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

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Abstract

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

The manuscript deals with conventional and innovative analytical approaches for assessing toxicity, by reviewing laboratory and field assays; illustrative real scale and laboratory applications integrate and exemplify the proposed approach.

Keywords

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

Highlights

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

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1. Introduction

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

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

This opinion paper aims to gather the main findings obtained by different research groups participating to ES1202 COST Action "Conceiving Wastewater Treatment in 2020 – Energetic, environmental and economic challenges" (Water_2020). The final goal is to present the strength of a multi-tiered method within the risk assessment of whole effluent approach detailing the potentialities of toxicity as a parameter for treated wastewater quality evaluation in the perspective of its reuse. Pros and cons of

conventional and innovative bioassays have been investigated, including the socio-economic aspects; some case studies are showed as well.

2. Main knowledge and open issues

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

155 ecosystems, aquatic organisms being important targets, as they are exposed to wastewater residues over
156 their entire life.
157 The Water Framework Directive defines “hazardous substances” substances or groups of substances that
158 are toxic, persistent and liable to bio-accumulate, and other substances or groups of substances, which
159 give rise to an equivalent level of concern. Hence, in the risk assessment process, the initial step would
160 be hazard identification. Further, the primary investigation should already concern the possible health
161 problems caused by the pollutants. This process uses the intrinsic properties of a chemical (persistence,
162 solubility, K_{ow}, volatilization, etc.) to determine expected adverse effects, and on the other hand, to
163 estimate the probability of adverse effects to occur. In addition, the physical-chemical data provide
164 information about the relevance of some exposure paths. As the next step, and already partly depending
165 on the nature of the substance(s) under scrutiny, proper analytical tools capable of providing deeper
166 information on exposure and effects are required: the most commonly applied are acute toxicity, sub-
167 chronic and chronic toxicity, abiotic and biotic degradability, bioaccumulation and biomagnification.
168 During an exposure assessment, the following questions must be answered: 1) To which pollutant doses
169 are humans and ecosystems exposed, throughout a given lapse of time? and 2) How many individuals,
170 species or populations are exposed? In case of dose-response assessments, quantitative data regarding
171 biological effects under different situations and types of exposure must be supplied. Either finally, risk
172 assessment can be carried out, comparing exposure and effects, quantitatively or qualitatively, thus
173 determining the probability of effects occurrence. Both hazard and risk assessments are mandatory to
174 guarantee scientific support for regulations (Tarazona and Vega, 2002).
175 Ecotoxicity tests can be classified based on design (field, laboratory, computer), level of biological
176 organization (population, assemblage/community, ecosystem), exposure period (acute, sub-chronic,
177 chronic) and endpoint (lethal, sub-lethal). Short-term (“acute”) tests are generally used preliminarily,
178 being the survival the most common endpoint. Long term (“chronic”) tests (involving the observation of

179 sub-lethal effects on organism growth or reproduction) are used afterwards, if results from short term
180 tests combined with large safety factors indicate possible risks to the environment.
181 The use of acute and chronic tests in ecotoxicology has been proposed in reports from EU´s REACH
182 (Registration, Evaluation, Authorization and Restriction of Chemicals) with aims to improve the
183 protection of human health and the environment through the better and earlier identification of the
184 inherent properties of chemical substances. However, despite the presence of mixtures of multiple
185 compounds in environmental media and samples, theoretical considerations and experimental findings
186 suggest that the overall risk may be driven by only a few components in these mixtures (Hashmi et al.,
187 2018) (Altenburger et al., 2015), (Backhaus and Karlsson, 2014), (Price et al., 2012). Furthermore,
188 routinely detected chemicals often cannot explain the observed biological responses (*e.g.*, (Escher et al.,
189 2013)) confirming the need to integrate biological and chemical results.
190 Wastewaters, due to their nature and origin (municipal, industrial, runoff, grey), evidently collect and
191 concentrate a multitude of chemicals, that form complex mixtures including microbial consortia.
192 Therefore, when assessing the overall impact of any given wastewater (either raw or treated), it is
193 essential to take into account both: i) the removal/discharge of specific micropollutants and ii) the toxicity
194 of parent compounds, metabolites, treatment by-products and, in any case, of the whole stream. For this
195 reason, in order to gain insight not only into the impact of individual micropollutants, but also into the
196 effects exerted by wastewater, as a whole, bio-analytical tools are necessary. The removal of a target
197 xenobiotic compound, indeed, does not necessarily mean that the treatment process is detoxifying,
198 because adverse effects may be a result of the conversion of chemicals into metabolites or breakdown
199 products more toxic than the parent compounds.
200

201 ## 3. Which responses should be measured? A focus on “real life”

3.1 Principles

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

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

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

226 Different morphological findings linked to the exposure to organic pollutants have been described in
227 cells and tissues, such as inflammation, necrosis, apoptosis, and sometimes hypertrophy or hyperplasia
228 (Chen et al., 2016)(Cheng et al., 2015) (Ray et al., 2015) (Kumar et al., 2017) (Santana et al.,
229 2018)(Vicario-Parés et al., 2018).
230 Tissues, organs and systems are involved in vertebrate toxicokinetics: integument, respiratory and
231 digestive organs are firstly subjected to pollutant exposure, due to their direct interface with the outside
232 (Fatima et al., 2014) (Alves et al., 2016) (Salamat and Zarie, 2016) (Strzyzewska et al., 2016); besides,
233 nervous, immune, and endocrine systems are mostly studied (Kumari and Khare, 2018) (Lonappan et al.,
234 2016) (Vogt et al., 2018) (Xu et al., 2018)(Gambardella et al., 2016)(Adeogun et al., 2016) (Hicks et al.,
235 2017) (Tulloch et al., 2016a).
236 At the organismal level, different single-species bioassays (of which some are standardized) can be
237 applied to identify hazards through all relevant exposure routes: soil, water, air and food. The results can
238 be applied in ecological risk assessments (ERA), to yield Species Sensitivity Distributions (SSDs), which
239 model species sensitivity to chemicals or other stressors (Posthuma et al., 2002)(Tulloch et al., 2016b)
240 (Liu et al., 2014). At the border between organismal and population levels, alterations on growth and
241 development, reproduction and behavior, are issues of concern: tests on fish, birds, terrestrial and aquatic
242 invertebrates, terrestrial and aquatic macrophytes, and microscopic plants are commonly part of
243 monitoring campaigns (Kapustka, 2003) (Moro et al., 2014) (Gauthier et al., 2016) (Schwindt, 2015) (Lu
244 et al., 2017) (Díaz-Gil et al., 2017) (Gür et al., 2016) (Gopalapillai et al., 2014) (van Wijngaarden and
245 Arts, 2018).
246 Although long-term single-species tests and laboratory multi-species tests can be performed to predict or
247 evaluate population dynamics, increasing attention has been paid to *in situ* toxicological studies. In
248 particular, laboratory scale assays (*in vitro* and *in vivo*) may not allow a relevant simulation of real cases,
249 due to the presence of other stressing conditions that influence the biological response. Thus, laboratory

250 scale experiments might dramatically stray from real situation. Mesocosms and field assays provide
251 information about real ecological effects (Tarazona and Vega, 2002) (Szöcs et al., 2015) (Hasenbein et
252 al., 2017) (Lemm and Feld, 2017). A critical aspect may consist in the multi-faceted scenario of
253 responses, which can present non-monotonic trends and can be affected by hormesis and adaptation
254 phenomena (Calabrese and Blain, 2011).
255
256 **3.2 Exploiting the biological responses**
257 As far as chemical substances are concerned, every mechanism of toxicity is initiated by the interaction
258 between the chemical(s) and the organism (through a MIE, Molecular Initiating Event) and can be
259 described according to the following sequence: exposure, bioavailability and formation of a bond with
260 the ligand. Consequently, two opposite scenarios can reveal either alteration or adaptation, both driven
261 by complex pathways. Sub-lethal responses can be evaluated by quantifying proper physiological
262 condition indices, linked to morphometry, biochemistry and growth. Considering the toxicity pathways,
263 a MIE is the starting point of xenobiotic metabolism pathways; afterwards, cells answer via specific and
264 reactive modes of action (MOAs). Bioassays may reveal xenobiotic metabolism pathways, MOAs, as
265 well as the induction of adaptive stress response, by capturing specific signals. Cell viability remains a
266 major phenomenon to take into account. System responses, as abovementioned, regard the whole
267 apparatuses (Escher and Leusch, 2012) (Escher et al., 2014).
268 The effects occurring after an exposure to chemicals at molecular and cellular level can be linked with
269 those exhibited at system and organism levels, thanks to the model of the Adverse Outcome Pathways.
270 It represents a tool for predicting adverse effects, based on mechanistic evidence (specific key events,
271 KEs, can be measured), without exploring chemical reactions and biotransformation; it connects the
272 responses of in vitro, in chemico and in silico experiments with the toxicity shown in vivo and is
273 applicable to a variety of living organisms, from invertebrates to mammals (Ankley et al., 2016) (Escher

et al., 2017). Recently, a possible keystone for assessing the risk connected to the exposure to chemicals and mixtures has been theorized, starting from the concept of exposome (Wild, 2012), which represents an index of the cumulative risk (Smith et al., 2015). In effect, this concept, used in epidemiology, holds the action of exposure to external stressors (throughout the entire life of an organism) and the internal events taking place in response to the exposure. The causal link between exposure and adverse effects may be investigated by merging the principles of AOPS and exposome, which complement each other; moreover, the overall external exposure, via environment and dietary intake (Aggregate Exposure Pathway) can be considered by this approach, since the AEP accounts for the key events taking place from the external exposure to the internal target. The challenge will consist mainly in the capability of measuring exogenous and endogenous chemicals (Escher et al., 2017).

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

298 contained in a sample, and the use of a reference chemical to express the toxicity exhibited by a sample,
299 can offer a way of predicting the actual hazard for the aquatic environment (see also Paragraph § 5.3).
300 Finally, it is worth underlining, that the concurrence of the concentration of a substance freely dissolved
301 in the bioassay medium, in the cell and also in the cellular membrane cannot be taken for granted.
302 Consequently, the interpretation of the assay results can be twisted, since the response of the biological
303 system is usually correlated to the nominal concentration, which can be overestimated, due, for instance,
304 to partial adsorption of lipids and proteins dispersed in the medium. Therefore, the actual bioavailability
305 is a function of chemical partitioning in vitro (Fischer et al., 2017).
306 *Xenobiotic metabolism pathways*. The induction of these pathways indicates the presence of pollutants,
307 although it may not get to cytotoxicity. Among the measured endpoints it is worth citing the induction
308 of cytochrome P 450 1 A2, the activation of aryl hydrocarbon (AhR) and pregnane X (PXR) receptors,
309 the bond with peroxisome proliferator-activated receptor gamma (PPARγ) (Escher and Leusch,
310 2012)(Frederic D.L. Leusch et al., 2014).
311 *Specific receptor-mediated modes of toxic action*. They include endocrine disruption, reproduction and
312 development impairment, and acetylcholinesterase inhibition (Escher et al., 2014) (Escher et al., 2015b).
313 As far as endocrine disruption is concerned, it is mandatory to select the mechanisms to investigate, by
314 taking into consideration the biological complexity of the target organism and its physiology;
315 developmental and reproductive toxicity, however, are unpredictable through the execution of in vitro
316 tests, since they are meta-cellular events (Leusch et al., 2014). Several mechanisms account for endocrine
317 impairment: the most commonly studied are the bonds with nuclear receptors (this super family includes
318 48 types, in case of humans), and the interactions with membrane receptors, cytosolic receptors, orphan
319 nuclear receptors. Moreover, epigenetic changes as well as regulation cascade processes, effects on
320 hormones and oxidative metabolism can be numbered among the modes of action of endocrine disrupting
321 compounds. The modes of action concern all the biological levels, from single cells to the whole

organisms, both with acute and chronic effects, including reproductive, immunological and neurological disorders, cancer, diabetes, obesity.

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

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

A possible side effect of endocrine disruption might be the acquisition of antibiotic resistance. Large environmental releases are caused by their intensive use and, often, overuse or misuse. Furthermore, it is worth noting that the majority of antibiotics can be only partially metabolized after medication, and, thus, are excreted directly into the wastewater. Main hotspots are soils fertilized with manure runoff water from farms (Sarmah et al., 2006), effluents of drug production units (Larsson et al., 2007) (Li et al., 2010), WWTP effluents and sludge and, consequently, the receiving waterbodies (Kümmerer, 2009a), (Michael et al., 2013) (Lofrano et al., 2017). Antibiotic resistance is mechanistically based on inactivation or modification of the antibiotic, an alteration in the target site of the antibiotic that reduces its binding capacity, a modification of the metabolic pathways to circumvent the antibiotic effect or a reduction in the intracellular antibiotic accumulation by decreasing the permeability and/or increasing the active efflux of the antibiotic (Schmieder and Edwards, 2012). Acquisition of antibiotic resistance may occur

346 by mutation of its own genes (vertical evolution) or by acquiring new genes from other strains or species
347 (horizontal gene transfer) (Blair et al., 2015). The latter is mediated by the so-called mobile genetic
348 elements (MGE) such as phages, plasmids, integrons and transposons. The pool of genetic material
349 maintained by the environmental bacterial communities, named the resistome, provides the molecular
350 functions for protecting bacteria against the majority of clinically important classes of antibiotics and
351 constitutes a reservoir of ARGs that can be mobilized into human pathogenic bacteria (Cantón, 2009),
352 (Allen et al., 2010). ARGs have gained increasing attention in recent years (Zhang et al., 2011),
353 (Kristiansson et al., 2011), (Schmieder and Edwards, 2012), (Yang et al., 2013); there is still a critical
354 lack of knowledge about the diversity, distribution and origin of resistance genes (Kümmerer, 2009b),
355 especially for the unculturable majority of environmental bacteria, of which less than 1% are estimated
356 to be culturable (Hugenholtz et al., 1998).
357 Recent developments in genomics, together with the decrease of equipment prices and the wide
358 availability of sophisticated tools (such as DNA micro-arrays) have contributed to a tremendous
359 exploitation of molecular techniques. Starting from genome sequencing, this led to the study of
360 expression profiling (m-RNA transcripts, miRNA, ncRNA), the so-called transcriptomics, until the
361 characterization of protein (proteomics), peptide (peptidomics) and metabolic profiles (metabolomics).
362 The application of these analyses to toxicology (toxicogenomics) has rapidly spread to the impact
363 assessment of chemicals, mixtures and effluents towards the whole ecosystems (with regard to water
364 matrices), thus turning the research field into ecotoxicogenomics. This novel discipline, by investigating
365 transcripts, proteins and metabolites, overcomes several gaps inherent to the traditional approach, such
366 as long response time and relationships between exposure duration and possible adverse effects.
367 Meanwhile, it is possible to gain information on basic biology of organisms, also highlighting common
368 patterns of modes of action (Snape et al., 2004), (Miracle and Ankley, 2005).

369 The identification of gene expression mechanisms due to stimulation of natural hormones and
370 xenobiotics has been studied by means of DNA microarrays. Research has been focused mainly on the
371 estrogen nuclear receptors, which behave as transcription factors, *i.e.*, they interfere with the DNA
372 transcription process. On the contrary, knowledge of the response elements in gene promoter regions is
373 still lacking (Iguchi et al., 2006), (Iguchi et al., 2007). Transcriptome differs from proteome, due to post-
374 translational modifications; each environmental stimulus affects these mechanisms, as well as gene
375 expression. The challenge is, thus, to find the link between the "protein expression signatures", which
376 are constituted by biomarker patterns and the modes of actions of chemicals. At the same time, however,
377 the physiological levels, from the sub-cellular up to the organism, must be scrutinized to investigation,
378 to avoid collecting a huge amount of protein sequences without getting any relative response (Lemos et
379 al., 2010), (Shepard et al., 2000).
380 *Reactive modes of action*. They cover crucial effects, such as mutagenicity, genotoxicity and reactive
381 oxygen species (ROS) formation.
382 The toxicity towards the DNA and the genetic processes exhibits a wide spectrum of effects, and,
383 therefore, can be investigated by means of several complementary tests. The observed phenomena
384 include genotoxicity (not directly transmissible), mutagenicity (heritable change in a genotype),
385 mechanisms of DNA repair, carcinogenesis, and genetic-related developmental toxicity.
386 Briefly, damage to DNA involves alkylation (which mainly induces H bonds alteration, errors in base-
387 pairing); hydroxylation (hence, errors in base-pairing), deamination (bringing on changes from cytosine
388 to uracil, then errors in base-pairing and base substitution) formation of base analogues (for instance by
389 replacement of H atoms with halogens) leading to errors in base-pairing and base substitutions, strand
390 breaks, intra/interstrand cross links. Large planar molecules can intercalate within the double helix,
391 without reacting but disrupting replication, recombination and repair. The mutations consist in point
392 mutations (referred to nucleotide substitutions), yielding to errors in amino acids coding, and

chromosomal mutations (consisting in deletion or insertion of several contiguous genes, inversion of genes on a chromosome, exchange of large segments of DNA between non-homologous chromosomes) which lead to several mistakes in amino acids coding and, thus, to major phenotypic consequences. Bioassays often exploit mechanisms of DNA repair, which aim, for instance, at restoring its pristine function or destroying the damaged cell by means of apoptosis. They are based on the action of multiple enzymatic reactions, which, allow to repair base excision, nucleotide excision, double strand breaks, and base mispairing. (Chatterjee and Walker, 2017) (Croom, 2016) (Stalter et al., 2016) (Claxton et al., 2010) (Dearfield et al., 2002) (Turkez et al., 2017) (Verheyen, 2017) (Cartus and Schrenk, 2017) (Basu, 2018). Production of reactive oxygen species (ROS) and free radicals can be associated with carcinogenesis, immunotoxicity, teratogenesis and genotoxicity.

Although oxidative processes and the subsequent generation of free radicals are normal in the cellular metabolism of organisms (Finkel and Holbrook, 2000), oxidative stress is a condition of imbalance between the antioxidant defense and the production of ROS, so that the defense is overcome by the formation of radicals (Halliwell and Gutteridge, 2007). This process may cause oxidative damage to membrane lipids, DNA and proteins, and lead to cellular dysfunction and tissue injury (Schieber and Chandel, 2014)(Valavanidis et al., 2006) (Sies, 2015) (Neale et al., 2017a) (Sies et al., 2017).

Oxidative stress can be induced through different mechanisms. They may affect the redox cycle by donating electrons to or withdrawing electrons from cell components. During metabolism, they may deplete glutathione (endogenous antioxidant) or even inactivate other endogenous antioxidants (Lushchak, 2011). In short, oxidative stress can act either through overproduction of free radicals or alteration of the antioxidant homeostasis (Abdollahi et al., 2004). Indeed, a close relationship was described between metal cytotoxicity, the total GSH content and the dissociation energy of the sulphur-metal bonds, confirming the involvement of antioxidant defense mechanisms in the toxic action of these ions (García-Fernández et al., 2002). Oxidative stress is also due to the alteration of antioxidant enzymes

417 as glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferase (GST), catalase
418 (CAT) and superoxide dismutase (SOD), which may lead to elevated lipid peroxidation (Bayoumi et al.,
419 2001), (Garcia-Fernandez et al., 2002), (Koivula and Eeva, 2010). Increased plasma and erythrocytes
420 concentration of thiobarbituric acid reactive substances (TBARs), changes in the antioxidant status, and
421 impaired activities of cellular enzymes like superoxide dismutase (SOD) and catalase (CAT) indicate
422 higher oxidative stress in pesticides sprayers. Hence, many researchers have associated exposure to
423 pesticides with oxidative stress (Wafa et al., 2013), although concentrations occurring in water are
424 usually too low to elicit this effect (Neale et al., 2017b).
425 Biomarkers can be chosen based on the biological damage they are linked to. For instance, membrane
426 disruption might be associated with malondialdehyde, ethylene and ethane, isoprostanes concentration;
427 ROS production affects glutathione, photosynthetic pigments, total phenols content. Other biomarkers
428 may indicate more general phenomena, such ageing, decay and cell integrity (putrescine, spermidine,
429 spermine) and undetermined stressors (proline) (Rhee et al., 2007) (Pisoschi and Pop, 2015).
430 For such a complex scenario, it is essential to use an array of biomarkers to detect oxidative stress.
431 Different antioxidants are involved in the protection against ROS through a close cooperation between
432 them, and antioxidant defense may respond differently depending on the species used (Costantini and
433 Verhulst, 2009). Hence, at least one marker of oxidative damage should be measured in order to draw
434 inferences about oxidative stress (Costantini and Verhulst, 2009). Previous studies have shown that
435 antioxidant enzymes, particularly GPx, CAT and SOD, and lipid peroxidation may function as useful
436 biomarkers of metal induced effects on the antioxidant system in different bird species (Espín et al.,
437 2014a), (Espín et al., 2014b), (Espín et al., 2016). Further studies on other taxa will yield a better
438 understanding of the toxicity mechanisms induced by metal in wild birds and the definition of
439 concentrations prone to cause effects on the antioxidant system.

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

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

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

462 In this section, attention will be focused on how the different biological responses or stresses potentially
463 caused by micropollutants present in wastewater can be assessed in lab-scale tests. It is crucial that such
464 assays can simulate the actual conditions occurring in case of receiving waterbodies and reused waters.
465 Due to the challenges in collecting representative samples without losses and the inherent high costs for
466 conducting proper toxicity assessments, a well thought-through sampling strategy and sample collection
467 and preparation are of major importance.
468

469 **4.1. Sampling strategy, sample collection and preparation**

470 Wastewater concentrations of a wide range of compounds exerting plenty of unwanted biological
471 responses vary considerably as a function of time, agglomerate type and treatment plant performance.
472 All these factors can be dramatically different from site to site. For instance, the hydraulic retention time
473 (HRT) and sludge retention time (SRT) are highly dependent on plant design (e.g. type and size of
474 treatment units and internal flow patterns, including sludge treatment and reject water) and changes in
475 volumetric loading (*e.g.*, due to storm water intrusion). When assessing acute toxicity, the worst-case
476 scenario would usually be appropriate. However, chronic effects would better require average or median
477 exposure conditions. (Ort et al., 2010) detailed and explained all these aspects, highlighting the
478 importance of the sampled volumes, collection duration, storage conditions and data elaboration.
479 If possible, (considering the storage time constrains) composite samples are preferred, taken by means
480 of automatic samplers, usually collecting a volume aliquot every 10 min over a certain period, typically
481 24 hours. This would normally cover at least 1 HRT at most WWTPs, and it would be within the
482 maximum recommended storage time (if stored refrigerated) for the most relevant compounds (ISO,
483 2012), (McCall et al., 2016). In case of a longer sampling period, more 24-hours composite samples can
484 be summed, possibly after a pre-treatment (see below). When planning the monitoring campaign,
485 depending on the final goals, the expected weekly and seasonal variations may be taken into account (Sui

et al., 2011), as well as the conditions of receiving waterbodies (see paragraph §5). For instance, the concentrations of some illegal drugs have been found to increase towards the weekends or in relation to popular events which draw the crowds (EMCDDA, 2016).

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

Several factors can undesirably influence the composition of wastewater from collection to analysis: i) Compounds may be adsorbed to or diffuse from the sampler tubing and container. Most of the larger WWTPs have an automatic sampling equipment installed, which should preferably be used to minimize these effects. ii) Depending on the WWTP scheme, the compounds of interest in effluent water samples will usually be in the range of µg/L to pg/L together with suspended solids and microorganisms (mg/L). Biodegradation preferably occurs in raw sewage and in the effluents, with respect to the receiving waterbodies. Hence, it is important to limit both biotic and abiotic processes after sampling. Sterile filtration (<0.2 µm) is an efficient way to stop biotransformation, though enzymes may still be present. It is also necessary prior to solid-phase extraction (SPE) to prevent clogging. 0.45 µm filters are more commonly used since they are less prone to blocking. Anyway, the choice of filter type is also crucial: polycarbonate or (low static charge) cellulose acetate filters may be preferred as *e.g.* nitrocellulose filters tend to bind proteins while nylon filters tend to bind proteins, DNA and RNA. Acidification with HCl or HNO_3 is often adopted, alone or after filtration, to preserve the sample. However, this may alter the speciation and stability of the compounds and, therefore, should be applied with care (Comerton et al., 2009). iii) Unaltered sample should be used in toxicity tests, but for sensitivity reasons (i.e. frequently, toxicity tests are short-term based assays) micropollutants in the filtered sample can be cleaned up and concentrated prior to application in bioassays by stepwise SPE and elution. The composition and

concentration of the eluate depend on parameters such as physico-chemical properties of the compounds themselves, type of SPE, sample volume and percolation rate, sorbent cartridge volume, type of elution solvent and elution volume (see (Comerton et al., 2009) for a more detailed discussion). Anyway, solid phase extraction cannot retain most metals and salts; likewise, the majority of volatile organic compounds escape, thus modifying the final composition of mixture in the assay medium (Shane and Leusch, 2018). For this reason, recently it has been proposed to test the raw sample just immediately after a pre-filtration for sterilizing it prior addition to concentrated cells suspension (Niss et al., 2018)

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

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

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

4.2. From research to standards: multifaceted approach in bioassays

Several national and international authorities and scientific and technical organizations are instrumental in compiling and evaluating toxicity tests such as the Organisation for Economic Cooperation and

534 Development (OECD), World Health Organization (WHO), Food and Agriculture Organization (FAO),
535 International Organization for Standardization (ISO), American Society for Testing and Materials
536 (ASTM), United States Environmental Protection Agency (USEPA), United States Army Corps of
537 Engineers (USACE), American Public Health Association (APHA), Association Française de
538 Normalisation (AFNOR), Deutsches Institut für Normung (DIN), Italian Association for Standardisation
539 in the Chemical Sector (UNICHIM). The level of worldwide methods harmonization is sometimes
540 limited, thus a plethora of standard protocols exist with overlapping normalisation actions that sometimes
541 can be conflicting in terms of sensitivity, meaning that each protocol has its own feasibility. Time-by-
542 time authors must clearly declare which method they follow, to assure data reproducibility and correct
543 interpretation.
544 Table S1 (Supplementary material) reports a list (necessarily not exhaustive) of the most commonly
545 adopted toxicity tests, by pointing out the issuing agency and the measured response at the cell, tissue,
546 organ, organism and ecosystem level. The principle of adverse outcome pathways, AOP (Perkins et al.,
547 2015) allows to start from the initiating event, which possibly causes an adverse effect and to explore the
548 whole biological pathway, up to the ecosystem level, by following a mechanistic approach. Endocrine
549 activity testing is an example of such an application. The available assays can highlight both the
550 interference with the hormone receptors, by means of agonistic/anti-agonistic activities, and, more
551 generally, the interference with hormone synthesis and release.
552 The architecture of an assay involves simple cases, such as the mere formation of a bond between a
553 ligand (either radio-labeled or bound with a fluorochrome) and an isolated receptor (thus gaining only
554 analytical information). Options that are more complex rely on specific endpoints, such as protein activity
555 (both in terms of protein synthesis and protein interactions with co-factors), cell proliferation and direct
556 receptor activation linked with a gene reporter. The aforementioned tests employ several different
557 techniques for the quantification of the biological activity. They range from basic approaches with UV-

VIS spectroscopy, to the most exploited tools like ELISA, radio-immunology, and fluorometry (including flow cytometry) (NIEHS, 2002), (Escher and Leusch, 2012), (Scognamiglio et al., 2016). Reporter gene-assays involve the use of cells (deriving from bacteria, yeasts, fish, humans and other mammals) to assess gene expression mediated by chemicals. The endpoints consist in cell proliferation in case of E-SCREEN (Scognamiglio et al., 2016), (Bicchi et al., 2009), (Schenk et al., 2010), (Selma, Etteieb, Atsushi, Kawachi, Junkyu, Han, Jamila, Tarhouni, Hiroko, 2014), while, most assays are based on gene expression, often after specific transfection. The main testing tools are the following: CALUX, CAFLUX, PALM, MELN, MVLN, T47D-kBluc, HELN, HGELN, MDA-kb2, PR reporter gene assay, YES, YAS, BLYES, BLYAS, BLYR (luciferase/fluorescent protein gene expression, β-galactosidase synthesis induction); they measure the binding with estrogen, androgen, progesterone, glucocorticoid, peroxisome proliferator activator receptors (Scognamiglio et al., 2016), (Bertanza et al., 2010), (Di Dea Bergamasco et al., 2011), (Bertanza et al., 2011), (Metcalfe et al., 2013), (Bain et al., 2014), (Wang et al., 2015), (Conley et al., 2015), (Bazin et al., 2016). Other tests are focused on the quantification of the production of proteins, such as vitellogenin, choriogenin, *zona radiata* protein after estrogenic stimulation (Ihara et al., 2015), (Xuereb et al., 2011), (Adeogun et al., 2016), (Cavallin et al., 2016). Steroidogenesis based tests look promising in providing additional information on disruption mechanisms ((Cavallin et al., 2016), (Garcia-Reyero et al., 2011)) although *in vivo* compensation of the effects which occur during *in vitro* assays is far from being defined. Among the *in vivo* assays aimed to evidence the gene expression induced by pollutants, there is the application of the genetically modified *Danio rerio* (green fluorescent protein expression, controlled by a thyroid hormone response promoter of *Xenopus laevis*) already applied to environmental samples (Terrien et al., 2011), (Scholz et al., 2013). An *in vitro* reporter gene assay ($ER\alpha$-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 ($ER\alpha$-luc assay) based

582 on U2OS-ERαcells, with luciferase as reporter (Quaedackers et al., 2001). The method to culture and
583 expose the cells and to assay luciferase activity has previously been described in (de Weert et al., 2008).
584 Measurement of the estrogenic activity of nonylphenol during biological degradation showed a decrease
585 of the estrogenic activity during microbial degradation, and, consequently, can be used to determine the
586 ecotoxicological risk of an environmental sample. An overview of pros and cons of the main assays
587 applied for ecotoxicological purposes, in case of water ecosystems, together with basic technical
588 information is reported in the paper recently published by (Brack et al., 2016).
589 Besides, the study of the effects of EDs on populations and communities requires the setting up of
590 mesocosm assays or the direct observation of real scenarios. It is worth noting that the pollutants (and
591 mixtures) are effective, in parallel, at increasing levels, up to the ecological aspects, hence yielding to
592 significant changes on the trophic web. This is the case, for instance, for *R. rutilus*, a planktivorous fish,
593 whose grazing capacity is deeply reduced by the exposure to EE2; the population of plankton, as a result,
594 can undergo a development (Hallgren et al., 2014).
595 Among the agencies and organizations which are facing the issue of endocrine disruption, the OECD
596 approach can be cited, since it prescribes further subsidiary levels of investigations, in order to draw a
597 complete profile of endocrine disruption (OECD, 2012). The five levels consist of: 1) acquirement of
598 existing data about chemical, physical and toxicological properties, 2) execution of *in vitro* assays aimed
599 to highlight endocrine pathways, 3) execution of *in vivo* assays aimed to highlight endocrine pathways,
600 4) execution of *in vivo* assays aimed to highlight adverse effects on endocrine endpoints, 5) execution of
601 *in vivo* assays aimed to highlight adverse effects on endocrine endpoints throughout the whole life of an
602 organism and across generations.
603 As far as genetic toxicity is concerned, the assays proposed in the scientific literature, the international
604 standards (in particular, issued by OECD and ISO) and available on the market (automatized, in most
605 cases) allow highlighting and quantifying multiple effects, from early, hence reversible modifications of

606 genetic material, up to irreversible damages, which can evolve to either apoptosis or neoplastic
607 formations. Therefore, the assays can be usefully integrated in a multi-layer frame, also due to the option
608 of testing organisms of growing biological complexity (prokaryotes and eukaryotes) and situated at
609 different levels of the trophic web (producers, consumers and decomposers). The detection of genetic
610 damage induced by various mechanisms is made possible by performing *in vitro* and *in vivo* tests. The
611 endpoints can involve a) gene mutations; b) chromosomal damage (to parts of the chromosomes); c)
612 genomic damage (loss/gain of entire chromosomes) c) epigenetics.
613 Among the large number of tests either standardized or just proposed for the evaluation of water matrices,
614 it is worth mentioning: a) the Ames test, for detecting point mutations in *S. typhimurium* bacterial strains;
615 it is based on the growth of histidine revertant bacteria over specific culture media, with or without the
616 addition of rat liver microsomal fractions. It is the most applied in case of environmental evaluations
617 (Bertanza et al., 2013), (Gonzalez-Gil et al., 2016), (Magdeburg et al., 2014), (Masood and Malik, 2013),
618 (Papa et al., 2016), (Sharif et al., 2016). It takes 48 hours to obtain a result. b) The micronuclei test, for
619 detecting chromosomal mutations (generally performed on root cells of *A. cepa*, throughout 72 hours)
620 (Bertanza et al., 2013), (Masood and Malik, 2013), (Papa et al., 2016); it is a biomarker of chromosomic
621 damage and genome instability. Its exposure depends on the employed organism. c) The Comet assay
622 (also called SCGE, Single Cell Gel Electrophoresis), for quantifying the primary DNA damage; it is
623 typically carried out on eukaryotic cells (Bertanza et al., 2013), (Gonzalez-Gil et al., 2016), (Papa et al.,
624 2016), (Sharif et al., 2016), (Penders et al., 2012). d) The reporter gene assays, which detect the SOS
625 response induced by DNA damage and have a duration of several hours; often automatized, they are less
626 sensitive and robust than the aforementioned tests (Magdeburg et al., 2014), (Weltens et al., 2012). e)
627 The GreenScreen assay (GSA) which employs cells of *S. cerevisiae;* it detects a DNA damage, based on
628 the quantification of a green fluorescent protein linked to the promoter of the RAD54 gene (Keenan et

al., 2007), (Zounkov et al., 2007). f) The sister chromatide exchange (SCE assay) based on mammal cells (Penders et al., 2012), (Ohe et al., 2009).

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

Therefore, it is important to clearly denominate the purpose of the assay in the frame of the toxicity testing. Enzymes like SOD, CAT, APOX, DHAR, MDHAR, GPOX and GR are members of the Halliwell-Asada-pathway (Halliwell and Gutteridge, 2007) detoxifying radicals and toxic oxygen species that might build up under xenobiotic stress (Lyubenova et al., 2009).

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

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

Among pharmaceuticals, antibiotics give seriously cause of concern, due to their indirect adverse effect on human health linked to the phenomenon of bacterial resistance. In clinical microbiology standardized

653 susceptibility tests they clearly dominate among the available methods, aimed to detect possible drug
654 resistance in common pathogens and to assure susceptibility to drugs for a particular infection (Jorgensen
655 and Ferraro, 2009). In these tests, resistance is detected by carrying out growth inhibition tests broth (*e.g*.
656 the macrobroth dilution test and the miniaturised broth dilution test) or by agar diffusion (*e.g*. the gradient
657 diffusion test and the disk diffusion test). In most of these tests (except the disk diffusion test) the lowest
658 concentration of antibiotic that prevents growth, represented by the minimum inhibitory concentration
659 (MIC), is quantified. A more detailed discussion of advantages and drawbacks of these methods is given
660 by (Jorgensen and Ferraro, 2009) and (Balouiri et al., 2016). Such culture-based approaches typically
661 require 1-2 days for fast-growing bacteria like *Escherichia coli* or *Salmonella* spp., and several weeks
662 for slow-growing bacteria, like *Mycobacterium tuberculosis*. However, the main drawback of cultural
663 methods is that the vast majority of strains present in environmental microbial communities (<1 %;
664 (Hugenholtz et al., 1998)) still cannot grow outside their host environment. Assessment of antibiotic
665 resistance in such communities based solely on cultivable bacteria will therefore easily generate
666 unrepresentative and biased results (Amann et al., 1995).
667 For that reason, tools for molecular detection of antibiotic resistance genes (ARG) have become
668 increasingly popular (Schmieder and Edwards, 2012), (Zhang et al., 2009), (Gilbride et al., 2006).
669 Polymerase chain reaction (PCR) assays such as multiplex PCR and quantitative real-time PCR (qPCR)
670 have frequently been applied to amplify and detect specific ARGs in environmental samples (Zhang et
671 al., 2009). Nevertheless, they only target well-studied pathogens or resistance-causing genes (as the
672 primers are based on known resistance genes only) and cannot easily be used for broad-spectrum
673 screening (Schmieder and Edwards, 2012). DNA microarray is a more powerful molecular method than
674 the PCR assays as it is able to detect the presence or absence of a large range of ARGs simultaneously
675 in a single assay (Gilbride et al., 2006). However, its use for environmental samples has been limited as
676 it is hampered by low detection limits (partially overcome if coupled with PCR) and the need for

677 complicated pre-treatment to reduce the presence of other compounds that inhibit DNA extraction and/or
678 target gene amplification (Zhang et al., 2009). Furthermore, both microarray and PCR based technologies
679 are not conclusive regarding the detection of resistance genes in metagenomes (Mullany, 2014).
680 Metagenomic analysis is one of the latest modern approaches for analysing complex microbial
681 communities and enables to describe the genetic potential of a community and to detect the
682 presence/absence of genes or genetic variations responsible for antibiotic resistance (Schmieder and
683 Edwards, 2012). Metagenomic analysis usually follows two different approaches, namely sequence-
684 based and functional. In the first case, a sample of DNA from the studied metagenome is extracted and
685 completely, but randomly, sequenced in relatively short contiguous sequence read lengths. These
686 sequences are then compared with known sequences that have accumulated over the years in public
687 databanks (reference sequences; e.g. (McArthur et al., 2013)) to identify resistance genes and/or
688 mutations that are known to cause resistance (Schmieder and Edwards, 2012). This approach has the
689 potential to identify all known resistance genes in a given metagenome. Though, important shortcomings
690 are that it can only identify known ARGs and that it gives no information on expression of the resistance
691 genes (Mullany, 2014). This is, however, overcome by the second approach, functional metagenomics,
692 in which the extracted DNA is shot-gun cloned into cloning vectors and subsequently expressed in a
693 cultivable surrogate host (usually *E. coli*) plated onto antibiotic-containing agar. If bacterial artificial
694 chromosomes (BACs) are used, a larger gene fragment can be inserted, potentially making it possible to
695 trace the phylogenetic origins of the original host bacteria (Mullany, 2014). These larger gene fragments
696 are also more likely to include antibiotic resistance that is encoded by multiple genes. Disadvantages of
697 using BACs is the low copy number (though, they are usually more stable than higher copy vectors) and
698 the need for the transcription and translation signals to be efficiently recognized by the host organism. If
699 vectors that only accept small inserts are used, the copy numbers are higher, and the host's transcription
700 and translation systems can be used, hence the drawbacks of using BACs are circumvented. However,

701 the small size of the insert will not normally allow information about the genetic background of the
702 resistance gene. However, if coupled with sequence-based metagenomics, this disadvantage can be
703 overcome to some extent. See (Mullany, 2014) and (Schmieder and Edwards, 2012) for a more thorough
704 discussion of advantages and drawbacks in metagenomics analyses.
705
706 **4.3 Criteria for selecting a bioassay**
707 Toxicity assessment can be prescribed by regulatory or voluntary requirements, generally, referring to
708 standardized methods to acquire, analyze and interpret data. On the contrary, when the final goal is a
709 deeper investigation of the impact of effluents (or chemicals/mixtures) on specific biological targets, at
710 different levels (from sub-cellular components to the whole community), either within a routine
711 monitoring or for the evaluation of a specific polluted site, several alternatives arise (Neale et al., 2017c).
712 The criteria underpinning the selection of a bioassay (or a battery of complementary assays) should
713 include the duration, the required volume (smaller volumes may favor the miniaturization, hence the
714 automation of the procedure), the price (capital expenses: building with related services, such as
715 hydraulics and electrics, instrumentations; operation expenses: consumables, personnel, license fees), the
716 throughput, the sensitivity (by taking into consideration possible non-monotonic responses), the
717 specificity, the requirement of trained and skillful operators, the possibility to measure
718 acute/chronic/transgenerational effects, the capability of evidencing toxicokinetic or, more generally,
719 specific metabolic pathways of interest (Campana and Wlodkowic, 2018) (Leusch et al., 2017b).
720 A pivotal role is played by the personnel cost, which differs highly among the countries: the European
721 example is revealing, varying the minimum wage per month from less than 250 € for Albania, to nearly
722 2,000 € for Luxembourg (Eurostat, 2016). Furthermore, the same test (e.g., Ames on *S. typhimurium*)
723 can be carried out either by adopting the conventional microbiological approach and cultivated bacteria
724 or using commercial kits, including also genetically modified microorganisms.

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

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

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

An encouraging step forward has been taken in the field of drinking water, by the German Federal Environment, with the recommendation of health-related indicator values (HRIV), which provide for thresholds, set as a function of availability and completeness of toxicological data. The key effects include genotoxicity, neurotoxicity and germ cell-damaging potential; further investigations may

749 profitably complement this battery (Kuckelkorn et al., 2018). A similar approach should be followed in
750 case of treated wastewater.
751 It is worth noting that, similarly to Green Chemistry, Green Toxicology proposes a list of principles,
752 which should be taken into account before planning the execution of a testing session: energy and
753 materials saving, use of harmless reagents, minimization of animal use (in accordance with the 3Rs –
754 reduction, refinement and replacement approach) are fundamental suggestions. A cultural change is.
755 required by companies and policy makers: computational tools might provide early information about
756 toxicity mechanisms of substances and health and safety. In silico and fully automated in vitro testing
757 might precede and complement a further multi-tiered assays battery (whose quantity and burden could
758 then be reduced) (Crawford et al., 2017) (Maertens and Hartung, 2018). Starting from the Quantitative
759 Structure–Activity Relationships (QSARs) and the QVIVE (quantitative in vitro-to-in vivo
760 extrapolation) (QIVIVE) approaches, by further implementations, it is possible to predict adverse
761 outcomes based on the effect concentrations (Ankley et al., 2010) (Tang et al., 2013). Nevertheless, a
762 definitive assessment of water quality cannot be reached by performing only in vitro tests (Shane and
763 Leusch, 2018).
764

765 # 5. Environmental risk assessment: challenges and limitations

766 ## 5.1 Traditional environmental risk assessment

767 Environmental Risk Assessment (ERA) deals with the interactions of agents or hazards, humans and
768 ecological resources. It describes human populations, ecological resources and agents, analyzes agents
769 and exposure potential, characterizes the potential for adverse effects, defines uncertainties, generates
770 options to deal with the risks, and communicates information about the risks to humans and ecosystems.
771 ERA is a process that evaluates the likelihood or probability that adverse effects may occur to

772 environmental values, because of human activities (i.e., a formal procedure for identifying and estimating
773 the risk of environmental damage). ERA provides information for making reasoned decisions by defining
774 the range of risks associated with various options, but it does not dictate a specific outcome. ERA also
775 provides a mechanism for managers to communicate forecasted risks associated with decisions, such that
776 stakeholders and the public are informed of the implications for environmental values.
777 Based on the toxicological data and measured environment concentrations found in the literature, the risk
778 for acute toxic effects is unlikely but chronic adverse effects cannot be excluded. Therefore, risk
779 characterization is one of the important tools to estimate the environmental risk, particularly in view that
780 co-occurrence of diverse micropollutants in environmental matrices may lead to additive, synergistic,
781 and antagonistic toxic effects which is difficult to predict if only concentration is available.
782

783 **5.2 Wastewater toxicity assessment, ranking, and reuse**

784 The problem of wastewater toxicity data management and interpretation is still a current issue, especially
785 when high toxicity levels are recorded and there are compulsory legislative threshold limits to comply
786 with (Libralato et al., 2010a), (Libralato et al., 2010b), (Libralato et al., 2016). Around the world,
787 countries have developed various toxicity-based methods to assess the quality of treated wastewater to
788 increase the accessibility to water and sanitation in order to avoid human health impacts and ecosystem
789 services impairment. Several procedures for discharge hazard estimation have been proposed generating
790 assessment toolboxes including limit-based threshold approaches, and toxicity score and index for data
791 integration and interpretation including expert judgment as well (Libralato et al., 2010a). The main goal
792 of wastewater ecotoxicity assessment and ranking should be to minimize the adverse impact onto the
793 receiving water body as well as treated wastewater recovery and reuse (Libralato et al., 2012). Apart
794 from the possibility of using toxicity tests to estimate potential hazardous effects on the ecosystem, they
795 can favour the protection and the optimization of wastewater treatment plant operation, by discriminating

796 the best available technologies (Libralato, 2013), (European Comission, 2014)). Consistent wastewater
797 toxicity assessment can increase the general level of sustainability in the management of water resources
798 pushing ahead both “zero emission” and “zero discharge” along with the precautionary principle
799 (OSPAR, 2005).
800 Toxicity is currently used to check effluent quality into various national legislation around the world to
801 be included in water monitoring and control programs like direct toxicity assessment (van Dam and
802 Chapman, 2001), whole effluent toxicity, integrating controlling of effluent, whole effluent
803 environmental risk, environmental effects monitoring (Power and Boumphrey, 2004), and whole effluent
804 assessment (OSPAR, 2005), (Protection and Assessment, 2000). Apart from any program peculiarities,
805 the main question is still how to use or “interpret” toxicity data keeping in mind that the objective is to
806 protect the environment and not the ‘‘white rat’’ testing species (Calow, 1994).
807 Generally, legislative requirements tend to refer to a toxicity limit based on a single test or a battery of
808 toxicity tests considering as result the worst registered data. This method is quite simple, but not
809 environmentally realistic, depending on the biological model-endpoint pairs considered and the weight-
810 of-evidence score attributable to each of them. Sometimes, the classification is attributed just on a
811 logarithmic (Bulich, 1982), (Sarakinos et al., 2000) or order of magnitude basis (Costan et al., 1993),
812 (Swedish EPA, 1997), (Tonkes et al., 1999), (Persoone et al., 2003) or expert judgment and regression
813 analysis pair (Vindimian et al., 1999). Some authors tried to overcome such drawbacks by identifying
814 tools to integrate and weight toxicity data on a statistical basis also according to the ecological relevance
815 of the considered endpoint (Libralato et al., 2010a). For example, Libralato et al. (Libralato et al., 2010a),
816 (Libralato et al., 2010b) applied the minimum significance distance (MSD) criterion to support general
817 decisions about the presence or absence of toxicity from wastewater samples on a database of more than
818 100 wastewater toxicity data including domestic, municipal and industrial discharges (Phillips et al.,
819 2001), (Thursby et al., 1997). This method enabled the consideration on a species-specific basis. Thus,

820 the relative sensitivity of the biological model made the assessment of toxicity independent to reference
821 wastewater as well. Moreover, expert judgement was reduced to a minimum just in relation to the choice
822 of the number of ranking classes and their extension in case of more toxic samples. This kind of approach
823 produced a toxicity score with classes (absent, low, medium, high and very high toxicity) composed of
824 two sub-scores. The first series of sub-scores (absent or low toxicity) was partly based on the percentage
825 of effect responses and partly on toxic unit values. The second series of sub-scores was entirely defined
826 on toxic unit values including a medium, high and extremely high toxicity threshold. The main limits of
827 this approach are related to the fact that each toxicity score is species-specific and databases including
828 wastewater toxicity data must be developed *ad hoc* also to support the data statistical reliability.
829 Further efforts are necessary to identify case-specific toxicity tests (country- or discharge-based),
830 supporting their round robin and toxicity data integration methods in the perspective of EU legislative
831 harmonization.
832

833 **5.3 How reliable is our risk assessment in the receiving water bodies?**

834 Within the Water Framework Directive (WFD) the term "ecological status" of a water body primarily
835 embraces the biological responses caused by other pollutants than micropollutants, but priority
836 micropollutants are taken into account through an environmental risk assessment (ERA) scheme by
837 implementing Environmental Quality Standards (EQS) that should not be exceeded in the environment
838 (EU, 2013). The EQS values are set by each member state based on the predicted no effect concentration
839 (PNEC) for each compound in water, sediment and/or biota. However, available ecotoxicity data are
840 often limited, especially for metabolites and transformation products. Therefore, traditional ERA, as
841 described by the European Commission Technical Guidance Document (TGD), allows the use of
842 assessment factors (AFs) to account for the uncertainty in deriving PNEC values based on acute toxicity
843 data and a limited number of species (EC, 2003). The intention of the use of AFs is to predict a

concentration below which an unacceptable effect will most likely not occur. Data on persistence in the environment (i.e. lack in biodegradability) and bioaccumulation should also be considered. An AF of 1000 is advised if only acute toxicity data are available for three trophic levels (algae, daphnids and fish). Only highly rarely sufficient data on long-term effects at several trophic levels and taxonomic groups exist for a given compound to be used for statistical extrapolation methods to derive a PNEC value. For biologically active compounds such as pharmaceuticals, this approach may, however, overlook sub-lethal and subtle subcellular effects that might occur in some species at much lower concentrations during chronic exposure. The complexity implied by the cocktail effects of compound mixtures and the large number of unknown transformation products during degradation in the environment warrants a switch to a more effects-oriented approach when assessing the environmental risk. Hence, the combined effects from all compounds in water or sediment samples are assessed using a set of toxicity tests targeting e.g. baseline toxicity, estrogenic and mutagenic activity and oxidative stress. The main drawback of this effects-oriented approach is that it is not able to identify the actual compound(s) that are asserting the observed effects. But if it is combined with the above-described MEC/PNEC (or MEC/EQS), any major discrepancies between the observed effects and the calculated MEC/PNEC values relevant to the respective effects may be used to identify "missing" contributing compounds and warrant more detailed analyses or studies. Still, true food web effects are not covered, leaving the question open whether an ecosystem hazard may be possible. Discharges from WWTPs are only one of many possible routes for micropollutants to enter the aquatic environment, and the environmental risk assessment (ERA) of discharges to a water body should take them all into account. Similar approaches as described above for the water body may be performed. Instead of measuring the actual environmental concentration (MEC), the environmental concentration is predicted (PEC) from concentrations in the effluent from the WWTP, the total discharged volumes and the immediate local dilution in the receiving waters. For compounds

867 that are persistent in the environment and/or bioaccumulate a more long-term and regional assessment
868 may be needed, including the potential accumulation in sediment.
869 Actually, any industrial agricultural, farming, commercial and recreational activity (including boats and
870 ships), as well as living units discharging wastewater to water bodies, standing both on freshwater and
871 marine environments, need to know the nature and the extent of impacts associated with their liquid
872 emissions. These issues are driving the need for a more detailed assessment of the impact of wastewater
873 discharges to support decision-making. The integrated assessment of biological effects of discharges in
874 the ecosystems is relevant and ecotoxicity tests are referred to as extremely useful tools for the
875 identification of environmental impacts (Mendonça et al., 2009). The use of the ecotoxicology can
876 provide an added value to hazard and risk assessment of discharges to the receiving water bodies.
877 Environmental management can take advantage from safe and non-toxic treated wastewater, supporting
878 its recovery and reuse, as in case of non-potable purposes. Ecotoxicity tests can identify the hazard and
879 be directly used in ecological risk assessment. Within the WFD, direct toxicity assessment of WWTP
880 discharges can contribute to attain or keep ecological quality objectives in water masses and finally
881 provide the postulated "good" quality of all water bodies in the EU.
882 Besides, the assessment of traditional acute and chronic (short- and long-term) toxicity tests, treated
883 wastewater evaluation in the perspective of its reclamation and reuse presents new potential ranking tools
884 like effect-based trigger values (EBTs), as abovementioned in paragraph § 3.2. EBTs can be derived
885 from the safe levels based on average daily intakes from existing toxicity databases according to with
886 various approaches to be explicitly declared each time (i.e. different algorithms produce different
887 thresholds). This means that EBTs approach is a chemically oriented approach based on specific
888 pollutants (e.g. androgenic (AR), estrogenic (ERα), glucocorticoid (GR), and progestagenic (PR)) rather
889 than on effects on bioindicators. About EBTs, discussion is still open on how to include the mixtures
890 (Escher et al., 2018), and how to cope with substantial difference between whole effluent testing (WET)

891 and bioanalytical assessment considering that EBTs are derived only for organic micropollutants. Thus,
892 they cannot be applied to wastewater in the case of other non-organic components (i.e. metals and other
893 inorganics) as the main causative agent (Escher et al., 2014) (Escher et al., 2018b). This means that
894 traditional toxicity tests integrating the effects of reclaimed wastewater to an exposed population cannot
895 be entirely substituted just with EBTs, at least according to their current definition. Moreover, also
896 traditional bioassays should be considered prevalently in their chronic exposure: quality standards must
897 be highly demanding for both traditional bioassays and the use of EBTs because once (ground)water is
898 contaminated the treatment/remediation could be very expensive or sometimes impossible to be carried
899 out.
900
901 **5.4 Socio-economic aspects**
902 Monitoring and predicting trace pollutant concentrations in the aquatic environment, together with their
903 possible subsequent toxicity, are vital in order to better assess the environmental impact as well as the
904 risks for human health. Thus, new effective tools for estimating the occurrence of these substances are
905 needed. A recent method is based on online search queries, though this only applies to those that are
906 widely known by the public. For example, considering pharmaceuticals, the prescription issuing in the
907 UK of several substances included in the EU watch list for water monitoring (2015/495, 2015) is
908 suggested to be correlated to online search queries (Mavragani et al., 2016). As the concentrations of
909 antibiotics in wastewater seem to follow the trend of prescriptions (Le-Minh et al., 2010), search traffic
910 data could be proven a valuable tools in predicting the occurrence of pollutants in wastewater.
911 The choice of proper removal treatment as well as the overall assessment of its environmental, economic,
912 and social impacts needs to be assessed with caution (Melvin and Leusch, 2016), and must necessarily
913 take into account pollutants loads, which, unfortunately, can be affected by extreme variability.
914 Therefore, all the cost items might be accurately overweighed, to avoid wastes of energy and material

915 resources, land consumption, and to reduce pollution towards other environmental matrices. Recently,
916 Life Cycle Assessment (LCA) has been applied to evaluate the economic and environmental viability of
917 processes aimed to remove trace pollutants from wastewater ((Hernández-Padilla et al., 2017), (Pintilie
918 et al., 2016)); this instrument provides standardized criteria to compare alternative options by taking into
919 account different impact categories.
920 In any case, the effective step towards the reduction of trace pollutants emissions and, consequently, their
921 effects on the environment is definitely a management at the source. Green chemistry principles (Anastas
922 and Warner, 1998) are the essential criteria for designing new production and supply chains, as well as
923 disposal and treatment. The example of pharmaceuticals is emblematic. Medical professionals and
924 patients should employ, if possible, products manufactured in accordance with the green pharmacy
925 principles, e.g. using pharmaceuticals that are designed to be better biodegradable ((Rastogi et al., 2014),
926 (Kümmerer and Clark, 2016)). Disposal of unused medicines is mostly carried out through household
927 waste (Bound et al., 2006), toilets and sinks ((Kotchen et al., 2009), (Straub, 2016), (Tijani et al., 2013)).
928 As many do not regard this as an environmental issue (Bound et al., 2006), it is evident, that public
929 awareness is vital, together with the need for better public information (Straub, 2016). Over the past
930 decades, attention has also focused on return policies advertisements (Bound et al., 2006) and the
931 importance of people information on the correct disposal (Bound et al., 2006), (Straub, 2016). As a
932 consequence, population willingness to pay for a better waste treatment system increases (Kotchen et al.,
933 2009), (Logar et al., 2014). Governments should implement the regulatory frameworks for improving
934 the whole water cycle management (Morris et al., 2017). According to the Polluter Pays Principle,
935 environmental damage should be decreased by introducing advanced treatment technologies, which
936 should be paid by the final users. Therefore, conventional tariff policies aiming to charge all households
937 as a function of wastewater production are not in accordance with the Polluter Pays Principle. It has been
938 shown, that increased charge rates and penalties do not contribute to more environmentally friendly

939 practices (Lu et al., 2016). Thus, in order to internalize the externalities of using products, which
940 potentially release micropollutants, the purchase cost should be increased in order to subsidize the
941 removal/remediation expenses. Revenues should be allocated to upgrade WWTPs, with the unfailing
942 support of national (and, possibly, international) policies which consider the global social and
943 environmental costs due to the use of such substances, together with the costs for water treatment (from
944 drinking water supply, to wastewater collection and purification).
945

946 # 6. Interpretation of eco-toxicity data: case studies

947 In recent years, some authors have applied toxicity tests to diverse applications. In this section, some
948 case studies are presented, which demonstrate the power and versatility of such investigations. For this
949 purpose, the examples chosen include a range of different scenarios, in terms of: employed bioassays
950 (crustaceans, algae, bacteria, etc.); tested matrices (e.g., municipal and complex wastewater); adopted
951 treatment systems (conventional activated sludge process, membrane bioreactor, ozonation,
952 photocatalysis, sonication, anaerobic process). Some of the aforementioned experiences have been
953 carried out at the full scale.
954 The pivotal role of bioassays in the integrated assessment of the environmental impact of wastewater is
955 clearly manifest in all the reported cases.
956

957 ## 6.1 Ecotoxicity removal from complex wastewaters: comparison among conventional and
958 advanced technologies

959 Currently, water quality standards and wastewater discharge limits in the European Union are mostly
960 based on a limited number of chemical parameters. The aim of The European Water Framework Directive
961 (European Parliament, 2000) is to obtain water bodies with a “good” biological quality. The biological

962 or ecological impact of complex industrial effluent discharges however, cannot be estimated using
963 chemical assays only, but should be measured using whole effluent toxicity (WET) tests (e.g. (OSPAR,
964 2005)).
965 A typical example of a complex industrial effluent is the water originating form tank truck cleaning
966 (TTC) activities. The TTC process mainly involves the cleaning of tank truck interiors. The wide
967 spectrum of transported cargo, ranging from food products to hazardous chemicals, results in wastewater
968 with a highly variable composition. De Schepper *et al.* (De Schepper et al., 2010) reported that a
969 significant residual toxicity was still present in biologically treated TTC effluent. A battery of acute
970 ecotoxicity assays, with *Raphidocelis subcapitata* (primary production), *Vibrio fischeri* (decomposition)
971 and *Daphnia magna* (primary consumption) was applied to assess the whole effluent toxicity. It was
972 found that the effluent of the full-scale treatment plant was extremely toxic to *R. subcapitata* with toxicity
973 values ranging from 800 to 3260 TU (toxic units).
974 The aim of a subsequent study was to investigate the removal of acute toxicity from TTC wastewater by
975 a series of key unit operations applied during the treatment of industrial wastewater, i.e. chemical
976 coagulation, activated sludge treatment and sorption by activated carbon (Dries et al., 2013). The
977 treatments steps were performed on a laboratory scale, in order to assess the full toxicity removal
978 potential of these technologies. The rapid *V. fischeri* bioluminescence inhibition test (applying a 30 min
979 contact time) was used to assess toxicity removal. Chemical pretreatment of the wastewater by
980 coagulation with $FeCl_3$ removed approx. 38% of the influent chemical oxygen demand (COD) and
981 reduced the bioluminescence inhibition by 8%. Biological treatment with activated sludge subsequently
982 removed another 77% of the remaining COD. This treatment step also reduced the bioluminescence
983 inhibition but the removal efficiency varied strongly from 5 to 92% for the different samples.

984 The ecotoxicity of the biotreated samples was also analyzed with the 72 h algal growth inhibition assay
985 using *R. subcapitata*. The TU values ranged from 610 to 5,470, confirming the very high algal growth
986 inhibition reported for the same type of wastewater by (De Schepper et al., 2010).
987 Powdered activated carbon (PAC) almost completely removed the remaining COD and inhibition in all
988 samples. The algal growth inhibition after PAC addition ranged from 23 to 82 TU, corresponding to a
989 reduction of more than 95%.
990 These results suggest that conventional technologies did not suffice for complete removal of toxicity
991 from TTC wastewater, and that advanced wastewater treatment technologies are required for a
992 satisfactory detoxification.
993

994 **6.2 Removal of estrogenicity from municipal wastewater: comparison between MBR and CAS**
995 **systems**

996 A monitoring campaign was conducted on a full scale municipal WWTP, consisting of 2 CAS and 1
997 MBR (ultrafiltration) parallel lines. The design size is 250,000 p.e. and the influent load is split about
998 50% on the MBR train and 25% on each CAS line. The plant is operated according to the modified
999 Ludzak-Ettinger process scheme, with chemical phosphorus removal (aluminium sulphate dosage into
1000 the biological reactors).
1001 Both chemical and biological analyses were carried out all along a 19 days period, in order to compare
1002 the CAS and MBR processes in terms of EDCs removal. The following target substances were measured:
1003 4-nonylphenol (NP), its parent compounds 4-nonylphenol monoethoxylate (NP1EO) and 4-nonylphenol
1004 diethoxylate (NP2EO), and bisphenol A (BPA). The same wastewater samples used for chemical
1005 analyses were submitted to the measurement of hormonal activity by means of human breast cancer
1006 MCF-7 based reporter gene assay, using 17β-estradiol (E2) as a standard.

1007 Removal efficiency and residual effluent concentration of target compounds were quite similar for both
1008 CAS and MBR lines, ranging between 70% (BPA) and 95% (NP1EO) and from 0,3 mg/L (NP1EO) to
1009 0,8 mg/L (NP), respectively. The CAS and MBR lines were operated at a sludge age of 9 and 15 d,
1010 respectively, the sewage temperature being around 23°C. The reason for the different plants to have
1011 similar performances can be explained based on the well-known relevance of these operating parameters:
1012 Clara et al. ((Clara et al., 2004), (Clara et al., 2005)) demonstrated that any increase of sludge age and
1013 temperature above 10 d and 10°C does not lead to noticeable improvements, regardless of the type of
1014 process (either CAS or MBR). Moreover, several Authors (e.g. (Koh et al., 2009), (McAdam et al., 2010),
1015 (Verlicchi et al., 2012), (Hicks et al., 2017)) evidenced the positive effect of an efficient nitrification on
1016 EDCs removal.
1017 Nevertheless, even if no appreciable difference in the EDCs effluent concentration was detected,
1018 biological measurements showed that the MBR effluent exerted a lower estrogenic activity
1019 (estrogenicity, expressed as Relative Light Units, and normalized towards protein concentration, was up
1020 to 50% lower in MBR effluent samples, ranging from 1.0 to 3.5 x 10^7 RLU/$mg_{protein}$). The higher
1021 performance of the MBR system is likely attributable to the more efficient retention of suspended solid,
1022 and, consequently, of specialized slow-growing bacteria and of the organics to be degraded (in case they
1023 are adsorbed onto the suspended solids).
1024 The findings confirm the irreplaceability of bioassays in the monitoring of any impact on the ecosystems
1025 (in this case, the biological reactor o fa WWTP). Detailed results are reported in (Bertanza et al., 2011).

1026

1027 **6.3 Removal of antibiotics and their effects of anaerobic and aerobic systems**

1028 As the working principle of antibiotics inhibits biological activities directly, their adverse/inhibitory
1029 effects on the biodegradation of organic compounds in the wastewater treatment plants are one of the

1030 main concerns. In order to evaluate the inhibitory impact of these compounds in biological systems, two
1031 different experimental approaches are commonly applied: short-term (acute) and long-term (chronic)
1032 tests. The short-term, acute tests usually involve a non-acclimated microbial population to the inhibitor.
1033 In long-term experiments with continuous feeding of the antibiotics, the test may reflect, aside from
1034 changes in substrate removal and utilization, adaptation and/or resistance of the microbial community
1035 or even shifts in microbial composition in response to continuous exposure ((Pala-Ozkok et al., 2014a),
1036 (Cetecioglu et al., 2016)). While Kümmerer and his colleagues (Kümmerer et al., 2004) argue that short-
1037 term assays would not be sufficient to investigate the effect of antibiotics on complex microbial systems
1038 because of different mechanisms associated with acute and chronic inhibition, Alighardashi *et al.*
1039 (Alighardashi et al., 2009) propose that the microbial community becomes well adapted to a synthetic
1040 substrate, which is a significantly different scenario from biomass in a full-scale plant under long-term
1041 exposure. Despite different opinions expressed in the literature, these two inhibition tests complement
1042 one another and reflect real-life inhibition schemes encountered in wastewater treatment.
1043 In the light of this knowledge, acute and chronic tests were applied to aerobic and anaerobic biological
1044 treatment systems with three selected antibiotics: sulfamethoxazole (SMX), tetracycline (TET) and
1045 erythromycin (ERY).
1046 For the aerobic acute tests; laboratory-scale fill-and-draw reactors with hydraulic retention time of one
1047 day were established and sustained at sludge ages of 10 and 2 days at steady state under aerobic
1048 conditions (Pala-Ozkok, 2012), and a series of fully aerated batch reactors for kinetic investigations of
1049 peptone-meat extract mixture biodegradation and acute/chronic inhibition of the selected antibiotics
1050 ((Ozkok et al., 2011), (Pala-Ozkok and Orhon, 2013), (Pala-Ozkok et al., 2014b)). Fill-and-draw reactors
1051 were fed with peptone-meat extract mixture at concentrations characterizing domestic wastewaters. To
1052 determine the acute and chronic inhibition effects of the selected antibiotics, batch experiments were
1053 conducted with 50 mg/L antibiotic additions (Pala-Ozkok, 2012). Respirometric tests were performed

to determine the effect of antibiotics on non-acclimated (acute effect) and acclimated (chronic) biomass, which yielded oxygen uptake rate (OUR) profiles. Obtained OUR profiles were used for simulation to determine the kinetic properties of each activated sludge biomass ((Pala-Ozkok and Orhon, 2013), (Pala-Ozkok et al., 2014b)). Reactors were monitored for COD, suspended solids (SS), volatile suspended solids (VSS) and polyhydroxyalkanoates (PHA) (Beun et al., 2000). The inhibitory impact of selected antibiotics was observed as a decrease in the amount of oxygen consumed in the OUR tests, which led to the conclusion that antibiotics have the property to block the microbial substrate consumption (Pala-Ozkok, 2012), (Ozkok et al., 2011). The kinetic evaluation revealed that antibiotic substances mainly increase endogenous decay levels, the half-saturation constant of the substrate and inhibit hydrolysis of different COD fractions (Pala-Ozkok, 2012), (Ozkok et al., 2011), (Pala-Ozkok and Orhon, 2013), (Pala-Ozkok et al., 2014b).

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 (Pala-Ozkok, 2012). 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 (Ozkok et al., 2011). The kinetic evaluation revealed that antibiotic substances mainly increase endogenous

decay levels, the half-saturation constant of the substrate and inhibit hydrolysis of different COD fractions (Pala-Ozkok, 2012).

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 (Cetecioglu et al., 2012) (Cetecioglu et al., 2015a). 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 (Cetecioglu et al., 2012). Almost complete methane inhibition was observed for antibiotics doses above 500 mg/L. The monitored effect was found coherent with uncompetitive inhibition, which similarly exerts a binding impact on substrate–enzyme complex. For VFA mixture (acetate, propionate, and butyrate fed system), at lower doses, the VFA mixture was completely removed but partially used, leading to reduced biogas and methane generation, suggesting the resemblance of uncompetitive inhibition (Cetecioglu et al., 2015a), (Cetecioglu, 2011).

Anaerobic chronic inhibition tests represented different results from acute tests. The experiments involved anaerobic sequencing batch reactors fed with a synthetic substrate mixture including glucose, starch, and volatile fatty acids, and operated in a sequence of different phases with gradually increasing antibiotics, for more than five months. TET exerted a terminal/lethal effect at 8.5 mg/L on the microbial community, which caused the inhibition of substrate/COD utilization and biogas production and leading

1102 to a total collapse of the reactor (Cetecioglu et al., 2013). The microbial activity could not be retrieved
1103 and re-started within a period of more than 10 days, even after stopping TET dosing. During the
1104 experiments, TET was partially removed either through biodegradation or conversion into its by-
1105 products. The adverse long-term effect was quite variable for fermenting heterotrophic and
1106 methanogenic fractions of the microbial community based on changes generating on the composition of
1107 remaining/residual organic substrate. The results revealed that anaerobic treatment was suitable for
1108 pharmaceutical industry wastewater with concentrations of up to 40 mg/L of SMX. Higher levels
1109 exserted toxic effects on the microbial community under anaerobic conditions, inducing the inhibition
1110 of substrate/COD utilization and biogas production and leading to a total collapse of the reactor. The
1111 adverse long-term impact was quite variable for fermentative bacteria and methanogenic archaeal
1112 fractions of the microbial community depend on changes inflicted on the composition of the residual
1113 organic substrate and mRNA expression of the key enzymes (Cetecioglu et al., 2015b). ERY fed reactors
1114 showed that methane production and VFA recovery are simultaneously possible up to 2 mg/L of ERY.
1115 ERY exerted a terminal effect at 3 mg/L on the biomass, and the activity could not be recovered after
1116 stopping ERY dosing (Cetecioglu, 2011).
1117 Also, another study was performed to reveal if anaerobic-aerobic biological treatment strategy is proper
1118 for antibiotic production waste streams. Although activated sludge treatment systems are inhibited by the
1119 low concentration of antibiotic mixture, the same aerobic system can tolerate higher concentrations of
1120 the same mixtures after an anaerobic pre-treatment (Cetecioglu, 2014).

1121

1122 **6.4 Removal of estrogenicity from textile wastewater by means of ozonation**

1123 A pilot scale ozonation plant was installed at the outlet flow of a CAS plant (design size 370,000 p.e.,
1124 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 (Bertanza et al., 2011)), only a slight additional reduction of hormonal activity was achieved (measured value = 2.90 x 10^7 RLU/$mg_{protein}$; removal efficiency = 60%). The difference between the chemical and the biological answer may be due to the formation of active by-products, metabolites and/or conjugates, able to exert an estrogenic activity comparable to those of parent compounds, and to the synergistic and additive effect among the different compounds.

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

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

Photocatalysis and ultrasound treatments have been widely investigated for the treatment of emerging pollutants in urban wastewaters, including EDCs, pharmaceuticals, personal care products, drugs ((Belgiorno et al., 2007), (Rizzo et al., 2009), (Carotenuto et al., 2014), (Lofrano et al., 2016)). Since

during the oxidation process some by-products (intermediates) are formed and the effluent may become more toxic than the untreated solutions or the parent compounds, respectively, the overall efficiency of the treatment process for this class of chemical pollutants strictly depends on the toxicity and estrogenic potency of treated effluents.

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

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

1173 The toxicity of the mixtures of three pharmaceuticals (2.5 mg/L, diclofenac, DCF, 2.5, 5 and 10 mg $L^{-}1$,
1174 amoxicillin, AMX, 2.5, 5 mg/L carbamazepine, CBZ) at different concentrations in contaminated urban
1175 wastewater treated by ultrasound has been evaluated by Naddeo *et al*. (Naddeo et al., 2009). Sonication
1176 decreased toxicity of contaminated WW sample to *R. subcapitata* and no significant effect on this
1177 decrease by either the sonication time or the applied power density was observed. *R. subcapitata* was
1178 found more sensitive than *D. magna.*
1179 Toxicity data about photocatalysis and ultrasound treatments are still in their infancy, especially for
1180 sonolysis where just few studies have been performed. From the available results it can be stated that
1181 photocatalysis can be suitable to fully remove toxicity at the discharge but focused research must be
1182 oriented specifically, not only on target compound removal but also on effluent toxicity goal. Moreover,
1183 toxicity investigation must comply with the international recognized approach, considering the
1184 integration of at least three species belonging to different phylogenetic levels [149], [150]..
1185

1186 ## 7. Conclusions

1187 This paper reports the shared opinions of the participants to COST Action ES1202 Conceiving
1188 Wastewater Treatment in 2020-Energetic, environmental and economic challenges (Water_2020) about
1189 the topic of toxicity of wastewater trace organic pollutants.
1190 Notwithstanding the valuable literature production, which, up to now, includes also hundreds of reviews,
1191 the choice to write another work about the topic of toxicity of wastewater organic trace pollutants arose
1192 from the awareness that there are still gaps between the different scientific sectors involved in this
1193 research. The debated subjects, indeed, pertain to several disciplines and have been connected based on
1194 the final goal to propose criteria for choosing the proper tools to assess and reduce the possible
1195 environmental impact of such pollutants on the human health and the aquatic ecosystems.

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

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

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References

2015/495, C.I.D. (EU), 2015. 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.

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

Adeogun, A.O., Onibonoje, K., Ibor, O.R., Omiwole, R.A., Chukwuka, A. V., Ugwumba, A.O., Ugwumba, A.A.A., Arukwe, A., 2016. 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, 10–21. doi:10.1016/j.aquatox.2016.02.002

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

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

Altenburger, R., Ait-Aissa, S., Antczak, P., Backhaus, T., Barcel??, D., Seiler, T.B., Brion, F., Busch, W., Chipman, K., de Alda, M.L., de Arag??o Umbuzeiro, G., Escher, B.I., Falciani, F., Faust, M., Focks, A., Hilscherova, K., Hollender, J., Hollert, H., J??ger, F., Jahnke, A., Kortenkamp, A., Krauss, M., Lemkine, G.F., Munthe, J., Neumann, S., Schymanski, E.L., Scrimshaw, M., Segner, H., Slobodnik, J., Smedes, F., Kughathas, S., Teodorovic, I., Tindall, A.J., Tollefsen, K.E., Walz, K.H., Williams, T.D., Van den Brink, P.J., van Gils, J., Vrana, B., Zhang, X., Brack, W., 2015. Future water quality monitoring - Adapting tools to deal with mixtures of pollutants in water

resource management. Sci. Total Environ. doi:10.1016/j.scitotenv.2014.12.057

Alves, R.M.S., Pereira, B.F., Ribeiro, R.G.L.G., Pitol, D.L., Ciamarro, C.., Valim, J.R.T., Caetano, F.H., 2016. The scale epithelium as a novel, non-invasive tool for environmental assessment in fish: Testing exposure to linear alkylbenzene sulfonate. Ecotoxicol. Environ. Saf. 129, 43–50. doi:10.1016/j.ecoenv.2016.03.010

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

Anastas, P.T., Warner, J.C., 1998. 12 Principles of Green Chemistry. Green Chem. Theory Pract. doi:10.1039/b513020b

Anderson, J.C., Park, B.J., Palace, V.P., 2016. Microplastics in aquatic environments: Implications for Canadian ecosystems. Environ. Pollut. doi:10.1016/j.envpol.2016.06.074

Ankley, G., Escher, B., Hartung, T., Shah, I., 2016. Pathway-Based Approaches for Environmental Monitoring and Risk Assessment. Chem. Res. Toxicol. doi:10.1021/acs.chemrestox.6b00321

Ankley, G.T., Bennett, R.S., Erickson, R.J., Hoff, D.J., Hornung, M.W., Johnson, R.D., Mount, D.R., Nichols, J.W., Russom, C.L., Schmieder, P.K., Serrrano, J.A., Tietge, J.E., Villeneuve, D.L., 2010. Adverse outcome pathways: A conceptual framework to support ecotoxicology research and risk assessment. Environ. Toxicol. Chem. doi:10.1002/etc.34

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

Backhaus, T., Karlsson, M., 2014. Screening level mixture risk assessment ofpharmaceuticals in STP

1265 effluents. Water Res. 49, 157–165. doi:10.1016/j.watres.2013.11.005

1266 Bain, P.A., Williams, M., Kumar, A., 2014. Assessment of multiple hormonal activities in wastewater
1267 at different stages of treatment. Environ. Toxicol. Chem. 33, 2297–2307. doi:10.1002/etc.2676

1268 Balouiri, M., Sadiki, M., Ibnsouda, S.K., 2016. Methods for in vitro evaluating antimicrobial activity:
1269 A review. J. Pharm. Anal. 6, 71–79. doi:10.1016/j.jpha.2015.11.005

1270 Barron, M.G., Lilavois, C.R., Martin, T.M., 2015. MOAtox: A comprehensive mode of action and
1271 acute aquatic toxicity database for predictive model development. Aquat. Toxicol. 161, 102–107.
1272 doi:10.1016/j.aquatox.2015.02.001

1273 Basu, A., 2018. DNA Damage, Mutagenesis and Cancer. Int. J. Mol. Sci. 19, 970.
1274 doi:10.3390/ijms19040970

1275 Bayoumi, A.E., García-Fernández, A.J., Ordóñez, C., Pérez-Pertejo, Y., Cubría, J.C., Reguera, R.M.,
1276 Balaña-Fouce, R., Ordóñez, D., 2001. Cyclodiene organochlorine insecticide-induced alterations
1277 in the sulfur-redox cycle in CHO-K1 cells. Comp. Biochem. Physiol. - C Toxicol. Pharmacol. 130,
1278 315–323. doi:10.1016/S1532-0456(01)00257-5

1279 Bazin, I., Seo, H. Bin, Suehs, C.M., Ramuz, M., de Waard, M., Gu, M.B., 2016. Profiling the
1280 biological effects of wastewater samples via bioluminescent bacterial biosensors combined with
1281 estrogenic assays. Environ. Sci. Pollut. Res. 1–9. doi:10.1007/s11356-016-6050-5

1282 Belgiorno, V., Rizzo, L., Fatta, D., Della Rocca, C., Lofrano, G., Nikolaou, A., Naddeo, V., Meric, S.,
1283 2007. Review on endocrine disrupting-emerging compounds in urban wastewater: occurrence and
1284 removal by photocatalysis and ultrasonic irradiation for wastewater reuse. Desalination 215, 166–
1285 176. doi:10.1016/j.desal.2006.10.035

1286 Beronius, A., Vandenberg, L.N., 2016. Using systematic reviews for hazard and risk assessment of

endocrine disrupting chemicals. Rev. Endocr. Metab. Disord. 16, 273–287. doi:10.1007/s11154-016-9334-7

Bertanza, G., Papa, M., Pedrazzani, R., Repice, C., Mazzoleni, G., Steimberg, N., Feretti, D., Ceretti, E., Zerbini, I., 2013. 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, 160–168. doi:10.1016/j.scitotenv.2013.03.108

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

Bertanza, G., Pedrazzani, R., Papa, M., Mazzoleni, G., Steimberg, N., Caimi, L., Montani, C., Dilorenzo, D., 2010. Removal of BPA and NPnEOs from Secondary Effluents of Municipal WWTPs by Means of Ozonation. Ozone Sci. Eng. 32, 204–208. doi:10.1080/01919511003795303

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

Bicchi, C., Schilirò, T., Pignata, C., Fea, E., Cordero, C., Canale, F., Gilli, G., 2009. 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, 1842–1851. doi:10.1016/j.scitotenv.2008.11.039

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

1310 Bound, J.P., Kitsou, K., Voulvoulis, N., 2006. Household disposal of pharmaceuticals and perception
1311 of risk to the environment. Environ. Toxicol. Pharmacol. 21, 301–307.
1312 doi:10.1016/j.etap.2005.09.006

1313 Brack, W., Ait-Aissa, S., Burgess, R.M., Busch, W., Creusot, N., Di Paolo, C., Escher, B.I., Mark
1314 Hewitt, L., Hilscherova, K., Hollender, J., Hollert, H., Jonker, W., Kool, J., Lamoree, M.,
1315 Muschket, M., Neumann, S., Rostkowski, P., Ruttkies, C., Schollee, J., Schymanski, E.L.,
1316 Schulze, T., Seiler, T.B., Tindall, A.J., De Aragão Umbuzeiro, G., Vrana, B., Krauss, M., 2016.
1317 Effect-directed analysis supporting monitoring of aquatic environments - An in-depth overview.
1318 Sci. Total Environ. doi:10.1016/j.scitotenv.2015.11.102

1319 Bulich, A.A., 1982. A practical and reliable method for monitoring the toxicity of aquatic samples.
1320 Process Biochem. 17, 45–47.

1321 Calabrese, E.J., Blain, R.B., 2011. The hormesis database: The occurrence of hormetic dose responses
1322 in the toxicological literature. Regul. Toxicol. Pharmacol. 61, 73–81.
1323 doi:10.1016/j.yrtph.2011.06.003

1324 Calow, P., 1994. ECOTOXICOLOGY: WHAT ARE WE TRYING TO PROTECT? Environ. Toxicol.
1325 Chem. 13, 1549. doi:10.1897/1552-8618(1994)13[1549:EWAWTT]2.0.CO;2

1326 Campana, O., Wlodkowic, D., 2018. Ecotoxicology Goes on a Chip: Embracing Miniaturized
1327 Bioanalysis in Aquatic Risk Assessment. Environ. Sci. Technol. 52, 932–946.
1328 doi:10.1021/acs.est.7b03370

1329 Cantón, R., 2009. Antibiotic resistance genes from the environment: a perspective through newly
1330 identified antibiotic resistance mechanisms in the clinical setting. Clin. Microbiol. Infect. 15 Suppl
1331 1, 20–25. doi:10.1111/j.1469-0691.2008.02679.x

1332 Carotenuto, M., Lofrano, G., Siciliano, A., Aliberti, F., Guida, M., 2014. TiO2 photocatalytic
1333 degradation of caffeine and ecotoxicological assessment of oxidation by-products. Glob. Nest J.
1334 16, 463–473.

1335 Cartus, A., Schrenk, D., 2017. Current methods in risk assessment of genotoxic chemicals. Food Chem.
1336 Toxicol. 106, 574–582. doi:10.1016/j.fct.2016.09.012

1337 Carvan, M.J., Di Giulio, R.T., 2015. Oxidative Stress Responses in Aquatic and Marine Fishes. pp.
1338 481–493. doi:10.1007/978-3-319-19096-9_26

1339 Cavallin, J.E., Jensen, K.M., Kahl, M.D., Villeneuve, D.L., Lee, K.E., Schroeder, A.L., Mayasich, J.,
1340 Eid, E.P., Nelson, K.R., Milsk, R.Y., Blackwell, B.R., Berninger, J.P., Lalone, C.A., Blanksma,
1341 C., Jicha, T., Elonen, C., Johnson, R., Ankley, G.T., 2016. Pathway-based approaches for
1342 assessment of real-time exposure to an estrogenic wastewater treatment plant effluent on fathead
1343 minnow reproduction. Environ. Toxicol. Chem. 35, 702–716. doi:10.1002/etc.3228

1344 Cetecioglu, Z., 2014. Aerobic inhibition assessment for anaerobic treatment effluent of antibiotic
1345 production wastewater. Environ. Sci. Pollut. Res. 21, 2856–2864. doi:10.1007/s11356-013-2243-3

1346 Cetecioglu, Z., 2011. Evaluation of anaerobic biodegradability characteristics of antibiotics and
1347 toxic/inhibitory effect on mixed microbial culture. Istanbul Technical University.

1348 Cetecioglu, Z., Ince, B., Gros, M., Rodriguez-Mozaz, S., Barceló, D., Ince, O., Orhon, D., 2015.
1349 Biodegradation and reversible inhibitory impact of sulfamethoxazole on the utilization of volatile
1350 fatty acids during anaerobic treatment of pharmaceutical industry wastewater. Sci. Total Environ.
1351 536, 667–674. doi:10.1016/j.scitotenv.2015.07.139

1352 Cetecioglu, Z., Ince, B., Gros, M., Rodriguez-Mozaz, S., Barceló, D., Orhon, D., Ince, O., 2013.
1353 Chronic impact of tetracycline on the biodegradation of an organic substrate mixture under

anaerobic conditions. Water Res. 47, 2959–2969. doi:10.1016/j.watres.2013.02.053

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

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

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

Chatterjee, N., Walker, G.C., 2017. Mechanisms of DNA damage, repair, and mutagenesis. Environ. Mol. Mutagen. 58, 235–263. doi:10.1002/em.22087

Chen, C., Liu, W., Wang, L., Li, J., Chen, Y., Jin, J., Kawan, A., Zhang, X., 2016. Pathological damage and immunomodulatory effects of zebrafish exposed to microcystin-LR. Toxicon 118, 13–20. doi:10.1016/j.toxicon.2016.04.030

Cheng, C.-H., Yang, F.-F., Ling, R.-Z., Liao, S.-A., Miao, Y.-T., Ye, C.-X., Wang, A.-L., 2015. Effects of ammonia exposure on apoptosis, oxidative stress and immune response in pufferfish (Takifugu obscurus). Aquat. Toxicol. 164, 61–71. doi:10.1016/j.aquatox.2015.04.004

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

Clara, M., Strenn, B., Ausserleitner, M., Kreuzinger, N., 2004. Comparison of the behaviour of selected

micropollutants in a membrane bioreactor and a conventional wastewater treatment plant. Water Sci. Technol. 50, 29–36.

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

Comerton, A.M., Andrews, R.C., Bagley, D.M., Baugros, J.B., Giroud, B., Dessalces, G., Grenier-Loustalot, M.F., Cren-Olivé, C., Benijts, T., Dams, R., Lambert, W., Leenheer, A. De, Brody, J.G., Rudel, R.A., Chen, J., Pawliszyn, J.B., Colborn, T., vom, S.S., Soto, A.M., Costa, L.G., Giordano, G., Guizzetti, M., Vitalone, A., Díaz-Cruz, M.S., Barceló, D., Doerr-MacEwen, N.A., Haight, M.E., Farré, M., Fatta, D., Nikolaou, A., Achilleos, A., Meriç, S., Gabet, V., Miège, C., Bados, P., Coquery, M., Giger, W., Gómez, M.J., Agüera, A., Mezcua, M., Hurtado, J., Mocholí, F., Fernández-Alba, A.R., Gros, M., Petrović, M., Barceló, D., Gros, M., Petrović, M., Barceló, D., Hua, W., Bennett, E.R., Letcher, R.J., Jahnke, A., Gandrass, J., Ruck, W., Jobling, S., Nolan, M., Tyler, C.R., Brighty, G., Sumpter, J.P., Kampioti, A.A., Cunha, A.C.B. da, Alda, M.L. de, Barceló, D., Kim, K.S., Oh, B.S., Kang, J.W., Chung, D.M., Cho, W.H., Choi, Y.K., Kolpin, D.W., Furlon, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B., Buxton, H.T., Larsson, D.J., Adolfsson-Erici, M., Parkkonen, J., Pettersson, M., Berg, A.H., Olsson, P.-E., Förlin, L., Leatherland, J.F., Lin, W.C., Chen, H.C., Ding, W.H., Lindqvist, N., Tuhkanen, T., Kronberg, L., Lishman, L., Loraine, G.A., Pettigrove, M.E., Monzón, A.L., Moreno, D. V., Padrón, M.T., Ferrera, Z.S., Rodríguez, J.J.S., Pavlović, D.M., Babić, S., Horvat, A.M., Kaštelan-Macan, M., Pérez, S., Barceló, D., Pichon, V., Pietrogrande, M.C., Basaglia, G., Purdom, C.E., Hardiman, P.A., Bye, V.J., Eno, N.C., Tyler, C.R., Sumpter, J.P., Radjenović, J., Petrović, M., Barceló, D., Reddersen, K., Heberer, T., Renew, J.E., Huang, C.H., Richardson, S.D., Rossi, D.T.,

Zhang, N., Safe, S., Safe, S., Schultz, M., Lëffler, D., Wagner, M., Ternes, T.A., Schwab, B.W., Hayes, E.P., Fiori, J.M., Mastrocco, F.J., Roden, N.M., Cragin, D., Meyerhoff, R.D., D'Aco, V.J., Anderson, P.D., Servos, M.R., Smith, M., McInnis, R., Burnison, B.K., Lee, B.H., Backus, S., Solomon, G.M., Schettler, T., Stackelberg, P.E., Furlong, E.T., Meyer, M.T., Zaugg, S.D., Henderson, A.K., Reissman, D.B., Steen, R.A., Hogenboom, A.C., Leonards, P.G., Peerboom, R.L., Cofino, W.P., Brinkmasn, U.T., Suchara, E.A., Budziak, D., Martendal, E., Costa, L.F., Carasek, E., Ternes, T.A., Weber, R.A., Pierik, F.H., Dohle, G.R., Burdorf, A., Wennmalm, A., Gunnarsson, B., Westerhoff, P., Yoon, Y., Snyder, S., Wert, E., Zhang, S., Zhang, Q., Darisaw, S., Ehie, O., Wang, G., Zrostlíková, J., Hajšlová, J., Poustka, J., Begany, P., Zuehlke, S., Duennbier, U., Heberer, T., 2009. 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, 3923–39. doi:10.1098/rsta.2009.0111

Conley, J.M., Evans, N., Mash, H., Rosenblum, L., Schenck, K., Glassmeyer, S., Furlong, E.T., Kolpin, D.W., Wilson, V.S., 2015. 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. doi:10.1016/j.scitotenv.2016.02.093

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

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

Crawford, S.E., Hartung, T., Hollert, H., Mathes, B., van Ravenzwaay, B., Steger-Hartmann, T., Studer, C., Krug, H.F., 2017. Green Toxicology: a strategy for sustainable chemical and material

development. Environ. Sci. Eur. doi:10.1186/s12302-017-0115-z

Croom, E.L., 2016. Mutational Effects, in: Analytical Chemistry. pp. 85–111. doi:10.1007/978-3-319-27449-2_3

Dearfield, K.L., Cimino, M.C., McCarroll, N.E., Mauer, I., Valcovic, L.R., 2002. Genotoxicity risk assessment: a proposed classification strategy. Mutat. Res. Toxicol. Environ. Mutagen. 521, 121–135. doi:10.1016/S1383-5718(02)00236-X

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

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

Denslow, N.D., Maruya, K.A., Leusch, F.D.L., 2016. Bioanalytical approaches in assessing transformation products, in: ACS Symposium Series. doi:10.1021/bk-2016-1242.ch004

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

Díaz-Gil, C., Cotgrove, L., Smee, S.L., Simón-Otegui, D., Hinz, H., Grau, A., Palmer, M., Catalán, I.A., 2017. Anthropogenic chemical cues can alter the swimming behaviour of juvenile stages of a temperate fish. Mar. Environ. Res. 125, 34–41. doi:10.1016/j.marenvres.2016.11.009

Dries, J., De Schepper, W., Geuens, L., Blust, R., 2013. Removal of ecotoxicity and COD from tank

truck cleaning wastewater. Water Sci. Technol. 68, 2202–2207. doi:10.2166/wst.2013.477

EC, 2003. 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.

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

Escher, B.I., Allinson, M., Altenburger, R., Bain, P.A., Balaguer, P., Busch, W., Crago, J., Denslow, N.D., Dopp, E., Hilscherova, K., Humpage, A.R., Kumar, A., Grimaldi, M., Jayasinghe, B.S., Jarosova, B., Jia, A., Makarov, S., Maruya, K.A., Medvedev, A., Mehinto, A.C., Mendez, J.E., Poulsen, A., Prochazka, E., Richard, J., Schifferli, A., Schlenk, D., Scholz, S., Shiraishi, F., Snyder, S., Su, G., Tang, J.Y.M., Burg, B. van der, Linden, S.C. van der, Werner, I., Westerheide, S.D., Wong, C.K.C., Yang, M., Yeung, B.H.Y., Zhang, X., Leusch, F.D.L., 2014. Benchmarking Organic Micropollutants in Wastewater, Recycled Water and Drinking Water with In Vitro Bioassays. Environ. Sci. Technol. 48, 1940–1956. doi:10.1021/es403899t

Escher, B.I., Aït-Aïssa, S., Behnisch, P.A., Brack, W., Brion, F., Brouwer, A., Buchinger, S., Crawford, S.E., Du Pasquier, D., Hamers, T., Hettwer, K., Hilscherová, K., Hollert, H., Kase, R., Kienle, C., Tindall, A.J., Tuerk, J., van der Oost, R., Vermeirssen, E., Neale, P.A., 2018a. Effect-based trigger values for in vitro and in vivo bioassays performed on surface water extracts supporting the environmental quality standards (EQS) of the European Water Framework Directive. Sci. Total Environ. 628–629, 748–765. doi:10.1016/j.scitotenv.2018.01.340

Escher, B.I., Aït-Aïssa, S., Behnisch, P.A., Brack, W., Brion, F., Brouwer, A., Buchinger, S., Crawford, S.E., Du Pasquier, D., Hamers, T., Hettwer, K., Hilscherová, K., Hollert, H., Kase, R., Kienle, C., Tindall, A.J., Tuerk, J., van der Oost, R., Vermeirssen, E., Neale, P.A., 2018b. Effect-based trigger values for in vitro and in vivo bioassays performed on surface water extracts

supporting the environmental quality standards (EQS) of the European Water Framework Directive. Sci. Total Environ. 628–629, 748–765. doi:10.1016/j.scitotenv.2018.01.340

Escher, B.I., Hackermï¿½ller, J., Polte, T., Scholz, S., Aigner, A., Altenburger, R., Bï¿½hme, A., Bopp, S.K., Brack, W., Busch, W., Chadeau-Hyam, M., Covaci, A., Eisentrï¿½ger, A., Galligan, J.J., Garcia-Reyero, N., Hartung, T., Hein, M., Herberth, G., Jahnke, A., Kleinjans, J., Klï¿½ver, N., Krauss, M., Lamoree, M., Lehmann, I., Luckenbach, T., Miller, G.W., Mï¿½ller, A., Phillips, D.H., Reemtsma, T., Rolle-Kampczyk, U., Schï¿½ï¿½rmann, G., Schwikowski, B., Tan, Y.M., Trump, S., Walter-Rohde, S., Wambaugh, J.F., 2017. From the exposome to mechanistic understanding of chemical-induced adverse effects. Environ. Int. doi:10.1016/j.envint.2016.11.029

Escher, B.I., Lawrence, M., Macova, M., Mueller, J.F., Poussade, Y., Robillot, C., Roux, A., Gernjak, W., 2011. Evaluation of Contaminant Removal of Reverse Osmosis and Advanced Oxidation in Full-Scale Operation by Combining Passive Sampling with Chemical Analysis and Bioanalytical Tools. Environ. Sci. Technol. 45, 5387–5394. doi:10.1021/es201153k

Escher, B.I., Leusch, F.D.L., 2012. Bioanalytical tools in water quality assessment. IWA Publishing, London, New York.

Escher, B.I., Neale, P.A., Leusch, F.D.L., 2015a. Effect-based trigger values for in vitro bioassays: Reading across from existing water quality guideline values. Water Res. 81, 137–148. doi:10.1016/j.watres.2015.05.049

Escher, B.I., Neale, P.A., Leusch, F.D.L., 2015b. Effect-based trigger values for in vitro bioassays: Reading across from existing water quality guideline values. Water Res. 81, 137–148. doi:10.1016/j.watres.2015.05.049

Escher, B.I., van Daele, C., Dutt, M., Tang, J.Y.M., Altenburger, R., 2013. Most Oxidative Stress Response In Water Samples Comes From Unknown Chemicals: The Need For Effect-Based

Water Quality Trigger Values. Environ. Sci. Technol. 47, 7002–7011. doi:10.1021/es304793h

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

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

Espín, S., Martínez-López, E., León-Ortega, M., Martínez, J.E., García-Fernández, A.J., 2014b. Oxidative stress biomarkers in Eurasian eagle owls (Bubo bubo) in three different scenarios of heavy metal exposure. Environ. Res. 131, 134–144. doi:10.1016/j.envres.2014.03.015

EU, 2013. Directive 2013/39/EU [WWW Document]. Off. J. Eur. Union. doi:2013/39/EU

European Comission, 2014. JRC Reference Report on Monitoring of Emissions to Air and Water from IED installations.

European Community, 2000. 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, 1–82. doi:10.1039/ap9842100196

European Parliament, 2000. Directive 2000/60/EC. Off. J. Eur. Communities L 269, 1–90. doi:10.1039/ap9842100196

Eurostat, 2016. Minimum wages,July 2016 [WWW Document].

Fatima, M., Usmani, N., Hossain, M.M., 2014. Heavy Metal in Aquatic Ecosystem Emphasizing its Effect on Tissue Bioaccumulation and Histopathology: A Review. J. Environ. Sci. Technol. 7, 1–15. doi:10.3923/jest.2014.1.15

1512 Finkel, T., Holbrook, N.J., 2000. Oxidants, oxidative stress and the biology of ageing. Nature 408,
1513 239–247. doi:10.1038/35041687

1514 Fischer, F.C., Henneberger, L., König, M., Bittermann, K., Linden, L., Goss, K.U., Escher, B.I., 2017.
1515 Modeling Exposure in the Tox21 in Vitro Bioassays. Chem. Res. Toxicol.
1516 doi:10.1021/acs.chemrestox.7b00023

1517 Gambardella, C., Ferrando, S., Gatti, A.M., Cataldi, E., Ramoino, P., Aluigi, M.G., Faimali, M.,
1518 Diaspro, A., Falugi, C., 2016. Review: Morphofunctional and biochemical markers of stress in sea
1519 urchin life stages exposed to engineered nanoparticles. Environ. Toxicol. 31, 1552–1562.
1520 doi:10.1002/tox.22159

1521 García-Fernández, A.J., Bayoumi, A.E., Pérez-Pertejo, Y., Motas, M., Reguera, R.M., Ordóñez, C.,
1522 Balaña-Fouce, R., Ordóñez, D., 2002. Alterations of the glutathione-redox balance induced by
1523 metals in CHO-K1 cells. Comp. Biochem. Physiol. - C Toxicol. Pharmacol. 132, 365–373.
1524 doi:10.1016/S1532-0456(02)00079-0

1525 Garcia-Fernandez, A.J., Bayoumi, A.E., Perez-Pertejo, Y., Romero, D., Ordonez, C., Reguera, R.M.,
1526 Balana-Fouce, R., Ordonez, D., 2002. Changes in glutathione-redox balance induced by
1527 hexachlorocyclohexane and lindane in CHO-K1 cells. Xenobiotica. 32, 1007–1016.
1528 doi:10.1080/00498250210000012628

1529 Garcia-Reyero, N., Lavelle, C.M., Escalon, B.L., Martinović, D., Kroll, K.J., Sorensen, P.W., Denslow,
1530 N.D., 2011. Behavioral and genomic impacts of a wastewater effluent on the fathead minnow.
1531 Aquat. Toxicol. 101, 38–48. doi:10.1016/j.aquatox.2010.08.014

1532 Gauthier, P.T., Norwood, W.P., Prepas, E.E., Pyle, G.G., 2016. Behavioural alterations from exposure
1533 to Cu, phenanthrene, and Cu-phenanthrene mixtures: linking behaviour to acute toxic mechanisms
1534 in the aquatic amphipod, Hyalella azteca. Aquat. Toxicol. 170, 377–383.

doi:10.1016/j.aquatox.2015.10.019

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

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

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

Gopalapillai, Y., Vigneault, B., Hale, B.A., 2014. Root length of aquatic plant, Lemna minor L., as an optimal toxicity endpoint for biomonitoring of mining effluents. Integr. Environ. Assess. Manag. 10, 493–497. doi:10.1002/ieam.1558

Gür, N., Türker, O.C., Böcük, H., 2016. Toxicity assessment of boron (B) by Lemna minor L. and Lemna gibba L. and their possible use as model plants for ecological risk assessment of aquatic ecosystems with boron pollution. Chemosphere 157, 1–9. doi:10.1016/j.chemosphere.2016.04.138

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

Halliwell, B., Gutteridge, J.M.C., 2007. Free Radicals in Biology and Medicine, Free Radical Biology and Medicine. doi:10.1016/0891-5849(91)90055-8

Hasenbein, S., Lawler, S.P., Connon, R.E., 2017. An assessment of direct and indirect effects of two

herbicides on aquatic communities. Environ. Toxicol. Chem. 36, 2234–2244. doi:10.1002/etc.3740

Hashmi, Muhammad Arslan Kamal, Escher, Beate I., Krauss, Martin, Teodorovic, Ivana, Brack, W., 2018. Effect-directed analysis (EDA) of Danube River water sample receiving untreated municipal wastewater from Novi Sad, Serbia. Sci. Total Environ. 624, 1072–1081. doi:https://doi.org/10.1016/j.scitotenv.2017.12.187

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

Hicks, K.A., Fuzzen, M.L.M., McCann, E.K., Arlos, M.J., Bragg, L.M., Kleywegt, S., Tetreault, G.R., McMaster, M.E., Servos, M.R., 2017. Reduction of Intersex in a Wild Fish Population in Response to Major Municipal Wastewater Treatment Plant Upgrades. Environ. Sci. Technol. 51, 1811–1819. doi:10.1021/acs.est.6b05370

Hodgson, E., 2004. A Textbook of Modern Toxicology, Third Edit. ed. John Wiley & Sons, Inc.

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

Iguchi, T., Watanabe, H., Katsu, Y., 2007. Toxicogenomics and ecotoxicogenomics for studying endocrine disruption and basic biology. Gen. Comp. Endocrinol. doi:10.1016/j.ygcen.2007.01.013

Iguchi, T., Watanabe, H., Katsu, Y., 2006. Application of ecotoxicogenomics for studying endocrine disruption in vertebrates and invertebrates. Environ. Health Perspect. 114, 101–105.

1579 doi:10.1289/ehp.8061

1580 Ihara, M., Kitamura, T., Kumar, V., Park, C.B., Ihara, M.O., Lee, S.J., Yamashita, N., Miyagawa, S.,
1581 Iguchi, T., Okamoto, S., Suzuki, Y., Tanaka, H., 2015. Evaluation of estrogenic activity of
1582 wastewater: Comparison among in vitro ER?? reporter gene assay, in vivo vitellogenin induction,
1583 and chemical analysis. Environ. Sci. Technol. 49, 6319–6326. doi:10.1021/acs.est.5b01027

1584 Ippolito, A., Giacchini, R., Parenti, P., Vighi, M., 2017. Natural variability of enzymatic biomarkers in
1585 freshwater invertebrates. Environ. Sci. Pollut. Res. 24, 732–742. doi:10.1007/s11356-016-7833-4

1586 ISO, 2012. ISO 5667-3:2012, Water quality – Sampling – Part 3: Preservation and handling of water
1587 samples.

1588 Jorgensen, J.H., Ferraro, M.J., 2009. Antimicrobial susceptibility testing: a review of general principles
1589 and contemporary practices. Clin. Infect. Dis. 49, 1749–55. doi:10.1086/647952

1590 Kapustka, L.A., 2003. Rationale for use of wildlife habitat characterization to improve relevance of
1591 ecological risk assessments. Hum. Ecol. Risk Assess. 9, 1425–1430. doi:Doi
1592 10.1080/10807030390250921

1593 Keenan, P.O., Knight, A.W., Billinton, N., Cahill, P. a, Dalrymple, I.M., Hawkyard, C.J., Stratton-
1594 Campbell, D., Walmsley, R.M., 2007. Clear and present danger? The use of a yeast biosensor to
1595 monitor changes in the toxicity of industrial effluents subjected to oxidative colour removal
1596 treatments. J. Environ. Monit. 9, 1394–401. doi:10.1039/b710406e

1597 Koh, Y.K.K., Chiu, T.Y., Boobis, A.R., Scrimshaw, M.D., Bagnall, J.P., Soares, A., Pollard, S.,
1598 Cartmell, E., Lester, J.N., 2009. Influence of operating parameters on the biodegradation of steroid
1599 estrogens and nonylphenolic compounds during biological wastewater treatment processes.
1600 Environ. Sci. Technol. 43, 6646–54. doi:10.1021/es901612v

1601 Koivula, M.J., Eeva, T., 2010. Metal-related oxidative stress in birds. Environ. Pollut.
1602 doi:10.1016/j.envpol.2010.03.013

1603 Kotchen, M., Kallaos, J., Wheeler, K., Wong, C., Zahller, M., 2009. Pharmaceuticals in wastewater:
1604 Behavior, preferences, and willingness to pay for a disposal program. J. Environ. Manage. 90,
1605 1476–1482. doi:10.1016/j.jenvman.2008.10.002

1606 Kristiansson, E., Fick, J., Janzon, A., Grabic, R., Rutgersson, C., Weijdegård, B., Söderström, H.,
1607 Joakim Larsson, D.G., 2011. Pyrosequencing of antibiotic-contaminated river sediments reveals
1608 high levels of resistance and gene transfer elements. PLoS One 6.
1609 doi:10.1371/journal.pone.0017038

1610 Kuckelkorn, J., Redelstein, R., Heide, T., Kunze, J., Maletz, S., Waldmann, P., Grummt, T., Seiler,
1611 T.B., Hollert, H., 2018. A hierarchical testing strategy for micropollutants in drinking water
1612 regarding their potential endocrine-disrupting effects—towards health-related indicator values.
1613 Environ. Sci. Pollut. Res. doi:10.1007/s11356-017-0155-3

1614 Kumar, N., Krishnani, K.K., Gupta, S.K., Singh, N.P., 2017. Cellular stress and histopathological tools
1615 used as biomarkers in Oreochromis mossambicus for assessing metal contamination. Environ.
1616 Toxicol. Pharmacol. 49, 137–147. doi:10.1016/j.etap.2016.11.017

1617 Kumari, K., Khare, A., 2018. Integration of Biomarker Approach in Pollution Monitoring Programme
1618 of Aquatic Ecosystem. pp. 331–354. doi:10.1007/978-981-10-7434-9_18

1619 Kümmerer, K., 2009a. Antibiotics in the aquatic environment - A review - Part I. Chemosphere.
1620 doi:10.1016/j.chemosphere.2008.11.086

1621 Kümmerer, K., 2009b. Chemosphere antibiotics in the aquatic environment – A review – Part II.
1622 Chemosphere 75, 435–441. doi:10.1016/j.chemosphere.2008.12.006

1623 Kümmerer, K., Alexy, R., Hüttig, J., Schöll, A., 2004. Standardized tests fail to assess the effects of
1624 antibiotics on environmental bacteria. Water Res. 38, 2111–2116.
1625 doi:10.1016/j.watres.2004.02.004

1626 Kümmerer, K., Clark, J., 2016. Green and Sustainable Chemistry, in: Sustainability Science. pp. 43–59.
1627 doi:10.1007/978-94-017-7242-6_4

1628 Larsson, D.G.J., de Pedro, C., Paxeus, N., 2007. Effluent from drug manufactures contains extremely
1629 high levels of pharmaceuticals. J. Hazard. Mater. 148, 751–755.
1630 doi:10.1016/j.jhazmat.2007.07.008

1631 Le-Minh, N., Khan, S.J., Drewes, J.E., Stuetz, R.M., 2010. Fate of antibiotics during municipal water
1632 recycling treatment processes. Water Res. doi:10.1016/j.watres.2010.06.020

1633 Lemm, J.U., Feld, C.K., 2017. Identification and interaction of multiple stressors in central European
1634 lowland rivers. Sci. Total Environ. 603–604, 148–154. doi:10.1016/j.scitotenv.2017.06.092

1635 Lemos, M.F.L., Soares, A.M.V.M., Correia, A.C., Esteves, A.C., 2010. Proteins in ecotoxicology -
1636 How, why and why not? Proteomics. doi:10.1002/pmic.200900470

1637 Leusch, F.D.L., de Jager, C., Levi, Y., Lim, R., Puijker, L., Sacher, F., Tremblay, L.A., Wilson, V.S.,
1638 Chapman, H.F., 2010. Comparison of Five in Vitro Bioassays to Measure Estrogenic Activity in
1639 Environmental Waters. Environ. Sci. Technol. 44, 3853–3860. doi:10.1021/es903899d

1640 Leusch, F.D.L., Khan, S.J., Gagnon, M.M., Quayle, P., Trinh, T., Coleman, H., Rawson, C., Chapman,
1641 H.F., Blair, P., Nice, H., Reitsema, T., 2014. Assessment of wastewater and recycled water
1642 quality: a comparison of lines of evidence from in vitro, in vivo and chemical analyses. Water
1643 Res. doi:10.1016/j.watres.2013.10.056

1644 Leusch, F.D.L., Khan, S.J., Laingam, S., Prochazka, E., Froscio, S., Trinh, T., Chapman, H.F.,

1645 Humpage, A., 2014. Assessment of the application of bioanalytical tools as surrogate measure of
1646 chemical contaminants in recycled water. Water Res. doi:10.1016/j.watres.2013.11.030

1647 Leusch, F.D.L., Neale, P.A., Hebert, A., Scheurer, M., Schriks, M.C.M., 2017a. Analysis of the
1648 sensitivity of in vitro bioassays for androgenic, progestagenic, glucocorticoid, thyroid and
1649 estrogenic activity: Suitability for drinking and environmental waters. Environ. Int. 99, 120–130.
1650 doi:10.1016/j.envint.2016.12.014

1651 Leusch, F.D.L., Neale, P.A., Hebert, A., Scheurer, M., Schriks, M.C.M., 2017b. Analysis of the
1652 sensitivity of in vitro bioassays for androgenic, progestagenic, glucocorticoid, thyroid and
1653 estrogenic activity: Suitability for drinking and environmental waters. Environ. Int. 99, 120–130.
1654 doi:10.1016/j.envint.2016.12.014

1655 Li, D., Yu, T., Zhang, Y., Yang, M., Li, Z., Liu, M., Qi, R., 2010. Antibiotic resistance characteristics
1656 of environmental bacteria from an oxytetracycline production wastewater treatment plant and the
1657 receiving river. Appl. Environ. Microbiol. 76, 3444–3451. doi:10.1128/AEM.02964-09

1658 Li, J.Y., Tang, J.Y.M., Jin, L., Escher, B.I., 2013. Understanding bioavailability and toxicity of
1659 sediment-associated contaminants by combining passive sampling with in vitro bioassays in an
1660 urban river catchment. Environ. Toxicol. Chem. doi:10.1002/etc.2387

1661 Libralato, G., 2013. Management and control of wastewater: An ecotoxicological approach, Handbook
1662 of Wastewater Treatment: Biological Methods, Technology and Environmental Impact.

1663 Libralato, G., Gentile, E., Volpi Ghirardini, A., 2016. Wastewater effects on Phaeodactylum
1664 tricornutum (Bohlin): Setting up a classification system. Ecol. Indic. 60, 31–37.
1665 doi:10.1016/j.ecolind.2015.06.014

1666 Libralato, G., Ghirardini Annamaria, V., Francesco, A., 2010a. How toxic is toxic? A proposal for

wastewater toxicity hazard assessment. Ecotoxicol. Environ. Saf. 73, 1602–1611. doi:10.1016/j.ecoenv.2010.03.007

Libralato, G., Volpi Ghirardini, A., Avezzù, F., 2012. To centralise or to decentralise: An overview of the most recent trends in wastewater treatment management. J. Environ. Manage. 94, 61–68. doi:10.1016/j.jenvman.2011.07.010

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

Liscio, C., Abdul-Sada, A., Al-Salhi, R., Ramsey, M.H., Hill, E.M., 2014. Methodology for profiling anti-androgen mixtures in river water using multiple passive samplers and bioassay-directed analyses. Water Res. doi:10.1016/j.watres.2014.03.039

Liu, Y., Wu, F., Mu, Y., Feng, C., Fang, Y., Chen, L., Giesy, J.P., 2014. Setting Water Quality Criteria in China: Approaches for Developing Species Sensitivity Distributions for Metals and Metalloids, in: Philosophical Transactions of the Royal Society B: Biological Sciences. pp. 35–57. doi:10.1007/978-3-319-04411-8_2

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

Lofrano, G., Libralato, G., Adinolfi, R., Siciliano, A., Iannece, P., Guida, M., Giugni, M., Volpi Ghirardini, A., Carotenuto, M., 2016. Photocatalytic degradation of the antibiotic chloramphenicol and effluent toxicity effects. Ecotoxicol. Environ. Saf. 123, 65–71. doi:10.1016/j.ecoenv.2015.07.039

1689 Lofrano, G., Pedrazzani, R., Libralato, G., Carotenuto, M., 2017. Advanced Oxidation Processes for
1690 Antibiotics Removal: A Review. Curr. Org. Chem. 21, 1054–1067.
1691 doi:10.2174/1385272821666170103162813

1692 Logar, I., Brouwer, R., Maurer, M., Ort, C., 2014. Cost-benefit analysis of the swiss national policy on
1693 reducing micropollutants in treated wastewater. Environ. Sci. Technol. 48, 12500–12508.
1694 doi:10.1021/es502338j

1695 Lonappan, L., Brar, S.K., Das, R.K., Verma, M., Surampalli, R.Y., 2016. Diclofenac and its
1696 transformation products: Environmental occurrence and toxicity - A review. Environ. Int. 96, 127–
1697 138. doi:10.1016/j.envint.2016.09.014

1698 Lu, B., Du, X., Huang, S., 2016. The economic and environmental implications of wastewater
1699 management policy in China: From the LCA perspective. J. Clean. Prod.
1700 doi:10.1016/j.jclepro.2016.10.113

1701 Lu, Y., Aitken, R.J., Lin, M., 2017. Detailed analysis of the male reproductive system in a potential
1702 bio-indicator species – The marine invertebrate Galeolaria caespitosa (Polychaeta: Serpulidae).
1703 PLoS One 12, e0174907. doi:10.1371/journal.pone.0174907

1704 Lushchak, V.I., 2011. Environmentally induced oxidative stress in aquatic animals. Aquat. Toxicol.
1705 doi:10.1016/j.aquatox.2010.10.006

1706 Lyubenova, L., Nehnevajova, E., Herzig, R., Schröder, P., 2009. Response of antioxidant enzymes in
1707 Nicotiana tabacum clones during phytoextraction of heavy metals. Environ. Sci. Pollut. Res. 16,
1708 573–581. doi:10.1007/s11356-009-0175-8

1709 Maertens, A., Hartung, T., 2018. Green Toxicology-know early about and avoid toxic product
1710 liabilities. Toxicol. Sci. doi:10.1093/toxsci/kfx243

1711 Magdeburg, A., Stalter, D., Schl??sener, M., Ternes, T., Oehlmann, J., 2014. Evaluating the efficiency
1712 of advanced wastewater treatment: Target analysis of organic contaminants and (geno-)toxicity
1713 assessment tell a different story. Water Res. 50, 35–47. doi:10.1016/j.watres.2013.11.041

1714 Maruya, K.A., Schlenk, D., Anderson, P.D., Denslow, N.D., Drewes, J.E., Olivieri, A.W., Scott, G.I.,
1715 Snyder, S.A., 2014. An Adaptive, Comprehensive Monitoring Strategy for Chemicals of
1716 Emerging Concern (CECs) in California's Aquatic Ecosystems. Integr. Environ. Assess. Manag.
1717 10, 69–77. doi:10.1002/ieam.1483

1718 Masood, F., Malik, A., 2013. Mutagenicity and genotoxicity assessment of industrial wastewaters.
1719 Environ. Sci. Pollut. Res. 20, 7386–7397. doi:10.1007/s11356-013-1756-0

1720 Mavragani, A., Sypsa, K., Sampri, A., Tsagarakis, K., 2016. Quantifying the UK Online Interest in
1721 Substances of the EU Watchlist for Water Monitoring: Diclofenac, Estradiol, and the Macrolide
1722 Antibiotics. Water 8, 542. doi:10.3390/w8110542

1723 McAdam, E.J., Bagnall, J.P., Koh, Y.K.K., Chiu, T.Y., Pollard, S., Scrimshaw, M.D., Lester, J.N.,
1724 Cartmell, E., 2010. Removal of steroid estrogens in carbonaceous and nitrifying activated sludge
1725 processes. Chemosphere 81, 1–6. doi:10.1016/j.chemosphere.2010.07.057

1726 McArthur, A.G., Waglechner, N., Nizam, F., Yan, A., Azad, M.A., Baylay, A.J., Bhullar, K., Canova,
1727 M.J., De Pascale, G., Ejim, L., Kalan, L., King, A.M., Koteva, K., Morar, M., Mulvey, M.R.,
1728 O'Brien, J.S., Pawlowski, A.C., Piddock, L.J. V, Spanogiannopoulos, P., Sutherland, A.D., Tang,
1729 I., Taylor, P.L., Thaker, M., Wang, W., Yan, M., Yu, T., Wright, G.D., 2013. The comprehensive
1730 antibiotic resistance database. Antimicrob. Agents Chemother. 57, 3348–3357.
1731 doi:10.1128/AAC.00419-13

1732 McCall, A.K., Bade, R., Kinyua, J., Lai, F.Y., Thai, P.K., Covaci, A., Bijlsma, L., van Nuijs, A.L.N.,
1733 Ort, C., 2016. Critical review on the stability of illicit drugs in sewers and wastewater samples.

1734 Water Res. doi:10.1016/j.watres.2015.10.040

1735 Melvin, S.D., Leusch, F.D.L., 2016. Removal of trace organic contaminants from domestic wastewater:
1736 A meta-analysis comparison of sewage treatment technologies. Environ. Int. 92, 183–188.
1737 doi:10.1016/j.envint.2016.03.031

1738 Mendonça, E., Picado, A., Paixão, S.M., Silva, L., Cunha, M.A., Leitão, S., Moura, I., Cortez, C.,
1739 Brito, F., 2009. Ecotoxicity tests in the environmental analysis of wastewater treatment plants:
1740 Case study in Portugal. J. Hazard. Mater. 163, 665–670. doi:10.1016/j.jhazmat.2008.07.012

1741 Metcalfe, C.D., Kleywegt, S., Letcher, R.J., Topp, E., Wagh, P., Trudeau, V.L., Moon, T.W., 2013. A
1742 multi-assay screening approach for assessment of endocrine-active contaminants in wastewater
1743 effluent samples. Sci. Total Environ. 454–455, 132–140. doi:10.1016/j.scitotenv.2013.02.074

1744 Michael, I., Rizzo, L., McArdell, C.S., Manaia, C.M., Merlin, C., Schwartz, T., Dagot, C., Fatta-
1745 Kassinos, D., 2013. Urban wastewater treatment plants as hotspots for the release of antibiotics in
1746 the environment: A review. Water Res. doi:10.1016/j.watres.2012.11.027

1747 Miracle, A.L., Ankley, G.T., 2005. Ecotoxicogenomics: Linkages between exposure and effects in
1748 assessing risks of aquatic contaminants to fish. Reprod. Toxicol.
1749 doi:10.1016/j.reprotox.2004.06.007

1750 Moro, I., Matozzo, V., Piovan, A., Moschin, E., Vecchia, F.D., 2014. Morpho-physiological effects of
1751 ibuprofen on Scenedesmus rubescens. Environ. Toxicol. Pharmacol. 38, 379–387.
1752 doi:10.1016/j.etap.2014.06.005

1753 Morris, L., Colombo, V., Hassell, K., Kellar, C., Leahy, P., Long, S.M., Myers, J.H., Pettigrove, V.,
1754 2017. Municipal wastewater effluent licensing: A global perspective and recommendations for
1755 best practice. Sci. Total Environ. 580, 1327–1339. doi:10.1016/j.scitotenv.2016.12.096

1756 Mullany, P., 2014. Functional metagenomics for the investigation of antibiotic resistance. Virulence 5,
1757 443–7. doi:10.4161/viru.28196

1758 Naddeo, V., Meriç, S., Kassinos, D., Belgiorno, V., Guida, M., 2009. Fate of pharmaceuticals in
1759 contaminated urban wastewater effluent under ultrasonic irradiation. Water Res. 43, 4019–4027.
1760 doi:10.1016/j.watres.2009.05.027

1761 Neale, P.A., Achard, M.E.S., Escher, B.I., Leusch, F.D.L., 2017a. Exploring the oxidative stress
1762 response mechanism triggered by environmental water samples. Environ. Sci. Process. Impacts
1763 19, 1126–1133. doi:10.1039/C6EM00541A

1764 Neale, P.A., Achard, M.E.S., Escher, B.I., Leusch, F.D.L., 2017b. Exploring the oxidative stress
1765 response mechanism triggered by environmental water samples. Environ. Sci. Process. Impacts
1766 19, 1126–1133. doi:10.1039/C6EM00541A

1767 Neale, P.A., Altenburger, R., Aït-Aïssa, S., Brion, F., Busch, W., de Aragão Umbuzeiro, G., Denison,
1768 M.S., Du Pasquier, D., Hilscherová, K., Hollert, H., Morales, D.A., Novák, J., Schlichting, R.,
1769 Seiler, T.B., Serra, H., Shao, Y., Tindall, A.J., Tollefsen, K.E., Williams, T.D., Escher, B.I.,
1770 2017c. Development of a bioanalytical test battery for water quality monitoring: Fingerprinting
1771 identified micropollutants and their contribution to effects in surface water. Water Res.
1772 doi:10.1016/j.watres.2017.07.016

1773 Neale, P.A., Brack, W., Aït-Aïssa, S., Busch, W., Hollender, J., Krauss, M., Maillot-Maréchal, E.,
1774 Munz, N.A., Schlichting, R., Schulze, T., Vogler, B., Escher, B.I., 2018. Solid-phase extraction as
1775 sample preparation of water samples for cell-based and other: In vitro bioassays. Environ. Sci.
1776 Process. Impacts. doi:10.1039/c7em00555e

1777 Neale, P.A., Escher, B.I., 2013. Coextracted dissolved organic carbon has a suppressive effect on the
1778 acetylcholinesterase inhibition assay. Environ. Toxicol. Chem. n/a-n/a. doi:10.1002/etc.2196

1779 Neale, P.A., Munz, N.A., Aït-Aïssa, S., Altenburger, R., Brion, F., Busch, W., Escher, B.I.,
1780 Hilscherovï¿½, K., Kienle, C., Novï¿½k, J., Seiler, T.B., Shao, Y., Stamm, C., Hollender, J.,
1781 2017d. Integrating chemical analysis and bioanalysis to evaluate the contribution of wastewater
1782 effluent on the micropollutant burden in small streams. Sci. Total Environ.
1783 doi:10.1016/j.scitotenv.2016.10.141

1784 Newman, M.C., Clements, W.H., 2008. Ecotoxicology: An comprehensive treatment, Ecotoxicology.
1785 doi:10.1002/ieam.5630040423

1786 NIEHS, 2002. Current status of test methods for detecting endocrine disruptors. Expert panel
1787 evaluation of the validation status of in vitro test methods for detecting endocrine disruptors.

1788 Niss, F., Rosenmai, A.K., Mandava, G., Örn, S., Oskarsson, A., Lundqvist, J., 2018. Toxicity bioassays
1789 with concentrated cell culture media—a methodology to overcome the chemical loss by
1790 conventional preparation of water samples. Environ. Sci. Pollut. Res. doi:10.1007/s11356-018-
1791 1656-4

1792 Novák, J., Vrana, B., Rusina, T., Okonski, K., Grabic, R., Neale, P.A., Escher, B.I., Macová, M., Ait-
1793 Aissa, S., Creusot, N., Allan, I., Hilscherová, K., 2018. Effect-based monitoring of the Danube
1794 River using mobile passive sampling. Sci. Total Environ. doi:10.1016/j.scitotenv.2018.02.201

1795 Ode, P., Schiff, K., 2009. Recommendations for the Development and Maintenance of a Reference
1796 Condition Management Program (RCMP_ to Support Biological Assessment of California's
1797 Wadeable Streams. Swamp.

1798 OECD, 2012. Conceptual framework for testing and assment of endocrine disrupters (as revised in
1799 2012).

1800 Ohe, T., Suzuki, A., Watanabe, T., Hasei, T., Nukaya, H., Totsuka, Y., Wakabayashi, K., 2009.

1801 Induction of SCEs in CHL cells by dichlorobiphenyl derivative water pollutants, 2-
1802 phenylbenzotriazole (PBTA) congeners and river water concentrates. Mutat. Res. - Genet.
1803 Toxicol. Environ. Mutagen. 678, 38–42. doi:10.1016/j.mrgentox.2009.06.003

1804 Ort, C., Lawrence, M.G., Rieckermann, J., Joss, A., 2010. Sampling for pharmaceuticals and personal
1805 care products (PPCPs) and illicit drugs in wastewater systems: Are your conclusions valid? A
1806 critical review. Environ. Sci. Technol. 44, 6024–6035. doi:10.1021/es100779n

1807 Osorio, V., Schriks, M., Vughs, D., de Voogt, P., Kolkman, A., 2018. A novel sample preparation
1808 procedure for effect-directed analysis of micro-contaminants of emerging concern in surface
1809 waters. Talanta. doi:10.1016/j.talanta.2018.04.058

1810 OSPAR, 2005. Whole effluent assessment.

1811 Ozkok, I.P., Yazan, T.K., Cokgor, E.U., Insel, G., Talinli, I., Orhon, D., 2011. Respirometric
1812 assessment of substrate binding by antibiotics in peptone biodegradation. J. Environ. Sci. Health.
1813 A. Tox. Hazard. Subst. Environ. Eng. 46, 1588–97. doi:10.1080/10934529.2011.609442

1814 Pala-Ozkok, I., 2012. Inhibitory Impact of Selected Antibiotics on Biodegradation Characteristic and
1815 Microbial Population Under Aerobic Conditions. Istanbul Technical University.

1816 Pala-Ozkok, I., Orhon, D., 2013. Chronic effect of erythromycin on substrate biodegradation kinetics of
1817 activated sludge. Biochem. Eng. J. 81, 29–39. doi:10.1016/j.bej.2013.10.002

1818 Pala-Ozkok, I., Rehman, A., Ubay-Cokgor, E., Jonas, D., Orhon, D., 2014a. Pyrosequencing reveals
1819 the inhibitory impact of chronic exposure to erythromycin on activated sludge bacterial
1820 community structure. Biochem. Eng. J. 90, 195–205. doi:10.1016/j.bej.2014.06.003

1821 Pala-Ozkok, I., Ubay-Cokgor, E., Cakar, Z.P., Orhon, D., 2014b. Acute impact of erythromycin on
1822 substrate utilization by activated sludge: Effect of sludge age. J. Chem. Technol. Biotechnol. 89,

1823 1091–1102. doi:10.1002/jctb.4208

1824 Papa, M., Ceretti, E., Viola, G.C.V., Feretti, D., Zerbini, I., Mazzoleni, G., Steimberg, N., Pedrazzani,
1825 R., Bertanza, G., 2016. The assessment of WWTP performance: Towards a jigsaw puzzle
1826 evaluation? Chemosphere 145, 291–300. doi:10.1016/j.chemosphere.2015.11.054

1827 Penders, E.J.M., Martijn, A.J., Spenkelink, A., Alink, G.M., Rietjens, I.M.C.M., Hoogenboezem, W.,
1828 2012. Genotoxicity testing of samples generated during UV/H 2 O 2 treatment of surface water for
1829 the production of drinking water using the Ames test in vitro and the Comet assay and the SCE
1830 test in vivo. J. Water Supply Res. Technol. 61, 435. doi:10.2166/aqua.2012.069

1831 Perkins, E., Garcia-Reyero, N., Edwards, S., Wittwehr, C., Villeneuve, D., Lyons, D., Ankley, G.,
1832 2015. The Adverse Outcome Pathway: A Conceptual Framework to Support Toxicity Testing in
1833 the Twenty-First Century. pp. 1–26. doi:10.1007/978-1-4939-2778-4_1

1834 Persoone, G., Marsalek, B., Blinova, I., Törökne, A., Zarina, D., Manusadzianas, L., Nalecz-Jawecki,
1835 G., Tofan, L., Stepanova, N., Tothova, L., Kolar, B., 2003. A practical and user-friendly toxicity
1836 classification system with microbiotests for natural waters and wastewaters. Environ. Toxicol. 18,
1837 395–402. doi:10.1002/tox.10141

1838 Petrović, M., Škrbić, B., Živančev, J., Ferrando-Climent, L., Barcelo, D., 2014. Determination of 81
1839 pharmaceutical drugs by high performance liquid chromatography coupled to mass spectrometry
1840 with hybrid triple quadrupole-linear ion trap in different types of water in Serbia. Sci. Total
1841 Environ. 468–469, 415–428. doi:10.1016/j.scitotenv.2013.08.079

1842 Phillips, B.M., Hunt, J.W., Anderson, B.S., Max Puckett, H., Fairey, R., Wilson, C.J., Tjeerdema, R.,
1843 2001. STATISTICAL SIGNIFICANCE OF SEDIMENT TOXICITY TEST RESULTS:
1844 THRESHOLD VALUES DERIVED BY THE DETECTABLE SIGNIFICANCE APPROACH.
1845 Environ. Toxicol. Chem. 20, 371. doi:10.1897/1551-5028(2001)020<0371:SSOSTT>2.0.CO;2

1846 Pintilie, L., Torres, C.M., Teodosiu, C., Castells, F., 2016. Urban wastewater reclamation for industrial
1847 reuse: An LCA case study. J. Clean. Prod. 139, 1–14. doi:10.1016/j.jclepro.2016.07.209

1848 Pisoschi, A.M., Pop, A., 2015. The role of antioxidants in the chemistry of oxidative stress: A review.
1849 Eur. J. Med. Chem. 97, 55–74. doi:10.1016/j.ejmech.2015.04.040

1850 Posthuma, L., Suter, W.G., Trass, P.T., 2002. Species sensitivity distributions in ecotoxicology,
1851 Ecotoxicology. doi:10.1017/CBO9781107415324.004

1852 Power, E.A., Boumphrey, R.S., 2004. International trends in bioassay use for effluent management.
1853 Ecotoxicology. doi:10.1023/B:ECTX.0000035290.89590.03

1854 Price, P., Han, X., Junghans, M., Kunz, P., Watts, C., Leverett, D., 2012. An application of a decision
1855 tree for assessing effects from exposures to multiple substances to the assessment of human and
1856 ecological effects from combined exposures to chemicals observed in surface waters and waste
1857 water effluents. Environ. Sci. Eur. 24, 34. doi:10.1186/2190-4715-24-34

1858 Protection, L.O.F., Assessment, R., 2000. Whole Effluent Toxicity Testing — Usefulness , Level of
1859 Protection , and Risk Assessment. Environ. Toxicol. Chem. 19, 3–13. doi:10.1897/1551-
1860 5028(2000)019<0003:wettul>2.3.co;2

1861 Quaedackers, M.E., Van Den Brink, C.E., Wissink, S., Schreurs, R.H., Gustafsson, J.A., Van Der Saag,
1862 P.T., Van Der Burg, B.B., 2001. 4-hydroxytamoxifen trans-represses nuclear factor-kappa B
1863 activity in human osteoblastic U2-OS cells through estrogen receptor (ER)alpha, and not through
1864 ER beta. Endocrinology 142, 1156–66. doi:10.1210/endo.142.3.8003

1865 Quesada, I., Fuentes, E., Viso-León, M.C., Soria, B., Ripoll, C., Nadal, A., 2002. Low doses of the
1866 endocrine disruptor bisphenol-A and the native hormone 17beta-estradiol rapidly activate
1867 transcription factor CREB. FASEB J. 16, 1671–1673. doi:10.1096/fj.02-0313fje

1868 Rastogi, T., Leder, C., Kümmerer, K., 2014. Designing green derivatives of ??-blocker Metoprolol: A
1869 tiered approach for green and sustainable pharmacy and chemistry. Chemosphere 111, 493–499.
1870 doi:10.1016/j.chemosphere.2014.03.119

1871 Ray, S., Mukherjee, S., Bhunia, N.S., Bhunia, A.S., Ray, M., 2015. Immunotoxicological Threats of
1872 Pollutants in Aquatic Invertebrates, in: Emerging Pollutants in the Environment - Current and
1873 Further Implications. InTech. doi:10.5772/60216

1874 Rhee, H.J., Kim, E.-J., Lee, J.K., 2007. Physiological polyamines: simple primordial stress molecules.
1875 J. Cell. Mol. Med. 11, 685–703. doi:10.1111/j.1582-4934.2007.00077.x

1876 Rizzo, L., Meric, S., Guida, M., Kassinos, D., Belgiorno, V., 2009. Heterogenous photocatalytic
1877 degradation kinetics and detoxification of an urban wastewater treatment plant effluent
1878 contaminated with pharmaceuticals. Water Res. 43, 4070–4078. doi:10.1016/j.watres.2009.06.046

1879 Salamat, N., Zarie, M., 2016. Fish histopathology as a tool for use in marine environment monitoring: a
1880 review. Comp. Clin. Path. 25, 1273–1278. doi:10.1007/s00580-014-2037-0

1881 Santana, M.S., Yamamoto, F.Y., Sandrini-Neto, L., Filipak Neto, F., Ortolani-Machado, C.F., Oliveira
1882 Ribeiro, C.A., Prodocimo, M.M., 2018. Diffuse sources of contamination in freshwater fish:
1883 Detecting effects through active biomonitoring and multi-biomarker approaches. Ecotoxicol.
1884 Environ. Saf. 149, 173–181. doi:10.1016/j.ecoenv.2017.11.036

1885 Sarakinos, H.C., Bermingham, N., White, P. a, Rasmussen, J.B., 2000. Correspondence between whole
1886 effluent toxicity and the presence of priority substances in complex industrial effluents. Environ.
1887 Toxicol. Chem. 19, 63–71. doi:10.1897/1551-5028(2000)019<0063:CBWETA>2.3.CO;2

1888 Sarmah, A.K., Meyer, M.T., Boxall, A.B.A., 2006. A global perspective on the use, sales, exposure
1889 pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment.

Chemosphere. doi:10.1016/j.chemosphere.2006.03.026

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

Schieber, M., Chandel, N.S., 2014. ROS Function in Redox Signaling and Oxidative Stress. Curr. Biol. 24, R453–R462. doi:10.1016/j.cub.2014.03.034

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

Scholz, S., Renner, P., Belanger, S.E., Busquet, F., Davi, R., Demeneix, B. a, Denny, J.S., Léonard, M., McMaster, M.E., Villeneuve, D.L., Embry, M.R., 2013. 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, 45–72. doi:10.3109/10408444.2012.737762

Schröder, P., 2007. Exploiting Plant Metabolism for Phytoremediation of Organic Xenobiotics, in: Willey, N. (Ed.), Phytoremediation: Methods and Reviews. Humana Press, Totowa, New Jersey, USA, pp. 251–265.

Schwindt, A.R., 2015. Parental effects of endocrine disrupting compounds in aquatic wildlife: Is there evidence of transgenerational inheritance? Gen. Comp. Endocrinol. 219, 152–164. doi:10.1016/j.ygcen.2015.01.020

Scognamiglio, V., Antonacci, A., Patrolecco, L., Lambreva, M.D., Litescu, S.C., Ghuge, S.A., Rea, G., 2016. Analytical tools monitoring endocrine disrupting chemicals. TrAC - Trends Anal. Chem.

1912 doi:10.1016/j.trac.2016.04.014

1913 Selma, Etteieb, Atsushi, Kawachi, Junkyu, Han, Jamila, Tarhouni, Hiroko, I., 2014. An evaluation of
1914 the environmental state of surface water quality using modified E-screen assay, in: C.A. Brebbia
1915 (Ed.), Water Pollution XII. Wit press, Southampton, UK.

1916 Sendra, M., S??nchez-Quiles, D., Blasco, J., Moreno-Garrido, I., Lubi??n, L.M., P??rez-Garc??a, S.,
1917 Tovar-S??nchez, A., 2017. Effects of TiO2 nanoparticles and sunscreens on coastal marine
1918 microalgae: Ultraviolet radiation is key variable for toxicity assessment. Environ. Int. 98, 62–68.
1919 doi:10.1016/j.envint.2016.09.024

1920 Shane, A.S., Leusch, F.D.L., 2018. In vitro bioassays: current status and future application for water
1921 management. State of the Sciene Report.

1922 Sharif, A., Ashraf, M., Anjum, A.A., Javeed, A., Altaf, I., Akhtar, M.F., Abbas, M., Akhtar, B.,
1923 Saleem, A., 2016. Pharmaceutical wastewater being composite mixture of environmental
1924 pollutants may be associated with mutagenicity and genotoxicity. Environ. Sci. Pollut. Res. 23,
1925 2813–2820. doi:10.1007/s11356-015-5478-3

1926 Shepard, J.L., Olsson, B., Tedengren, M., Bradley, B.P., 2000. Protein expression signatures identified
1927 in Mytilus edulis exposed to PCBs, copper and salinity stress, in: Marine Environmental Research.
1928 pp. 337–340. doi:10.1016/S0141-1136(00)00065-9

1929 Shyamasundar, S., Ng, C.T., Yue, L., Yung, L., Dheen, T., Bay, H., 2015. Epigenetic mechanisms in
1930 nanomaterial-induced toxicity. Epigenomics 7, 395–411. doi:10.2217/epi.15.3

1931 Sies, H., 2015. Oxidative stress: a concept in redox biology and medicine. Redox Biol. 4, 180–183.
1932 doi:10.1016/j.redox.2015.01.002

1933 Sies, H., Berndt, C., Jones, D.P., 2017. Oxidative Stress. Annu. Rev. Biochem. 86, 715–748.

1934 doi:10.1146/annurev-biochem-061516-045037

1935 Smith, M.T., de la Rosa, R., Daniels, S.I., 2015. Using exposomics to assess cumulative risks and
1936 promote health. Environ. Mol. Mutagen. doi:10.1002/em.21985

1937 Snape, J.R., Maund, S.J., Pickford, D.B., Hutchinson, T.H., 2004. Ecotoxicogenomics: The challenge
1938 of integrating genomics into aquatic and terrestrial ecotoxicology. Aquat. Toxicol. 67, 143–154.
1939 doi:10.1016/j.aquatox.2003.11.011

1940 Stalter, D., O'Malley, E., von Gunten, U., Escher, B.I., 2016. Fingerprinting the reactive toxicity
1941 pathways of 50 drinking water disinfection by-products. Water Res. 91, 19–30.
1942 doi:10.1016/j.watres.2015.12.047

1943 Straub, J.O., 2016. Reduction in the environmental exposure of pharmaceuticals through diagnostics,
1944 Personalised Healthcare and other approaches. A mini review and discussion paper. Sustain.
1945 Chem. Pharm. doi:10.1016/j.scp.2015.12.001

1946 Strzyzewska, E., Szarek, J., Babinska, I., 2016. Morphologic evaluation of the gills as a tool in the
1947 diagnostics of pathological conditions in fish and pollution in the aquatic environment: a review.
1948 Vet. Med. (Praha). 61, 123–132. doi:10.17221/8763-VETMED

1949 Sui, Q., Huang, J., Deng, S., Chen, W., Yu, G., 2011. Seasonal variation in the occurrence and removal
1950 of pharmaceuticals and personal care products in different biological wastewater treatment
1951 processes. Environ. Sci. Technol. 45, 3341–3348. doi:10.1021/es200248d

1952 Sunanda, M., Rao, J. C. S., Neelima, P., Simhachalam, G., 2016. Toxicity and effects of chlorpyrifos in
1953 a non-target organism (Fish)-A review. J. Atoms Mol. 6.3, 966–976.

1954 Swedish EPA, 1997. Characterisation of discharges from chemical industry – The stork project. Report
1955 No 4766. Stockholm, Sweden.

1956 Szöcs, E., Van den Brink, P.J., Lagadic, L., Caquet, T., Roucaute, M., Auber, A., Bayona, Y., Liess,
1957 M., Ebke, P., Ippolito, A., ter Braak, C.J.F., Brock, T.C.M., Schäfer, R.B., 2015. Analysing
1958 chemical-induced changes in macroinvertebrate communities in aquatic mesocosm experiments: a
1959 comparison of methods. Ecotoxicology 24, 760–769. doi:10.1007/s10646-015-1421-0

1960 Tang, J.Y.M., Busetti, F., Charrois, J.W.A., Escher, B.I., 2014. Which chemicals drive biological
1961 effects in wastewater and recycled water? Water Res. 60, 289–299.
1962 doi:10.1016/j.watres.2014.04.043

1963 Tang, J.Y.M., McCarty, S., Glenn, E., Neale, P.A., Warne, M.S.J., Escher, B.I., 2013. Mixture effects
1964 of organic micropollutants present in water: Towards the development of effect-based water
1965 quality trigger values for baseline toxicity. Water Res. 47, 3300–3314.
1966 doi:10.1016/j.watres.2013.03.011

1967 Tarazona, J. V., Vega, M.M., 2002. Hazard and risk assessment of chemicals for terrestrial ecosystems.
1968 Toxicology. doi:10.1016/S0300-483X(02)00279-2

1969 Terrien, X., Fini, J.B., Demeneix, B.A., Schramm, K.W., Prunet, P., 2011. Generation of fluorescent
1970 zebrafish to study endocrine disruption and potential crosstalk between thyroid hormone and
1971 corticosteroids. Aquat. Toxicol. 105, 13–20. doi:10.1016/j.aquatox.2011.04.007

1972 Thursby, G.B., Heltshe, J., Scott, K.J., 1997. REVISED APPROACH TO TOXICITY TEST
1973 ACCEPTABILITY CRITERIA USING A STATISTICAL PERFORMANCE ASSESSMENT.
1974 Environ. Toxicol. Chem. 16, 1322. doi:10.1897/1551-5028(1997)016<1322:RATTTA>2.3.CO;2

1975 Tijani, J.O., Fatoba, O.O., Petrik, L.F., 2013. A review of pharmaceuticals and endocrine-disrupting
1976 compounds: Sources, effects, removal, and detections. Water. Air. Soil Pollut. 224.
1977 doi:10.1007/s11270-013-1770-3

1978 Tonkes, M., De Graaf, P.J.F., Graansma, J., 1999. Assessment of complex industrial effluents in the
1979 Netherlands using a whole effluent toxicity (or wet) approach, in: Water Science and Technology.
1980 pp. 55–61. doi:10.1016/S0273-1223(99)00253-X

1981 Tulloch, A.I.T., Sutcliffe, P., Naujokaitis-Lewis, I., Tingley, R., Brotons, L., Ferraz, K.M.P.M.B.,
1982 Possingham, H., Guisan, A., Rhodes, J.R., 2016a. Conservation planners tend to ignore improved
1983 accuracy of modelled species distributions to focus on multiple threats and ecological processes.
1984 Biol. Conserv. 199, 157–171. doi:10.1016/j.biocon.2016.04.023

1985 Tulloch, A.I.T., Sutcliffe, P., Naujokaitis-Lewis, I., Tingley, R., Brotons, L., Ferraz, K.M.P.M.B.,
1986 Possingham, H., Guisan, A., Rhodes, J.R., 2016b. Conservation planners tend to ignore improved
1987 accuracy of modelled species distributions to focus on multiple threats and ecological processes.
1988 Biol. Conserv. 199, 157–171. doi:10.1016/j.biocon.2016.04.023

1989 Turkez, H., Arslan, M.E., Ozdemir, O., 2017. Genotoxicity testing: progress and prospects for the next
1990 decade. Expert Opin. Drug Metab. Toxicol. 13, 1089–1098. doi:10.1080/17425255.2017.1375097

1991 Valavanidis, A., Vlahogianni, T., Dassenakis, M., Scoullos, M., 2006. Molecular biomarkers of
1992 oxidative stress in aquatic organisms in relation to toxic environmental pollutants. Ecotoxicol.
1993 Environ. Saf. 64, 178–189. doi:10.1016/j.ecoenv.2005.03.013

1994 van Dam, R.A., Chapman, J.C., 2001. Direct toxicity assessment (DTA) for water quality guidelines in
1995 Australia and New Zealand. Australas. J. Ecotoxicol. 7, 175–198.

1996 van der Oost, R., Sileno, G., Janse, T., Nguyen, M.T., Besselink, H., Brouwer, A., 2017. SIMONI
1997 (Smart Integrated Monitoring) as a novel bioanalytical strategy for water quality assessment: Part
1998 II–field feasibility survey. Environ. Toxicol. Chem. doi:10.1002/etc.3837

1999 van Wijngaarden, R.P.A., Arts, G.H.P., 2018. Is the tier-1 effect assessment for herbicides protective

for aquatic algae and vascular plant communities? Environ. Toxicol. Chem. 37, 175–183. doi:10.1002/etc.3936

Verheyen, G.R., 2017. Testing the Mutagenicity Potential of Chemicals. J. Genet. Genome Res. 4. doi:10.23937/2378-3648/1410029

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

Vicario-Parés, U., Lacave, J.M., Reip, P., Cajaraville, M.P., Orbea, A., 2018. Cellular and molecular responses of adult zebrafish after exposure to CuO nanoparticles or ionic copper. Ecotoxicology 27, 89–101. doi:10.1007/s10646-017-1873-5

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

Vogt, É.L., Model, J.F.A., Vinagre, A.S., 2018. Effects of Organotins on Crustaceans: Update and Perspectives. Front. Endocrinol. (Lausanne). 9, 1552–1562. doi:10.3389/fendo.2018.00065

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

Wang, J., Eldridge, M., Menn, F. min, Dykes, T., Sayler, G., 2015. 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, 2088–2099. doi:10.1007/s10646-015-1556-z

2022 Welshons, W. V., Nagel, S.C., Vom Saal, F.S., 2006. Large effects from small exposures. III.
2023 Endocrine mechanisms mediating effects of bisphenol A at levels of human exposure.
2024 Endocrinology. doi:10.1210/en.2005-1159

2025 Weltens, R., Vanermen, G., Tirez, K., Robbens, J., Deprez, K., Michiels, L., 2012. Screening tests for
2026 hazard classification of complex waste materials - Selection of methods. Waste Manag. 32, 2208–
2027 2217. doi:10.1016/j.wasman.2012.05.013

2028 WHO-UNEP, 2012. State of the Science of Endocrine Disrupting Chemicals 2012. Geneva (CH).

2029 Wild, C.P., 2012. The exposome: From concept to utility. Int. J. Epidemiol. doi:10.1093/ije/dyr236

2030 Windsor, F.M., Ormerod, S.J., Tyler, C.R., 2018. Endocrine disruption in aquatic systems: up-scaling
2031 research to address ecological consequences. Biol. Rev. 93, 626–641. doi:10.1111/brv.12360

2032 Xia, J., Fu, S., Cao, Z., Peng, J., Peng, J., Dai, T., Cheng, L., 2013. Ecotoxicological effects of
2033 waterborne PFOS exposure on swimming performance and energy expenditure in juvenile
2034 goldfish (Carassius auratus). J. Environ. Sci. (China) 25, 1672–1679. doi:10.1016/S1001-
2035 0742(12)60219-8

2036 Xu, H., Zhang, X., Li, H., Li, C., Huo, X.-J., Hou, L.-P., Gong, Z., 2018. Immune response induced by
2037 major environmental pollutants through altering neutrophils in zebrafish larvae. Aquat. Toxicol.
2038 201, 99–108. doi:10.1016/j.aquatox.2018.06.002

2039 Xuereb, B., Bezin, L., Chaumot, A., Budzinski, H., Augagneur, S., Tutundjian, R., Garric, J., Geffard,
2040 O., 2011. Vitellogenin-like gene expression in freshwater amphipod Gammarus fossarum (Koch,
2041 1835): Functional characterization in females and potential for use as an endocrine disruption
2042 biomarker in males. Ecotoxicology 20, 1286–1299. doi:10.1007/s10646-011-0685-2

2043 Yang, Y., Li, B., Ju, F., Zhang, T., 2013. Exploring variation of antibiotic resistance genes in activated

sludge over a four-year period through a metagenomic approach. Environ. Sci. Technol. 47, 10197–10205. doi:10.1021/es4017365

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

Zhang, X.X., Zhang, T., Fang, H.H.P., 2009. Antibiotic resistance genes in water environment. Appl. Microbiol. Biotechnol. doi:10.1007/s00253-008-1829-z

Zhuang, W., Gao, X., 2014. Methods, Mechanisms and Typical Bio-Indicators of Engineered Nanoparticle Ecotoxicology: An Overview. Clean - Soil, Air, Water. doi:10.1002/clen.201200559

Zounkov, R., Odr Š, P.K., Doležˇ Alov, L., Hilscherov, K.R., Marš Lek, B., Bl Ha, L.K., 2007. ECOTOXICITY AND GENOTOXICITY ASSESSMENT OF CYTOSTATIC PHARMACEUTICALS. Environ. Toxicol. Chem. 26, 2208–2214. doi:10.1897/07-137R.1