5.5 Socio-economic aspects

Monitoring and predicting trace pollutant concentrations in the aquatic environment, together with their possible subsequent toxicity, are vital 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 [173] is suggested to be correlated to online search queries [174]. As the concentrations of antibiotics in wastewater seem to follow the trend

of prescriptions [175], 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 [176], 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 [177], [178]; 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 [179] 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 [180], [181]. Disposal of unused medicines is mostly carried out through household waste [182], toilets and sinks [183], [184], [185]. As many do not regard this as an environmental issue [182], it is evident, that public awareness is vital, together with the need for better public information [184]. Over the past decades, attention has also focused on return policies advertisements [182] and the importance of people information on the correct disposal [182], [184]. As a consequence, population willingness to pay for a better waste treatment system increases [183], [186]. Governments should implement the regulatory frameworks for improving the whole water cycle management [187]. 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 [188]. 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 (cladocer 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 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 [189] 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. [156]).

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.* [190] 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 *D. 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 [191]. The treatments steps were performed on a laboratory scale, 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 5,470, confirming the very high algal growth inhibition reported for the same type of wastewater by De Schepper *et al.* [190].

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 d, 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. [192], [193] demonstrated that any increase of sludge age and temperature above 10 d and 10°C does not lead to noticeable improvements, regardless of the type of process (either CAS or MBR). Moreover, several Authors (e.g. [194], [195], [196], [197]) 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 x 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 bio-assays in the monitoring of any impact on the ecosystems (in this case, the biological reactor of a WWTP). Detailed results are reported in [92].

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 [198], [199]. While Kümmerer and his colleagues [200] 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.* [201] 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 [202], 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 ([203], [204], [205]). 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 [202]. Respirometric tests were performed to determine the effect of antibiotics on unacclimated (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 ([204], [205]). Reactors were monitored for COD, suspended solids (SS), volatile suspended solids (VSS) and polyhydroxyalkanoates (PHA) [206]. 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 [202], [203]. 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 [202], [203], [204], [205].

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 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 [202]. 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. Respirometric tests were performed to determine the effect of antibiotics on unacclimated (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. 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 [203]. 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 [202].

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 (1–1000 mg/L) of the selected antibiotics [207], [208]. The batch reactors were kept running for six 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 [207]. 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 [208], [209].

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 [210]. 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 [211]. 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 [209].

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 [212].

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 mgCOD/L, 5 mgBOD_5/L, 12 mgTSS/L, 6.5 mgTKN/L; 4 mgNH_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 = 1,460 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 mgO_3/L, with an HRT of 20 min.

The estrogenicity of wastewater was reduced from 7.35 down to 3.25 x 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 [92]), 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 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 [213], [214], [215], [216]. 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*. [215]. 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 24h and 48 h. *R. subcapitata* showed to be more sensitive to by-products than *L. sativa.*

A set of bioassays (*D. magna, R. 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 photo-catalytically 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 gTiO_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 [217]. The residual toxicity of photo-catalytically 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 *V. fischeri* ($p<0.05$) was obtained at 1.6 g/L of TiO_2 with a residual toxicity of 8 ± 6% (5min) and 10 ± 4% (15min). Lower TiO_2 concentrations showed toxicities ranging between 45–62% (5min) and 53–76% (15min) [216].

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*. [218]. 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 [150], [151].

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 organic trace 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.

Some aspects appear particularly worth noting:

- bioassays must be chosen considering the meaning of the biological response;
- laboratory and in situ bioassays must be integrated, by considering their specific reliability and applicability;
- trace pollutants can cause unpredictable and non-linear responses by biological systems;
- wastewater variability in terms of pollutants concentrations and flowrates deeply affects any toxicity assessment;

- the choice of the optimal WWTPs process schemes for the abatement of trace pollutants must be based on the global assessment of environmental and socio-economic impacts.

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References

[1] H.P. Gelbke, M. Kayser, A. Poole, OECD test strategies and methods for endocrine disruptors, in: Toxicology, 2004: pp. 17–25. doi:10.1016/j.tox.2004.06.034.

[2] J. Xia, S. Fu, Z. Cao, J. Peng, J. Peng, T. Dai, L. Cheng, Ecotoxicological effects of waterborne PFOS exposure on swimming performance and energy expenditure in juvenile goldfish (*Carassius auratus*), J. Environ. Sci. (China). 25 (2013) 1672–1679. doi:10.1016/S1001-0742(12)60219-8.

[3] G. Sunanda, M., Rao, J. C. S., Neelima, P., Simhachalam, Toxicity and effects of chlorpyrifos in a non-target organism (Fish)-A review, J. Atoms Mol. 6.3 (2016) 966–976.

[4] European Community, Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000 establishing a framework for Community action in the field of water policy, Off. J. Eur. Parliam. L327 (2000) 1–82. Brussels. doi:10.1039/ap9842100196.

[5] H.F. Hemond, E.J. Fechner, Chemical fate and transport in the environment, 2015. doi:10.1016/B978-0-12-398256-8.00004-9.

[6] Y. Wayne, G., Landis, Ruth, M., Sofield, Ming-Ho, Introduction to Environmental toxicology: molecular structures to ecological landscapes, 4th ed., CRC Press, Boca Raton, USA, 2011.

[7] J. V. Tarazona, M.M. Vega, Hazard and risk assessment of chemicals for terrestrial ecosystems, Toxicology. 181–182 (2002) 187–191. doi:10.1016/S0300-483X(02)00279-2.

[8] T.E. McKone, R.L. Maddalena, Plant uptake of organic pollutants from soil: bioconcentration estimates based on models and experiments, Environ. Toxicol. Chem. 26 (2007) 2494–2504. doi:10.1897/06-269.1.

[9] M.F.L. Lemos, A.M.V.M. Soares, A.C. Correia, A.C. Esteves, Proteins in ecotoxicology - How,

why and why not?, Proteomics. 10 (2010) 873–887. doi:10.1002/pmic.200900470.

[10] R.M. Burgess, K.T. Ho, W. Brack, M. Lamoree, Effects-directed analysis (EDA) and toxicity identification evaluation (TIE): Complementary but different approaches for diagnosing causes of environmental toxicity, Environ. Toxicol. Chem. 32 (2013) 1935–1945. doi:10.1002/etc.2299.

[11] M. Adeel, X. Song, Y. Wang, D. Francis, Y. Yang, Environmental impact of estrogens on human, animal and plant life: A critical review, Environ. Int. 99 (2017) 107–119. doi:10.1016/j.envint.2016.12.010.

[12] W. Brack, S. Ait-Aissa, R.M. Burgess, W. Busch, N. Creusot, C. Di Paolo, B.I. Escher, L. Mark Hewitt, K. Hilscherova, J. Hollender, H. Hollert, W. Jonker, J. Kool, M. Lamoree, M. Muschket, S. Neumann, P. Rostkowski, C. Ruttkies, J. Schollee, E.L. Schymanski, T. Schulze, T.B. Seiler, A.J. Tindall, G. De Aragão Umbuzeiro, B. Vrana, M. Krauss, Effect-directed analysis supporting monitoring of aquatic environments - An in-depth overview, Sci. Total Environ. 544 (2016) 1073–1118. doi:10.1016/j.scitotenv.2015.11.102.

[13] European Comission, Regulation (EC) 1907/2006 of the European Parliament and of the Council of 18 December 2006 - REACH, Off. J. Eur. Union. (2006) 396–849. Brussels. doi:http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2006:396:0001:0849:EN:PDF.

[14] M. Petrović, B. Škrbić, J. Živančev, L. Ferrando-Climent, D. Barcelo, Determination of 81 pharmaceutical drugs by high performance liquid chromatography coupled to mass spectrometry with hybrid triple quadrupole-linear ion trap in different types of water in Serbia, Sci. Total Environ. 468–469 (2014) 415–428. doi:10.1016/j.scitotenv.2013.08.079.

[15] M. Auffan, J.-Y. Bottero, C. Chaneac, J. Rose, Inorganic manufactured nanoparticles: how their physicochemical properties influence their biological effects in aqueous environments., Nanomedicine (Lond). 5 (2010) 999–1007. doi:10.2217/nnm.10.61.

[16] W. Zhuang, X. Gao, Methods, Mechanisms and typical bio-indicators of engineered nanoparticle ecotoxicology: an overview, Clean - Soil, Air, Water. 42 (2014) 377–385. doi:10.1002/clen.201200559.

[17] S. Shyamasundar, C.T. Ng, L. Yue, L. Yung, T. Dheen, H. Bay, Epigenetic mechanisms in nanomaterial-induced toxicity, Epigenomics. 7 (2015) 395–411. doi:10.2217/epi.15.3.

[18] J.C. Anderson, B.J. Park, V.P. Palace, Microplastics in aquatic environments: Implications for Canadian ecosystems, Environ. Pollut. 218 (2016) 269–280. doi:10.1016/j.envpol.2016.06.074.

[19] M. Sendra, D. Sánchez-Quiles, J. Blasco, I. Moreno-Garrido, L.M. Lubián, S. Pérez-García, A. Tovar-Sánchez, Effects of TiO_2 nanoparticles and sunscreens on coastal marine microalgae: Ultraviolet radiation is key variable for toxicity assessment, Environ. Int. 98 (2017) 62–68. doi:10.1016/j.envint.2016.09.024.

[20] P. Schröder, B. Helmreich, B. Skrbic, M. Carballa, M. Papa, C. Pastore, Z. Emre, A. Oehmen, A. Langenhoff, M. Molinos, J. Dvarioniene, C. Huber, K.P. Tsagarakis, E. Martinez-Lopez, S.M. Pagano, C. Vogelsang, G. Mascolo, Status of hormones and painkillers in wastewater effluents across several European states - considerations for the EU watch list concerning estradiols and diclofenac, Environ. Sci. Pollut. Res. 23 (2016) 12835–12866. doi:10.1007/s11356-016-6503-x.

[21] R. Altenburger, S. Ait-Aissa, P. Antczak, T. Backhaus, D. Barceló, T.B. Seiler, F. Brion, W. Busch, K. Chipman, M.L. de Alda, G. de Aragão Umbuzeiro, B.I. Escher, F. Falciani, M. Faust, A. Focks, K. Hilscherova, J. Hollender, H. Hollert, F. Jäger, A. Jahnke, A. Kortenkamp, M. Krauss, G.F. Lemkine, J. Munthe, S. Neumann, E.L. Schymanski, M. Scrimshaw, H. Segner, J. Slobodnik, F. Smedes, S. Kughathas, I. Teodorovic, A.J. Tindall, K.E. Tollefsen, K.H. Walz, T.D. Williams, P.J. Van den Brink, J. van Gils, B. Vrana, X. Zhang, W. Brack, Future water

quality monitoring - Adapting tools to deal with mixtures of pollutants in water resource management, Sci. Total Environ. 512–513 (2015) 540–551. doi:10.1016/j.scitotenv.2014.12.057.

[22] T. Backhaus, M. Karlsson, Screening level mixture risk assessment ofpharmaceuticals in STP effluents, Water Res. 49 (2014) 157–165. doi:10.1016/j.watres.2013.11.005.

[23] P. Price, X. Han, M. Junghans, P. Kunz, C. Watts, D. Leverett, An application of a decision tree for assessing effects from exposures to multiple substances to the assessment of human and ecological effects from combined exposures to chemicals observed in surface waters and waste water effluents, Environ. Sci. Eur. 24 (2012) 34. doi:10.1186/2190-4715-24-34.

[24] B.I. Escher, C. Van Daele, M. Dutt, J.Y.M. Tang, R. Altenburger, Most oxidative stress response in water samples comes from unknown chemicals: The need for effect-based water quality trigger values, Environ. Sci. Technol. 47 (2013) 7002–7011. doi:10.1021/es304793h.

[25] M.C. Newman, W.H. Clements, Ecotoxicology: An comprehensive treatment, 2008. doi:10.1002/ieam.5630040423.

[26] L. Posthuma, W.G. Suter, P.T. Trass, Species sensitivity distributions in ecotoxicology, 2002. doi:10.1017/CBO9781107415324.004.

[27] J.S. Wang, Wuncheng, Gorsuck, Joseph W., Hughes, ed., Plants for environmental studies, CRC Lewis Publishers, Boca Raton, New York, USA, 1997.

[28] L.A. Kapustka, Rationale for use of wildlife habitat characterization to improve relevance of ecological risk assessments, Hum. Ecol. Risk Assess. 9 (2003) 1425–1430. doi:Doi 10.1080/10807030390250921.

[29] EEA, European waters - assessment of status and pressures. Report no. 8/2012, 2012.

[30] E.J. Calabrese, R.B. Blain, The hormesis database: The occurrence of hormetic dose responses

in the toxicological literature, Regul. Toxicol. Pharmacol. 61 (2011) 73–81. doi:10.1016/j.yrtph.2011.06.003.

[31] A. Beronius, L.N. Vandenberg, Using systematic reviews for hazard and risk assessment of endocrine disrupting chemicals, Rev. Endocr. Metab. Disord. 16 (2016) 273–287. doi:10.1007/s11154-016-9334-7.

[32] R.J. Kavlock, G.P. Daston, C. DeRosa, P. Fenner-Crisp, L.E. Gray, S. Kaattari, G. Lucier, M. Luster, M.J. Mac, C. Maczka, R. Miller, J. Moore, R. Rolland, G. Scott, D.M. Sheehan, T. Sinks, H.A. Tilson, Research needs for the risk assessment of health and environmental effects of endocrine disruptors: a report of the U.S. EPA-sponsored workshop., Environ. Health Perspect. 104 Suppl (1996) 715–40. doi:10.1289/ehp.96104s4715.

[33] T. Damstra, S. Barlow, A. Bergman, R. Kavlock, G. Van Der Kraak, Global assessment of the state-of-the-science of endocrine disruptors., WHO publication No. WHO/PCS/EDC/02.2. (2002) 180. doi:10.1089/15305620252933437.

[34] A.C. Gore, V.A. Chappell, S.E. Fenton, J.A. Flaws, A. Nadal, G.S. Prins, J. Toppari, R.T. Zoeller, Executive Summary to EDC-2: The Endocrine Society's second scientific statement on endocrine-disrupting chemicals, Endocr. Rev. 36 (2015) 593–602. doi:10.1210/er.2015-1093.

[35] W. V. Welshons, S.C. Nagel, F.S. Vom Saal, Large effects from small exposures. III. Endocrine mechanisms mediating effects of bisphenol A at levels of human exposure, Endocrinology. 147 (2006). doi:10.1210/en.2005-1159.

[36] I. Quesada, E. Fuentes, M.C. Viso-León, B. Soria, C. Ripoll, A. Nadal, Low doses of the endocrine disruptor bisphenol-A and the native hormone 17beta-estradiol rapidly activate transcription factor CREB., FASEB J. 16 (2002) 1671–1673. doi:10.1096/fj.02-0313fje.

[37] WHO-UNEP, State of the Science of endocrine disrupting chemicals 2012, Geneva (CH), 2012.

[38] NIEHS, Current status of test methods for detecting endocrine disruptors. Expert panel evaluation of the validation status of in vitro test methods for detecting endocrine disruptors, 2002.

[39] E.J. Routledge, D. Sheahan, C. Desbrow, G.C. Brighty, M. Waldock, J.P. Sumpter, Identification of estrogenic chemicals in STW effluent. 2. In vivo responses in trout and roach, Environ. Sci. Technol. 32 (1998) 1559–1565. doi:10.1021/es970796a.

[40] H.E. Witters, C. Vangenechten, P. Berckmans, Detection of estrogenic activity in Flemish surface waters using an in vitro recombinant assay with yeast cells, in: Water Sci. Technol., 2001: pp. 117–123.

[41] L.D. Claxton, G.A. De Umbuzeiro, D.M. DeMarini, The salmonella mutagenicity assay: The stethoscope of genetic toxicology for the 21st century, Environ. Health Perspect. 118 (2010) 1515–1522. doi:10.1289/ehp.1002336.

[42] J.R. Snape, S.J. Maund, D.B. Pickford, T.H. Hutchinson, Ecotoxicogenomics: The challenge of integrating genomics into aquatic and terrestrial ecotoxicology, Aquat. Toxicol. 67 (2004) 143–154. doi:10.1016/j.aquatox.2003.11.011.

[43] A.L. Miracle, G.T. Ankley, Ecotoxicogenomics: Linkages between exposure and effects in assessing risks of aquatic contaminants to fish, Reprod. Toxicol. 19 (2005) 321–326. doi:10.1016/j.reprotox.2004.06.007.

[44] T. Iguchi, H. Watanabe, Y. Katsu, Application of ecotoxicogenomics for studying endocrine disruption in vertebrates and invertebrates, Environ. Health Perspect. 114 (2006) 101–105. doi:10.1289/ehp.8061.

[45] T. Iguchi, H. Watanabe, Y. Katsu, Toxicogenomics and ecotoxicogenomics for studying endocrine disruption and basic biology, Gen. Comp. Endocrinol. 153 (2007) 25–29. doi:10.1016/j.ygcen.2007.01.013.

[46] J.L. Shepard, B. Olsson, M. Tedengren, B.P. Bradley, Protein expression signatures identified in Mytilus edulis exposed to PCBs, copper and salinity stress, in: Mar. Environ. Res., 2000: pp. 337–340. doi:10.1016/S0141-1136(00)00065-9.

[47] T. Finkel, N.J. Holbrook, Oxidants, oxidative stress and the biology of ageing., Nature. 408 (2000) 239–247. doi:10.1038/35041687.

[48] B. Halliwell, J.M.C. Gutteridge, Free radicals in biology and medicine, 2007. doi:10.1016/0891-5849(91)90055-8.

[49] M.R. Rotilio, G., Rossi, L., deMartino, A., Ferreira, A. M. D. S., Ciriolo, Free radicals, metalions and oxidative stress: chemical mechanisms of damage and protection in living systems, J. Braz. Chem. Soc. 6 (1995) 221–227.

[50] A. Valavanidis, T. Vlahogianni, M. Dassenakis, M. Scoullos, Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants, Ecotoxicol. Environ. Saf. 64 (2006) 178–189. doi:10.1016/j.ecoenv.2005.03.013.

[51] V.I. Lushchak, Environmentally induced oxidative stress in aquatic animals, Aquat. Toxicol. 101 (2011) 13–30. doi:10.1016/j.aquatox.2010.10.006.

[52] M. Abdollahi, A. Ranjbar, S. Shadnia, S. Nikfar, A. Rezaie, Pesticides and oxidative stress: a review, Med. Sci. Monit. 10 (2004) RA141-A147.

[53] A.J. García-Fernández, A.E. Bayoumi, Y. Pérez-Pertejo, M. Motas, R.M. Reguera, C. Ordóñez, R. Balaña-Fouce, D. Ordóñez, Alterations of the glutathione-redox balance induced by metals in

CHO-K1 cells, Comp. Biochem. Physiol. - C Toxicol. Pharmacol. 132 (2002) 365–373. doi:10.1016/S1532-0456(02)00079-0.

[54] A.E. Bayoumi, A.J. García-Fernández, C. Ordóñez, Y. Pérez-Pertejo, J.C. Cubría, R.M. Reguera, R. Balaña-Fouce, D. Ordóñez, Cyclodiene organochlorine insecticide-induced alterations in the sulfur-redox cycle in CHO-K1 cells, Comp. Biochem. Physiol. - C Toxicol. Pharmacol. 130 (2001) 315–323. doi:10.1016/S1532-0456(01)00257-5.

[55] A.J. Garcia-Fernandez, A.E. Bayoumi, Y. Perez-Pertejo, D. Romero, C. Ordonez, R.M. Reguera, R. Balana-Fouce, D. Ordonez, Changes in glutathione-redox balance induced by hexachlorocyclohexane and lindane in CHO-K1 cells., Xenobiotica. 32 (2002) 1007–1016. doi:10.1080/00498250210000012628.

[56] M.J. Koivula, T. Eeva, Metal-related oxidative stress in birds, Environ. Pollut. 158 (2010) 2359–2370. doi:10.1016/j.envpol.2010.03.013.

[57] T. Wafa, K. Nadia, N. Amel, C. Ikbal, T. Insaf, K. Asma, M.A. Hedi, H. Mohamed, Oxidative stress, hematological and biochemical alterations in farmers exposed to pesticides., J. Environ. Sci. Health. B. 48 (2013) 1058–69. doi:10.1080/03601234.2013.824285.

[58] D. Costantini, S. Verhulst, Does high antioxidant capacity indicate low oxidative stress?, Funct. Ecol. 23 (2009) 506–509. doi:10.1111/j.1365-2435.2009.01546.x.

[59] S. Espín, E. Martínez-López, P. Jiménez, P. María-Mojica, A.J. García-Fernández, Effects of heavy metals on biomarkers for oxidative stress in Griffon vulture (*Gyps fulvus*), Environ. Res. 129 (2014) 59–68. doi:10.1016/j.envres.2013.11.008.

[60] S. Espín, E. Martínez-López, M. León-Ortega, J.E. Martínez, A.J. García-Fernández, Oxidative stress biomarkers in Eurasian eagle owls (*Bubo bubo*) in three different scenarios of heavy metal

exposure, Environ. Res. 131 (2014) 134–144. doi:10.1016/j.envres.2014.03.015.

[61] S. Espín, E. Martínez-López, P. Jiménez, P. María-Mojica, A.J. García-Fernández, Interspecific differences in the antioxidant capacity of two Laridae species exposed to metals, Environ. Res. 147 (2016) 115–124. doi:10.1016/j.envres.2016.01.029.

[62] A.K. Sarmah, M.T. Meyer, A.B.A. Boxall, A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment, Chemosphere. 65 (2006) 725–759. doi:10.1016/j.chemosphere.2006.03.026.

[63] D.G.J. Larsson, C. de Pedro, N. Paxeus, Effluent from drug manufactures contains extremely high levels of pharmaceuticals, J. Hazard. Mater. 148 (2007) 751–755. doi:10.1016/j.jhazmat.2007.07.008.

[64] D. Li, T. Yu, Y. Zhang, M. Yang, Z. Li, M. Liu, R. Qi, Antibiotic resistance characteristics of environmental bacteria from an oxytetracycline production wastewater treatment plant and the receiving river, Appl. Environ. Microbiol. 76 (2010) 3444–3451. doi:10.1128/AEM.02964-09.

[65] K. Kümmerer, Antibiotics in the aquatic environment - A review - Part I, Chemosphere. 75 (2009) 417–434. doi:10.1016/j.chemosphere.2008.11.086.

[66] I. Michael, L. Rizzo, C.S. McArdell, C.M. Manaia, C. Merlin, T. Schwartz, C. Dagot, D. Fatta-Kassinos, Urban wastewater treatment plants as hotspots for the release of antibiotics in the environment: A review, Water Res. 47 (2013) 957–995. doi:10.1016/j.watres.2012.11.027.

[67] G. Lofrano, R. Pedrazzani, G. Libralato, M. Carotenuto, Advanced oxidation processes for antibiotics removal: a review, Curr. Org. Chem. 21 (2017) 1–14. doi:10.2174/1385278216661701031628 13.

[68] E. Kristiansson, J. Fick, A. Janzon, R. Grabic, C. Rutgersson, B. Weijdegård, H. Söderström,

D.G. Joakim Larsson, Pyrosequencing of antibiotic-contaminated river sediments reveals high levels of resistance and gene transfer elements, PLoS One. 6 (2011). doi:10.1371/journal.pone.0017038.

[69] R. Schmieder, R. Edwards, Insights into antibiotic resistance through metagenomic approaches, Future Microbiol. 7 (2012) 73–89. doi:10.2217/fmb.11.135.

[70] X.X. Zhang, T. Zhang, H.H.P. Fang, Antibiotic resistance genes in water environment, Appl. Microbiol. Biotechnol. 82 (2009) 397–414. doi:10.1007/s00253-008-1829-z.

[71] J.M.A. Blair, M.A. Webber, A.J. Baylay, D.O. Ogbolu, L.J. V. Piddock, Molecular mechanisms of antibiotic resistance, Nat. Rev. Microbiol. 13 (2015) 42–51. doi:10.1038/nrmicro3380.

[72] R. Cantón, Antibiotic resistance genes from the environment: a perspective through newly identified antibiotic resistance mechanisms in the clinical setting., Clin. Microbiol. Infect. 15 Suppl 1 (2009) 20–25. doi:10.1111/j.1469-0691.2008.02679.x.

[73] H.K. Allen, J. Donato, H.H. Wang, K.A. Cloud-Hansen, J. Davies, J. Handelsman, Call of the wild: antibiotic resistance genes in natural environments, Nat.Rev.Microbiol. 8 (2010) 251–259. doi:10.1038/nrmicro2312.

[74] T. Zhang, X.-X. Zhang, L. Ye, Plasmid metagenome reveals high levels of antibiotic resistance genes and mobile genetic elements in activated sludge., PLoS One. 6 (2011) e26041. doi:10.1371/journal.pone.0026041.

[75] Y. Yang, B. Li, F. Ju, T. Zhang, Exploring variation of antibiotic resistance genes in activated sludge over a four-year period through a metagenomic approach, Environ. Sci. Technol. 47 (2013) 10197–10205. doi:10.1021/es4017365.

[76] K. Kümmerer, Chemosphere antibiotics in the aquatic environment – A review – Part II,

Chemosphere. 75 (2009) 435–441. doi:10.1016/j.chemosphere.2008.12.006.

[77] P. Hugenholtz, B.M. Goebel, N.R. Pace, Erratum: Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity (Journal of Bacteriology (1998) 180:18 (4765-4774)), J. Bacteriol. 180 (1998) 6793. doi:0021-9193/98/$04.00+0.

[78] ISO, ISO 5667-3:2012, Water quality – Sampling – Part 3: Preservation and handling of water samples, 2012.

[79] A.K. McCall, R. Bade, J. Kinyua, F.Y. Lai, P.K. Thai, A. Covaci, L. Bijlsma, A.L.N. van Nuijs, C. Ort, Critical review on the stability of illicit drugs in sewers and wastewater samples, Water Res. 88 (2016) 933–947. doi:10.1016/j.watres.2015.10.040.

[80] Q. Sui, J. Huang, S. Deng, W. Chen, G. Yu, Seasonal variation in the occurrence and removal of pharmaceuticals and personal care products in different biological wastewater treatment processes, Environ. Sci. Technol. 45 (2011) 3341–3348. doi:10.1021/es200248d.

[81] EMCDDA, Assessing illicit drugs in wastewater: advances in wastewater-based drug epidemiology, 2016.

[82] C. Ort, M.G. Lawrence, J. Rieckermann, A. Joss, Sampling for pharmaceuticals and personal care products (PPCPs) and illicit drugs in wastewater systems: Are your conclusions valid? A critical review, Environ. Sci. Technol. 44 (2010) 6024–6035. doi:10.1021/es100779n.

[83] A.M. Comerton, R.C. Andrews, D.M. Bagley, , Practical overview of analytical methods for endocrine-disrupting compounds, pharmaceuticals and personal care products in water and wastewater., Philos. Trans. A. Math. Phys. Eng. Sci. 367 (2009) 3923–39. doi:10.1098/rsta.2009.0111.

[84] E.Perkins, N.Garcia-Reyero, S. Edwards, C. Wittwehr, D. Villeneuve, D. Lyons, and G. Ankley.

The adverse outcome pathway: A conceptual framework to support toxicity testing in the twenty-first century, Methods Pharmacol. Toxicol. 54 (2015). doi:10.1007/978-1-4939-2778-4_1.

[85] L. Escher, Beate I., Frederic, Bioanalytical tools in water quality assessment, IWA Publishing, London, New York, 2012.

[86] V. Scognamiglio, A. Antonacci, L. Patrolecco, M.D. Lambreva, S.C. Litescu, S.A. Ghuge, G. Rea, Analytical tools monitoring endocrine disrupting chemicals, TrAC - Trends Anal. Chem. 80 (2016) 555–567. doi:10.1016/j.trac.2016.04.014.

[87] C. Bicchi, T. Schilirò, C. Pignata, E. Fea, C. Cordero, F. Canale, G. Gilli, Analysis of environmental endocrine disrupting chemicals using the E-screen method and stir bar sorptive extraction in wastewater treatment plant effluents, Sci. Total Environ. 407 (2009) 1842–1851. doi:10.1016/j.scitotenv.2008.11.039.

[88] B. Schenk, M. Weimer, S. Bremer, B. van der Burg, R. Cortvrindt, A. Freyberger, G. Lazzari, C. Pellizzer, A. Piersma, W.R. Schäfer, A. Seiler, H. Witters, M. Schwarz, The ReProTect Feasibility Study, a novel comprehensive in vitro approach to detect reproductive toxicants, Reprod. Toxicol. 30 (2010) 200–218. doi:10.1016/j.reprotox.2010.05.012.

[89] I. Selma, Etteieb, Atsushi, Kawachi, Junkyu, Han, Jamila, Tarhouni, Hiroko, An evaluation of the environmental state of surface water quality using modified E-screen assay, in: C.A. Brebbia (Ed.), Water Pollut. XII, Wit press, Southampton, UK, 2014.

[90] G. Bertanza, R. Pedrazzani, M. Papa, G. Mazzoleni, N. Steimberg, L. Caimi, C. Montani, D. Dilorenzo, Removal of BPA and NPnEOs from secondary effluents of municipal WWTPs by means of ozonation, Ozone Sci. Eng. 32 (2010) 204–208. doi:10.1080/01919511003795303.

[91] A.M. Di Dea Bergamasco, M. Eldridge, J. Sanseverino, F.F. Sodré, C.C. Montagner, I.C. Pescara, W.F. Jardim, G.D.A. Umbuzeiro, Bioluminescent yeast estrogen assay (BLYES) as a sensitive tool to monitor surface and drinking water for estrogenicity, J. Environ. Monit. 13 (2011) 3288. doi:10.1039/c1em10464k.

[92] G. Bertanza, R. Pedrazzani, M. Dal Grande, M. Papa, V. Zambarda, C. Montani, N. Steimberg, G. Mazzoleni, D. Di Lorenzo, Effect of biological and chemical oxidation on the removal of estrogenic compounds (NP and BPA) from wastewater: An integrated assessment procedure, Water Res. 45 (2011) 2473–2484. doi:10.1016/j.watres.2011.01.026.

[93] C.D. Metcalfe, S. Kleywegt, R.J. Letcher, E. Topp, P. Wagh, V.L. Trudeau, T.W. Moon, A multi-assay screening approach for assessment of endocrine-active contaminants in wastewater effluent samples, Sci. Total Environ. 454–455 (2013) 132–140. doi:10.1016/j.scitotenv.2013.02.074.

[94] P.A. Bain, M. Williams, A. Kumar, Assessment of multiple hormonal activities in wastewater at different stages of treatment, Environ. Toxicol. Chem. 33 (2014) 2297–2307. doi:10.1002/etc.2676.

[95] J. Wang, M. Eldridge, F. min Menn, T. Dykes, G. Sayler, Standardized application of yeast bioluminescent reporters as endocrine disruptor screen for comparative analysis of wastewater effluents from membrane bioreactor and traditional activated sludge, Ecotoxicology. 24 (2015) 2088–2099. doi:10.1007/s10646-015-1556-z.

[96] J.M. Conley, N. Evans, H. Mash, L. Rosenblum, K. Schenck, S. Glassmeyer, E.T. Furlong, D.W. Kolpin, V.S. Wilson, Comparison of in vitro estrogenic activity and estrogen concentrations in source and treated waters from 25 U.S. drinking water treatment plants, Sci. Total Environ. (2015). doi:10.1016/j.scitotenv.2016.02.093.

[97] I. Bazin, H. Bin Seo, C.M. Suehs, M. Ramuz, M. de Waard, M.B. Gu, Profiling the biological effects of wastewater samples via bioluminescent bacterial biosensors combined with estrogenic assays, Environ. Sci. Pollut. Res. (2016) 1–9. doi:10.1007/s11356-016-6050-5.

[98] M. Ihara, T. Kitamura, V. Kumar, C.B. Park, M.O. Ihara, S.J. Lee, N. Yamashita, S. Miyagawa, T. Iguchi, S. Okamoto, Y. Suzuki, H. Tanaka, Evaluation of estrogenic activity of wastewater: Comparison among in vitro ER?? reporter gene assay, in vivo vitellogenin induction, and chemical analysis, Environ. Sci. Technol. 49 (2015) 6319–6326. doi:10.1021/acs.est.5b01027.

[99] B. Xuereb, L. Bezin, A. Chaumot, H. Budzinski, S. Augagneur, R. Tutundjian, J. Garric, O. Geffard, Vitellogenin-like gene expression in freshwater amphipod *Gammarus fossarum* (Koch, 1835): Functional characterization in females and potential for use as an endocrine disruption biomarker in males, Ecotoxicology. 20 (2011) 1286–1299. doi:10.1007/s10646-011-0685-2.

[100] A.O. Adeogun, K. Onibonoje, O.R. Ibor, R.A. Omiwole, A. V. Chukwuka, A.O. Ugwumba, A.A.A. Ugwumba, A. Arukwe, Endocrine-disruptor molecular responses, occurrence of intersex and gonado-histopathological changes in tilapia species from a tropical freshwater dam (Awba Dam) in Ibadan, Nigeria, Aquat. Toxicol. 174 (2016) 10–21. doi:10.1016/j.aquatox.2016.02.002.

[101] J.E. Cavallin, K.M. Jensen, M.D. Kahl, D.L. Villeneuve, K.E. Lee, A.L. Schroeder, J. Mayasich, E.P. Eid, K.R. Nelson, R.Y. Milsk, B.R. Blackwell, J.P. Berninger, C.A. Lalone, C. Blanksma, T. Jicha, C. Elonen, R. Johnson, G.T. Ankley, Pathway-based approaches for assessment of real-time exposure to an estrogenic wastewater treatment plant effluent on fathead minnow reproduction, Environ. Toxicol. Chem. 35 (2016) 702–716. doi:10.1002/etc.3228.

[102] N. Garcia-Reyero, C.M. Lavelle, B.L. Escalon, D. Martinović, K.J. Kroll, P.W. Sorensen, N.D. Denslow, Behavioral and genomic impacts of a wastewater effluent on the fathead minnow, Aquat. Toxicol. 101 (2011) 38–48. doi:10.1016/j.aquatox.2010.08.014.

[103] X. Terrien, J.B. Fini, B.A. Demeneix, K.W. Schramm, P. Prunet, Generation of fluorescent zebrafish to study endocrine disruption and potential crosstalk between thyroid hormone and corticosteroids, Aquat. Toxicol. 105 (2011) 13–20. doi:10.1016/j.aquatox.2011.04.007.

[104] S. Scholz, P. Renner, S.E. Belanger, F. Busquet, R. Davi, B. a Demeneix, J.S. Denny, M. Léonard, M.E. McMaster, D.L. Villeneuve, M.R. Embry, Alternatives to in vivo tests to detect endocrine disrupting chemicals (EDCs) in fish and amphibians--screening for estrogen, androgen and thyroid hormone disruption., Crit. Rev. Toxicol. 43 (2013) 45–72. doi:10.3109/10408444.2012.737762.

[105] M.E. Quaedackers, C.E. Van Den Brink, S. Wissink, R.H. Schreurs, J.A. Gustafsson, P.T. Van Der Saag, B.B. Van Der Burg, 4-hydroxytamoxifen trans-represses nuclear factor-kappa B activity in human osteoblastic U2-OS cells through estrogen receptor (ER)alpha, and not through ER beta., Endocrinology. 142 (2001) 1156–66. doi:10.1210/endo.142.3.8003.

[106] J. de Weert, A. de la Cal, H. van den Berg, A. Murk, A. Langenhoff, H. Rijnaarts, T. Grotenhuis, Bioavailability and biodegradation of nonylphenol in sediment determined with chemical and bioanalysis., Environ. Toxicol. Chem. 27 (2008) 778–785. doi:10.1897/07-367.1.

[107] P. Hallgren, A. Nicolle, L.A. Hansson, C. Brönmark, L. Nikoleris, M. Hyder, A. Persson, Synthetic estrogen directly affects fish biomass and may indirectly disrupt aquatic food webs, Environ. Toxicol. Chem. 33 (2014) 930–936. doi:10.1002/etc.2528.

[108] OECD, Conceptual framework for testing and assment of endocrine disrupters (as revised in 2012), 2012.

[109] G. Bertanza, M. Papa, R. Pedrazzani, C. Repice, G. Mazzoleni, N. Steimberg, D. Feretti, E. Ceretti, I. Zerbini, EDCs, estrogenicity and genotoxicity reduction in a mixed (domestic+textile) secondary effluent by means of ozonation: A full-scale experience, Sci. Total Environ. 458–460

(2013) 160–168. doi:10.1016/j.scitotenv.2013.03.108.

[110] L. Gonzalez-Gil, M. Papa, D. Feretti, E. Ceretti, G. Mazzoleni, N. Steimberg, R. Pedrazzani, G. Bertanza, J.M. Lema, M. Carballa, Is anaerobic digestion effective for the removal of organic micropollutants and biological activities from sewage sludge?, Water Res. 102 (2016) 211–220. doi:10.1016/j.watres.2016.06.025.

[111] A. Magdeburg, D. Stalter, M. Schl??sener, T. Ternes, J. Oehlmann, Evaluating the efficiency of advanced wastewater treatment: Target analysis of organic contaminants and (geno-)toxicity assessment tell a different story, Water Res. 50 (2014) 35–47. doi:10.1016/j.watres.2013.11.041.

[112] F. Masood, A. Malik, Mutagenicity and genotoxicity assessment of industrial wastewaters, Environ. Sci. Pollut. Res. 20 (2013) 7386–7397. doi:10.1007/s11356-013-1756-0.

[113] M. Papa, E. Ceretti, G.C.V. Viola, D. Feretti, I. Zerbini, G. Mazzoleni, N. Steimberg, R. Pedrazzani, G. Bertanza, The assessment of WWTP performance: Towards a jigsaw puzzle evaluation?, Chemosphere. 145 (2016) 291–300. doi:10.1016/j.chemosphere.2015.11.054.

[114] A. Sharif, M. Ashraf, A.A. Anjum, A. Javeed, I. Altaf, M.F. Akhtar, M. Abbas, B. Akhtar, A. Saleem, Pharmaceutical wastewater being composite mixture of environmental pollutants may be associated with mutagenicity and genotoxicity, Environ. Sci. Pollut. Res. 23 (2016) 2813–2820. doi:10.1007/s11356-015-5478-3.

[115] E.J.M. Penders, A.J. Martijn, A. Spenkelink, G.M. Alink, I.M.C.M. Rietjens, W. Hoogenboezem, Genotoxicity testing of samples generated during UV/H_2O_2 treatment of surface water for the production of drinking water using the Ames test in vitro and the Comet assay and the SCE test in vivo, J. Water Supply Res. Technol. 61 (2012) 435. doi:10.2166/aqua.2012.069.

[116] R. Weltens, G. Vanermen, K. Tirez, J. Robbens, K. Deprez, L. Michiels, Screening tests for

hazard classification of complex waste materials - Selection of methods, Waste Manag. 32 (2012) 2208–2217. doi:10.1016/j.wasman.2012.05.013.

[117] P.O. Keenan, A.W. Knight, N. Billinton, P. a Cahill, I.M. Dalrymple, C.J. Hawkyard, D. Stratton-Campbell, R.M. Walmsley, Clear and present danger? The use of a yeast biosensor to monitor changes in the toxicity of industrial effluents subjected to oxidative colour removal treatments., J. Environ. Monit. 9 (2007) 1394–401. doi:10.1039/b710406e.

[118] R. Zounkov, P.K. Odr Š, L. Dolež Alov, K.R. Hilscherov, B. Marš Lek, L.K. Bl Ha, Ecotoxicity and genotoxicity assessment of cytostatic pharmaceuticals, Environ. Toxicol. Chem. 26 (2007) 2208–2214. doi:10.1897/07-137R.1.

[119] T. Ohe, A. Suzuki, T. Watanabe, T. Hasei, H. Nukaya, Y. Totsuka, K. Wakabayashi, Induction of SCEs in CHL cells by dichlorobiphenyl derivative water pollutants, 2-phenylbenzotriazole (PBTA) congeners and river water concentrates, Mutat. Res. - Genet. Toxicol. Environ. Mutagen. 678 (2009) 38–42. doi:10.1016/j.mrgentox.2009.06.003.

[120] A. Ippolito, R. Giacchini, P. Parenti, M. Vighi, Natural variability of enzymatic biomarkers in freshwater invertebrates, Environ. Sci. Pollut. Res. 24 (2017) 732–742. doi:10.1007/s11356-016-7833-4.

[121] L. Lyubenova, E. Nehnevajova, R. Herzig, P. Schröder, Response of antioxidant enzymes in *Nicotiana tabacum* clones during phytoextraction of heavy metals, Environ. Sci. Pollut. Res. 16 (2009) 573–581. doi:10.1007/s11356-009-0175-8.

[122] P. Schröder, Exploiting plant metabolism for phytoremediation of organic xenobiotics, in: N. Willey (Ed.), Phytoremediation Methods Rev., Humana Press, Totowa, New Jersey, USA, 2007: pp. 251–265.

[123] J.H. Jorgensen, M.J. Ferraro, Antimicrobial susceptibility testing: a review of general principles and contemporary practices., Clin. Infect. Dis. 49 (2009) 1749–55. doi:10.1086/647952.

[124] M. Balouiri, M. Sadiki, S.K. Ibnsouda, Methods for in vitro evaluating antimicrobial activity: A review, J. Pharm. Anal. 6 (2016) 71–79. doi:10.1016/j.jpha.2015.11.005.

[125] R.I. Amann, W. Ludwig, K.H. Schleifer, Phylogenetic identification and in situ detection of individual microbial cells without cultivation., Microbiol. Rev. 59 (1995) 143–69. doi:10.1016/j.jip.2007.09.009.

[126] K.A. Gilbride, D.Y. Lee, L.A. Beaudette, Molecular techniques in wastewater: Understanding microbial communities, detecting pathogens, and real-time process control, J. Microbiol. Methods. 66 (2006) 1–20. doi:10.1016/j.mimet.2006.02.016.

[127] P. Mullany, Functional metagenomics for the investigation of antibiotic resistance., Virulence. 5 (2014) 443–7. doi:10.4161/viru.28196.

[128] A.G. McArthur, N. Waglechner, F. Nizam, A. Yan, M.A. Azad, A.J. Baylay, K. Bhullar, M.J. Canova, G. De Pascale, L. Ejim, L. Kalan, A.M. King, K. Koteva, M. Morar, M.R. Mulvey, J.S. O'Brien, A.C. Pawlowski, L.J. V Piddock, P. Spanogiannopoulos, A.D. Sutherland, I. Tang, P.L. Taylor, M. Thaker, W. Wang, M. Yan, T. Yu, G.D. Wright, The comprehensive antibiotic resistance database, Antimicrob. Agents Chemother. 57 (2013) 3348–3357. doi:10.1128/AAC.00419-13.

[129] Eurostat, Minimum wages, July 2016, (2016). http://ec.europa.eu/eurostat/statistics-explained/index.php/File:Minimum_wages,_July_2016_([1])_(EUR_per_month)_YB16.png

[130] EC, Council Directive 92/32/EEC of 30 April amending for the seventh Directive 67/548/EC on the approximation of the laws, regulations and administrative provisions relating to the

classification, packaging and labeling of dangerous substances, Brussels, 1992.

[131] EC, Commission Directive 93/67/EEC of 20 July, laying down the principles for the assessment of risks to man and the environment of substances notified in accordance with Council Directive 67/548/EEC, Brussels, 1993.

[132] EC, Technical guidance documents in support of the risk assessment Directive 93/67/EEC for new substances notified in accordance with the requirements of Council Directive 67/548/EEC, Brussels.

[133] EC, Council Regulation (EC) 793/93 of 23 March on the evaluation and control of the risks of existing substances, 1993.

[134] EC, Commission Regulation (EC) 1488/94 of 28 June, laying down the principles for the assessment of risks to man and the environment of existing substances in accordance with Council Regulation (EEC) No. 793/93, Brussels, 1994.

[135] EC, Commission Directive 98/8/EEC of 16 February concerning the placing of the biocidal products on the market, Brussels, 1998.

[136] J.J. Jiang, C.L. Lee, M. Der Fang, Emerging organic contaminants in coastal waters: Anthropogenic impact, environmental release and ecological risk, Mar. Pollut. Bull. 85 (2014) 391–399. doi:10.1016/j.marpolbul.2013.12.045.

[137] P.H. Chou, Y.L. Lin, T.C. Liu, K.Y. Chen, Exploring potential contributors to endocrine disrupting activities in Taiwan's surface waters using yeast assays and chemical analysis, Chemosphere. 138 (2015) 814–820. doi:10.1016/j.chemosphere.2015.08.016.

[138] C. Yan, Y. Yang, J. Zhou, M. Liu, M. Nie, H. Shi, L. Gu, Antibiotics in the surface water of the Yangtze Estuary: Occurrence, distribution and risk assessment, Environ. Pollut. 175 (2013) 22–

29. doi:10.1016/j.envpol.2012.12.008.

[139] Y. Sun, H. Huang, Y. Sun, C. Wang, X.L. Shi, H.Y. Hu, T. Kameya, K. Fujie, Ecological risk of estrogenic endocrine disrupting chemicals in sewage plant effluent and reclaimed water, Environ. Pollut. 180 (2013) 339–344. doi:10.1016/j.envpol.2013.05.006.

[140] B. Wang, F. Dong, S. Chen, M. Chen, Y. Bai, J. Tan, F. Li, Q. Wang, Phenolic endocrine disrupting chemicals in an urban receiving river (Panlong river) of Yunnan-Guizhou plateau: Occurrence, bioaccumulation and sources, Ecotoxicol. Environ. Saf. 128 (2016) 133–142. doi:10.1016/j.ecoenv.2016.02.018.

[141] P.-D. Hansen, Risk assessment of emerging contaminants in aquatic systems, TrAC Trends Anal. Chem. 26 (2007) 1095–1099. doi:10.1016/j.trac.2007.10.001.

[142] D.J. Caldwell, F. Mastrocco, P.D. Anderson, R. Länge, J.P. Sumpter, Predicted-no-effect concentrations for the steroid estrogens estrone, 17β-estradiol, estriol, and 17α-ethinylestradiol, Environ. Toxicol. Chem. 31 (2012) 1396–1406. doi:10.1002/etc.1825.

[143] Y. Valcárcel, S. González Alonso, J.L. Rodríguez-Gil, A. Gil, M. Catalá, Detection of pharmaceutically active compounds in the rivers and tap water of the Madrid Region (Spain) and potential ecotoxicological risk, Chemosphere. 84 (2011) 1336–1348. doi:10.1016/j.chemosphere.2011.05.014.

[144] M.D. Hernando, M. Mezcua, A.R. Fernández-Alba, D. Barceló, Environmental risk assessment of pharmaceutical residues in wastewater effluents, surface waters and sediments, Talanta. 69 (2006) 334–342. doi:10.1016/j.talanta.2005.09.037.

[145] Y.J. Lee, S.E. Lee, D.S. Lee, Y.H. Kim, Risk assessment of human antibiotics in Korean aquatic environment, Environ. Toxicol. Pharmacol. 26 (2008) 216–221. doi:10.1016/j.etap.2008.03.014.

[146] EMEA, Guideline on the environmental risk assessment of medical products for human use, 2006.

[147] L. Wang, G.G. Ying, J.L. Zhao, X.B. Yang, F. Chen, R. Tao, S. Liu, L.J. Zhou, Occurrence and risk assessment of acidic pharmaceuticals in the Yellow River, Hai River and Liao River of north China, Sci. Total Environ. 408 (2010) 3139–3147. doi:10.1016/j.scitotenv.2010.04.047.

[148] B. Quinn, F. Gagné, C. Blaise, An investigation into the acute and chronic toxicity of eleven pharmaceuticals (and their solvents) found in wastewater effluent on the cnidarian, *Hydra attenuata*, Sci. Total Environ. 389 (2008) 306–314. doi:10.1016/j.scitotenv.2007.08.038.

[149] D.S. Jones, L.W. Barnthouse, G.W. Suter, R.A. Efroymson, J.M. Field, J.J. Beauchamp, Ecological risk assessment in a large river-reservoir: 3. Benthic invertebrates, Environ. Toxicol. Chem. 18 (1999) 599–609.

[150] G. Libralato, V. Ghirardini Annamaria, A. Francesco, How toxic is toxic? A proposal for wastewater toxicity hazard assessment, Ecotoxicol. Environ. Saf. 73 (2010) 1602–1611. doi:10.1016/j.ecoenv.2010.03.007.

[151] G. Libralato, A. Volpi Ghirardini, F. Avezzù, Toxicity removal efficiency of decentralised sequencing batch reactor and ultra-filtration membrane bioreactors, Water Res. 44 (2010) 4437–4450. doi:10.1016/j.watres.2010.06.006.

[152] G. Libralato, E. Gentile, A. Volpi Ghirardini, Wastewater effects on *Phaeodactylum tricornutum* (Bohlin): Setting up a classification system, Ecol. Indic. 60 (2016) 31–37. doi:10.1016/j.ecolind.2015.06.014.

[153] G. Libralato, A. Volpi Ghirardini, F. Avezzù, To centralise or to decentralise: An overview of the most recent trends in wastewater treatment management, J. Environ. Manage. 94 (2012) 61–

68. doi:10.1016/j.jenvman.2011.07.010.

[154] G. Libralato, Management and control of wastewater: An ecotoxicological approach, 2013.

[155] European Comission, JRC Reference Report on monitoring of emissions to air and water from IED installations, Brussels, 2014.

[156] OSPAR, Whole effluent assessment, 2005.

[157] R.A. van Dam, J.C. Chapman, Direct toxicity assessment (DTA) for water quality guidelines in Australia and New Zealand, Australas. J. Ecotoxicol. 7 (2001) 175–198.

[158] E.A. Power, R.S. Boumphrey, International trends in bioassay use for effluent management, Ecotoxicology. 13 (2004) 377–398. doi:10.1023/B:ECTX.0000035290.89590.03.

[159] L.O.F. Protection, R. Assessment, Whole Effluent Toxicity Testing — Usefulness , Level of Protection , and Risk Assessment, Environ. Toxicol. Chem. 19 (2000) 3–13. doi:10.1897/1551-5028(2000)019<0003:wettul>2.3.co;2.

[160] P. Calow, Ecotoxicology: what are we trying to protect?, Environ. Toxicol. Chem. 13 (1994) 1549. doi:10.1897/1552-8618(1994)13[1549:EWAWTT]2.0.CO;2.

[161] A.A. Bulich, A practical and reliable method for monitoring the toxicity of aquatic samples., Process Biochem. 17 (1982) 45–47.

[162] H.C. Sarakinos, N. Bermingham, P. a White, J.B. Rasmussen, Correspondence between whole effluent toxicity and the presence of priority substances in complex industrial effluents, Environ. Toxicol. Chem. 19 (2000) 63–71. doi:10.1897/1551-5028(2000)019<0063:CBWETA>2.3.CO;2.

[163] G. Costan, N. Bermingham, C. Blaise, J.F. Ferard, Potential ecotoxic effects probe (PEEP): A novel index to assess and compare the toxic potential of industrial effluents, Environ. Toxicol. Water Qual. 8 (1993) 115–140. doi:10.1002/tox.2530080202.

[164] Swedish EPA, Characterisation of discharges from chemical industry – The stork project. Report No 4766, Stockholm, Sweden, 1997.

[165] M. Tonkes, P.J.F. De Graaf, J. Graansma, Assessment of complex industrial effluents in the Netherlands using a whole effluent toxicity (or wet) approach, in: Water Sci. Technol., 1999: pp. 55–61. doi:10.1016/S0273-1223(99)00253-X.

[166] G. Persoone, B. Marsalek, I. Blinova, A. Törökne, D. Zarina, L. Manusadzianas, G. Nalecz-Jawecki, L. Tofan, N. Stepanova, L. Tothova, B. Kolar, A practical and user-friendly toxicity classification system with microbiotests for natural waters and wastewaters, Environ. Toxicol. 18 (2003) 395–402. doi:10.1002/tox.10141.

[167] É. Vindimian, J. Garric, P. Flammarion, An index of effluent aquatic toxicity designed by partial least squares regression, using acute and chronic tests and expert judgements, … Toxicol. …. 18 (1999) 2386–2391. doi:10.1002/etc.5620181037.

[168] B.M. Phillips, J.W. Hunt, B.S. Anderson, H. Max Puckett, R. Fairey, C.J. Wilson, R. Tjeerdema, Statistical significance of sediment toxicity test results: threshold values derived by the detectable significance approach, Environ. Toxicol. Chem. 20 (2001) 371. doi:10.1897/1551-5028(2001)020<0371:SSOSTT>2.0.CO;2.

[169] G.B. Thursby, J. Heltshe, K.J. Scott, Revised approach to toxicity test acceptability criteria using a statistical performance assessment, Environ. Toxicol. Chem. 16 (1997) 1322. doi:10.1897/1551-5028(1997)016<1322:RATTTA>2.3.CO;2.

[170] EU, Directive 2013/39/EU, Off. J. Eur. Union. (2013) L 226/1-L226/17. doi:2013/39/EU.

[171] EC, Technical guidance document on risk assessment in support of Commision Directve 93/67/EEC, Commission Regulation (EC) No 1488/94, Directive 98/8/EC. Part II, 2003.

[172] E. Mendonça, A. Picado, S.M. Paixão, L. Silva, M.A. Cunha, S. Leitão, I. Moura, C. Cortez, F. Brito, Ecotoxicity tests in the environmental analysis of wastewater treatment plants: Case study in Portugal, J. Hazard. Mater. 163 (2009) 665–670. doi:10.1016/j.jhazmat.2008.07.012.

[173] C.I.D. (EU) 2015/495, Establishing a watch list of substances for Union-wide monitoring in the field of water policy pursuant to Directive 2008/105/EC of the European Parliament and of the Council, Brussels, 2015.

[174] A. Mavragani, K. Sypsa, A. Sampri, K. Tsagarakis, Quantifying the UK Online interest in substances of the EU Watchlist for water monitoring: diclofenac, estradiol, and the macrolide antibiotics, Water. 8 (2016) 542. doi:10.3390/w8110542.

[175] N. Le-Minh, S.J. Khan, J.E. Drewes, R.M. Stuetz, Fate of antibiotics during municipal water recycling treatment processes, Water Res. 44 (2010) 4295–4323. doi:10.1016/j.watres.2010.06.020.

[176] S.D. Melvin, F.D.L. Leusch, Removal of trace organic contaminants from domestic wastewater: A meta-analysis comparison of sewage treatment technologies, Environ. Int. 92 (2016) 183–188. doi:10.1016/j.envint.2016.03.031.

[177] F. Hernández-Padilla, M. Margni, A. Noyola, L. Guereca-Hernandez, C. Bulle, Assessing wastewater treatment in Latin America and the Caribbean: Enhancing life cycle assessment interpretation by regionalization and impact assessment sensibility, J. Clean. Prod. 142 (2017) 2140–2153. doi:10.1016/j.jclepro.2016.11.068.

[178] L. Pintilie, C.M. Torres, C. Teodosiu, F. Castells, Urban wastewater reclamation for industrial reuse: An LCA case study, J. Clean. Prod. 139 (2016) 1–14. doi:10.1016/j.jclepro.2016.07.209.

[179] P.T. Anastas, J.C. Warner, 12 Principles of Green Chemistry, Green Chem. Theory Pract. (1998)

30. doi:10.1039/b513020b.

[180] T. Rastogi, C. Leder, K. Kümmerer, Designing green derivatives of β-blocker Metoprolol: A tiered approach for green and sustainable pharmacy and chemistry, Chemosphere. 111 (2014) 493–499. doi:10.1016/j.chemosphere.2014.03.119.

[181] K. Kümmerer, J. Clark, Green and sustainable chemistry, in: Sustain. Sci., 2016: pp. 43–59. doi:10.1007/978-94-017-7242-6_4.

[182] J.P. Bound, K. Kitsou, N. Voulvoulis, Household disposal of pharmaceuticals and perception of risk to the environment, Environ. Toxicol. Pharmacol. 21 (2006) 301–307. doi:10.1016/j.etap.2005.09.006.

[183] M. Kotchen, J. Kallaos, K. Wheeler, C. Wong, M. Zahller, Pharmaceuticals in wastewater: Behavior, preferences, and willingness to pay for a disposal program, J. Environ. Manage. 90 (2009) 1476–1482. doi:10.1016/j.jenvman.2008.10.002.

[184] J.O. Straub, Reduction in the environmental exposure of pharmaceuticals through diagnostics, Personalised healthcare and other approaches. A mini review and discussion paper, Sustain. Chem. Pharm. 3 (2016) 1–7. doi:10.1016/j.scp.2015.12.001.

[185] J.O. Tijani, O.O. Fatoba, L.F. Petrik, A review of pharmaceuticals and endocrine-disrupting compounds: Sources, effects, removal, and detections, Water. Air. Soil Pollut. 224 (2013). doi:10.1007/s11270-013-1770-3.

[186] I. Logar, R. Brouwer, M. Maurer, C. Ort, Cost-benefit analysis of the swiss national policy on reducing micropollutants in treated wastewater, Environ. Sci. Technol. 48 (2014) 12500–12508. doi:10.1021/es502338j.

[187] L. Morris, V. Colombo, K. Hassell, C. Kellar, P. Leahy, S.M. Long, J.H. Myers, V. Pettigrove,

Municipal wastewater effluent licensing: A global perspective and recommendations for best practice, Sci. Total Environ. 580 (2017) 1327–1339. doi:10.1016/j.scitotenv.2016.12.096.

[188] B. Lu, X. Du, S. Huang, The economic and environmental implications of wastewater management policy in China: From the LCA perspective, J. Clean. Prod. (2016). doi:10.1016/j.jclepro.2016.10.113.

[189] European Parliament, Directive 2000/60/EC, Off. J. Eur. Communities. L 269 (2000) 1–90, Brussels, doi:10.1039/ap9842100196.

[190] W. De Schepper, J. Dries, L. Geuens, R. Blust, Wastewater treatment plant modeling supported toxicity identification and evaluation of a tank truck cleaning effluent, Ecotoxicol. Environ. Saf. 73 (2010) 702–709. doi:10.1016/j.ecoenv.2010.02.005.

[191] J. Dries, W. De Schepper, L. Geuens, R. Blust, Removal of ecotoxicity and COD from tank truck cleaning wastewater, Water Sci. Technol. 68 (2013) 2202–2207. doi:10.2166/wst.2013.477.

[192] M. Clara, B. Strenn, M. Ausserleitner, N. Kreuzinger, Comparison of the behaviour of selected micropollutants in a membrane bioreactor and a conventional wastewater treatment plant, Water Sci. Technol. 50 (2004) 29–36.

[193] M. Clara, N. Kreuzinger, B. Strenn, O. Gans, H. Kroiss, The solids retention time - A suitable design parameter to evaluate the capacity of wastewater treatment plants to remove micropollutants, Water Res. 39 (2005) 97–106. doi:10.1016/j.watres.2004.08.036.

[194] Y.K.K. Koh, T.Y. Chiu, A.R. Boobis, M.D. Scrimshaw, J.P. Bagnall, A. Soares, S. Pollard, E. Cartmell, J.N. Lester, Influence of operating parameters on the biodegradation of steroid estrogens and nonylphenolic compounds during biological wastewater treatment processes,

Environ. Sci. Technol. 43 (2009) 6646–54. doi:10.1021/es901612v.

[195] E.J. McAdam, J.P. Bagnall, Y.K.K. Koh, T.Y. Chiu, S. Pollard, M.D. Scrimshaw, J.N. Lester, E. Cartmell, Removal of steroid estrogens in carbonaceous and nitrifying activated sludge processes, Chemosphere. 81 (2010) 1–6. doi:10.1016/j.chemosphere.2010.07.057.

[196] P. Verlicchi, M. Al Aukidy, E. Zambello, Occurrence of pharmaceutical compounds in urban wastewater: Removal, mass load and environmental risk after a secondary treatment-A review, Sci. Total Environ. 429 (2012) 123–155. doi:10.1016/j.scitotenv.2012.04.028.

[197] K.A. Hicks, M.L.M. Fuzzen, E.K. McCann, M.J. Arlos, L.M. Bragg, S. Kleywegt, G.R. Tetreault, M.E. McMaster, M.R. Servos, Reduction of intersex in a wild fish population in response to major municipal wastewater treatment plant upgrades, Environ. Sci. Technol. 51 (2017) 1811–1819. doi:10.1021/acs.est.6b05370.

[198] I. Pala-Ozkok, A. Rehman, E. Ubay-Cokgor, D. Jonas, D. Orhon, Pyrosequencing reveals the inhibitory impact of chronic exposure to erythromycin on activated sludge bacterial community structure, Biochem. Eng. J. 90 (2014) 195–205. doi:10.1016/j.bej.2014.06.003.

[199] Z. Cetecioglu, B. Ince, D. Orhon, O. Ince, Anaerobic sulfamethoxazole degradation is driven by homoacetogenesis coupled with hydrogenotrophic methanogenesis, Water Res. 90 (2016) 79–89. doi:10.1016/j.watres.2015.12.013.

[200] K. Kümmerer, R. Alexy, J. Hüttig, A. Schöll, Standardized tests fail to assess the effects of antibiotics on environmental bacteria, Water Res. 38 (2004) 2111–2116. doi:10.1016/j.watres.2004.02.004.

[201] A. Alighardashi, D. Pandolfi, O. Potier, M.N. Pons, Acute sensitivity of activated sludge bacteria to erythromycin, J. Hazard. Mater. 172 (2009) 685–692. doi:10.1016/j.jhazmat.2009.07.051.

[202] I. Pala-Ozkok, Inhibitory Impact of selected antibiotics on biodegradation characteristic and microbial population under aerobic conditions, Istanbul Technical University, 2012.

[203] I.P. Ozkok, T.K. Yazan, E.U. Cokgor, G. Insel, I. Talinli, D. Orhon, Respirometric assessment of substrate binding by antibiotics in peptone biodegradation., J. Environ. Sci. Health. A. Tox. Hazard. Subst. Environ. Eng. 46 (2011) 1588–97. doi:10.1080/10934529.2011.609442.

[204] I. Pala-Ozkok, D. Orhon, Chronic effect of erythromycin on substrate biodegradation kinetics of activated sludge, Biochem. Eng. J. 81 (2013) 29–39. doi:10.1016/j.bej.2013.10.002.

[205] I. Pala-Ozkok, E. Ubay-Cokgor, Z.P. Cakar, D. Orhon, Acute impact of erythromycin on substrate utilization by activated sludge: Effect of sludge age, J. Chem. Technol. Biotechnol. 89 (2014) 1091–1102. doi:10.1002/jctb.4208.

[206] J.J. Beun, E. V Verhoef, M.C.M. Van Loosdrecht, J.J. Heijnen, Stoichiometry and kinetics of poly-beta-hydroxybutyrate metabolism under denitrifying conditions in activated sludge cultures, Biotechnol. Bioeng. 68 (2000) 496–507. doi:10.1002/(SICI)1097-0290(20000220)67:4<379::AID-BIT1>3.0.CO;2-2.

[207] Z. Cetecioglu, B. Ince, D. Orhon, O. Ince, Acute inhibitory impact of antimicrobials on acetoclastic methanogenic activity, Bioresour. Technol. 114 (2012) 109–116. doi:10.1016/j.biortech.2012.03.020.

[208] Z. Cetecioglu, B. Ince, O. Ince, D. Orhon, Acute effect of erythromycin on metabolic transformations of volatile fatty acid mixture under anaerobic conditions, Chemosphere. 124 (2015) 129–135. doi:10.1016/j.chemosphere.2014.12.004.

[209] Z. Cetecioglu, Evaluation of anaerobic biodegradability characteristics of antibiotics and toxic/inhibitory effect on mixed microbial culture, Istanbul Technical University, 2011.

[210] Z. Cetecioglu, B. Ince, M. Gros, S. Rodriguez-Mozaz, D. Barceló, D. Orhon, O. Ince, Chronic impact of tetracycline on the biodegradation of an organic substrate mixture under anaerobic conditions, Water Res. 47 (2013) 2959–2969. doi:10.1016/j.watres.2013.02.053.

[211] Z. Cetecioglu, B. Ince, M. Gros, S. Rodriguez-Mozaz, D. Barceló, O. Ince, D. Orhon, Biodegradation and reversible inhibitory impact of sulfamethoxazole on the utilization of volatile fatty acids during anaerobic treatment of pharmaceutical industry wastewater, Sci. Total Environ. 536 (2015) 667–674. doi:10.1016/j.scitotenv.2015.07.139.

[212] Z. Cetecioglu, Aerobic inhibition assessment for anaerobic treatment effluent of antibiotic production wastewater, Environ. Sci. Pollut. Res. 21 (2014) 2856–2864. doi:10.1007/s11356-013-2243-3.

[213] V. Belgiorno, L. Rizzo, D. Fatta, C. Della Rocca, G. Lofrano, A. Nikolaou, V. Naddeo, S. Meric, Review on endocrine disrupting-emerging compounds in urban wastewater: occurrence and removal by photocatalysis and ultrasonic irradiation for wastewater reuse, Desalination. 215 (2007) 166–176. doi:10.1016/j.desal.2006.10.035.

[214] L. Rizzo, S. Meric, M. Guida, D. Kassinos, V. Belgiorno, Heterogenous photocatalytic degradation kinetics and detoxification of an urban wastewater treatment plant effluent contaminated with pharmaceuticals, Water Res. 43 (2009) 4070–4078. doi:10.1016/j.watres.2009.06.046.

[215] M. Carotenuto, G. Lofrano, A. Siciliano, F. Aliberti, M. Guida, TiO_2 photocatalytic degradation of caffeine and ecotoxicological assessment of oxidation by-products, Glob. Nest J. 16 (2014) 463–473.

[216] G. Lofrano, G. Libralato, R. Adinolfi, A. Siciliano, P. Iannece, M. Guida, M. Giugni, A. Volpi Ghirardini, M. Carotenuto, Photocatalytic degradation of the antibiotic chloramphenicol and

effluent toxicity effects, Ecotoxicol. Environ. Saf. 123 (2016) 65–71. doi:10.1016/j.ecoenv.2015.07.039.

[217] G. Lofrano, M. Carotenuto, C.S. Uyguner-Demirel, A. Vitagliano, A. Siciliano, M. Guida, An integrated chemical and ecotoxicological assessment for the photocatalytic degradation of vancomycin, Environ. Technol. 35 (2014) 1234–1242. doi:10.1080/09593330.2013.865085.

[218] V. Naddeo, S. Meriç, D. Kassinos, V. Belgiorno, M. Guida, Fate of pharmaceuticals in contaminated urban wastewater effluent under ultrasonic irradiation, Water Res. 43 (2009) 4019–4027. doi:10.1016/j.watres.2009.05.027.