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CERTIFICAN:

Que el trabajo descrito en la presente memoria, titulado "Emerging Pollutants in Wastewater: Aquatic Toxicity and Ozonation", ha sido realizado bajo nuestra dirección por D. José Benito Carbajo Elena en el Área de Ingeniería Química del Departamento de Química Analítica, Química Física e Ingeniería Química de la Universidad de Alcalá, excepto parte del trabajo experimental recogido en los Capítulos 4 y 6 que ha sido llevado a cabo en el Departamento de Biología y Ciencias Ambientales de la Universidad de Gothenburg en Suecia. Asimismo, autorizan su presentación para que sea defendido como Tesis Doctoral.

Y para que conste y surta los efectos oportunos, firman el presente en Alcalá de Henares a 14 de julio de 2015.

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PhD. Thesis

Emerging Pollutants in Wastewater: Aquatic Toxicity and Ozonation

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SUMMARY

The increasing worldwide contamination of freshwater ecosystems with thousands of chemical compounds is one of the key environmental problems facing humanity. While concentrations of so-called Persistent Organic Pollutants (POPs) and Priority Pollutants are declining, numerous emerging substances, such as pharmaceuticals, personal care and house cleaning products amongst others, are commonly detected in the aquatic environment as complex mixtures. Aquatic toxicity of two mixtures of emerging substances with inherent antimicrobial properties (personal care product preservatives and antibiotics) were assessed on indigenous biological communities of the aquatic compartments (activated sludge microorganisms and natural limnic biofilms) in order to provide ecologically a more realistic data and to improve the knowledge about their environmental risk. The results showed that the preservative mixture (iodopropynyl butylcarbamate, bronopol, diazolidinyl urea, benzalkonium chloride, zinc pyrithione, propylparaben, triclosan and a mixture of methylchloroisothiazolinone and methylisothiazolinone) displayed a potential risk to the microorganisms present in an STP aeration tank and consequently, to the process performance of activated sludge. Among them, benzalkonium chloride is the most problematic of the studied preservatives as is the risk driver of the mixture. The result from a screening level risk assessment of antibiotics (doxycycline, erythromycin, ofloxacin, sulfamethoxazole and trimethoprim) towards bacterial periphytic communities showed potential risk to the aquatic ecosystem for the mixture under a Spanish STP scenario and for single ofloxacin, the risk-driver of the mixture toxicity.

Emerging pollutants enter the aquatic environment mainly through conventional STP, where most of them are not efficiently removed. One way of minimizing the input of these micropollutants into surface waters is to integrate an additional treatment step at STPs such as ozonation. In the present study, continuous ozonation is shown as a suitable technology for upgrading conventional STPs both as a pre-treatment stage and a polishing step of activated sludge process. Ozonation effectively removed compounds that pose environmental risk (benzalkonium chloride, ofloxacin and six-antibiotic

mixture) in synthetic water and real wastewater as a result of the combined attack of molecular ozone and hydroxyl radicals, being the optimum ozone dose strongly water matrix-dependent. Ozonation did not lead to a complete mineralization of the organic compounds with the consequent accumulation of transformation products (TPs), which were identified using mass spectrometry coupled to liquid chromatography (LC-ESI-MS(TOF), LC-ESI-MS(QTOF)). The further oxidation of TPs gave rise to low molecular weight by-products such as carboxylic acids. These ozone refractory compounds are easily assimilable and consequently, constitute a special concern for the proliferation of microbes downstream of an ozonated-wastewater discharge point. The current study demonstrated that copper-catalysed continuous ozonation, in both synthetic and real wastewater, significantly improves organic acid mineralization, which is mainly due to its high performance in oxalic acid depletion. Nonetheless, the water matrix has a notable influence on the optimum catalyst dose necessary to achieve a given degree of mineralization.

In addition to the chemical analysis, aquatic toxicity of ozone treated waters should also be taken into account to assess ozonation in a comprehensive manner. Ecotoxicity assessment was conducted combining different levels of biological complexity with the aim of providing an accurate indication of the toxic effects of ozonated wastewaters on exposure biological systems: bioassay batteries of single species belonging to different trophic levels (the bacteria *Vibrio fischeri* and *Pseudomonas putida*, the protozoan *Tetrahymena thermophila*, the alga *Pseudokirchneriella subcapitata* and the crustacean *Daphnia magna*) and indigenous biological communities (microorganisms from an STP aeration tank and natural limnic biofilms). The results indicated that during ozonation, the aquatic toxicity of wastewater decreases in proportion to the disappearance of the studied emerging pollutants. It can be assumed that toxicity is dominated by the parent compounds, and the TPs were not relevant for the aquatic hazard assessment. However, the degradation of emerging substances that interacts with nanoparticles, such as benzalkonium chloride, caused an increase in toxic-metal leaching from the nanomaterial and consequently, led to a toxicity enhancement of treated wastewater.

RESUMEN

El incremento de la contaminación en los ecosistemas acuáticos de todo el mundo, con miles de compuestos químicos, es uno de los mayores problemas ambientales a los que se enfrenta la humanidad. Mientras que la concentración de los llamados contaminantes orgánicos persistentes (COPs) y los contaminantes prioritarios está disminuyendo, numerosos compuestos emergentes tales como medicamentos, productos de cuidado personal y limpieza entre otros, son habitualmente detectados en el medio acuático como mezclas complejas. La toxicidad acuática de dos mezclas de contaminantes emergentes con inherentes propiedades antimicrobianas (conservantes de productos de cuidado personal y antibióticos) ha sido evaluada mediante comunidades biológicas autóctonas de los potenciales compartimentos acuáticos receptores (fango activo y bio-películas bentónicas de aguas continentales). El objetivo es proporcionar datos más realistas desde un punto de vista ecológico e incrementar el conocimiento sobre los riesgos ambientales que pueden presentar. Los resultados mostraron que la mezcla de conservantes (butilcarbamato de iodopropinilo, bronopol, diazolidinil urea, cloruro de benzalconio, piritionato de cinc, propilparabeno, triclosan y una mezcla comercial de metilcloroisotiazolinona y metilisotiazolinona) presenta un riesgo para los microorganismos presentes en un tanque de aireación de una estación de depuración de aguas residuales (EDAR) y por lo tanto, para el correcto funcionamiento del proceso de fango activo. Entre todos ellos, el cloruro de benzalconio es el conservante más peligroso al ser considerado el principal causante del riesgo que presenta la toxicidad de la mezcla. La evaluación de riesgos de los antibióticos (doxiciclina, eritromicina, ofloxacina, sulfametoxazol y trimetoprima) demostró que tanto su mezcla de acuerdo a las concentraciones presentes en una EDAR española como de manera individual ofloxacino (principal responsable de la toxicidad de la mezcla) muestran un potencial peligro sobre la biota presente en los ecosistemas acuáticos continentales.

Los contaminantes emergentes entran en el medio acuático principalmente a través de los efluentes de las EDAR convencionales, en las que muchos de ellos no se

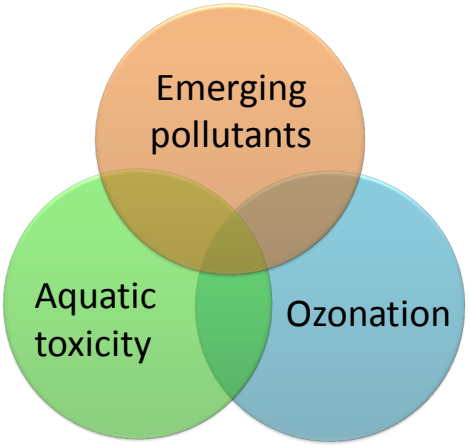
eliminan eficazmente. Una alternativa para solucionar este hecho es integrar una etapa adicional como la ozonización en la línea de flujo de las EDAR. En este estudio, la ozonización en continuo se mostró como una tecnología óptima para mejorar el rendimiento de la degradación de contaminantes emergentes de las EDAR convencionales como pre- o como post-tratamiento del fango activo. La ozonización eliminó eficazmente los micro-contaminantes que presentan riesgos ambientales (cloruro de benzalconio, ofloxacino y la mezcla de los seis antibióticos) como resultado del ataque combinado de la molécula de ozono y los radicales hidroxilos. Degradaciones totales fueron alcanzadas tanto en aguas residuales sintéticas como en reales, si bien, la dosis óptima de ozono estuvo fuertemente influenciada por la matriz del agua. A pesar de ello, la ozonización no logró mineralizar los compuestos orgánicos, con la consiguiente acumulación de productos de transformación, muchos de los cuales fueros identificados mediante cromatografía líquida acoplada a espectrometría de masas (LC-ESI-MS(TOF), LC-ESI-MS(QTOF)). La mayor oxidación de estos productos de transformación dio lugar a sub-productos de reacción de bajo peso molecular como ácidos carboxílicos. Estos compuestos refractarios a la ozonización son fácilmente asimilables por lo que constituyen una especial preocupación por la proliferación microbiana aguas abajo del punto de descarga de las aguas ozonizadas. El presente estudio demostró que la ozonización catalítica en continuo basada en cobre, mejora significativamente la mineralización de los ácidos orgánicos debido a su alto rendimiento en la degradación de oxalato. A pesar de que este hecho tiene lugar tanto en agua residual sintética como real, la dosis óptima del catalizador para alcanzar un determinado grado de mineralización estuvo fuertemente influenciada por el tipo de matriz.

Se ha tenido en cuenta también la toxicidad acuática de las aguas tratadas, con el objetivo de optimizar el tratamiento de ozonización. La evaluación de la toxicidad se llevó a cabo con organismos pertenecientes a diferentes niveles de complejidad biológica, con el fin de proporcionar una indicación precisa sobre los efectos causados en los sistemas biológicos expuestos: baterías de bioensayos uni-especie que forman parte de diferentes niveles de la cadena trófica (las bacterias *Vibrio fischeri* y

Pseudomonas putida, el protozoo *Tetrahymena thermophila*, el alga *Pseudokirchneriella subcapitata* y el crustáceo *Daphnia magna*) así como comunidades autóctonas (fango activo y bio-películas bentónicas de aguas continentales). Los resultados indicaron que durante la ozonización, el potencial tóxico de las aguas residuales causado por los compuestos emergentes disminuye en proporción a la disminución de su concentración. Puede ser asumido entonces que la toxicidad de las aguas ozonizadas está dominada por los compuestos iniciales, y que, en estos casos, los productos de transformación no han sido relevantes para la evaluación de los riesgos sobre el medio acuático. Sin embargo, la degradación de contaminantes emergentes que interaccionan con nanomateriales, como cloruro de benzalcónico, causó un aumento en los lixiviados de metales tóxicos procedentes de la nanopartícula, provocando de manera indirecta un aumento de la toxicidad de las aguas residuales tratadas.

Chapter 1

Introduction



INTRODUCTION

1.1. Background

1.1.1. Chemical pollution: Emerging pollutants

Thousands of chemicals play an important role in our daily activities (Fig. 1.1). They allow new technologies to be developed and help us to maintain our health and improve our quality of life. As a result of their widespread use, these substances enter the natural environment which causes the increasing worldwide contamination of aquatic ecosystems with thousands of industrial and everyday chemicals (Schwarzenbach *et al.*, 2006). As the global population increases and economies in many regions grow considerably (UNESCO, 2014), production of chemicals is also predicted to increase. Currently, more than 70 new chemicals are registered every hour by the American Chemical Society (McKinney *et al.*, 2012), many of which may get transported into water bodies at some stage in their lifecycle (Eggen *et al.*, 2014).

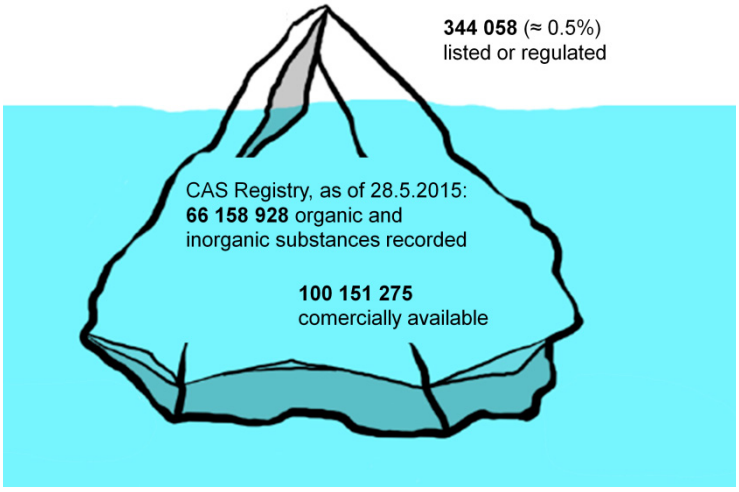


Fig. 1.1 The roughly 350 000 substances listed or regulated are only the tip of the iceberg. CAS: Chemical Abstracts Service.

For this transfer of chemicals to water bodies, several routes need to be considered (Fig. 1.2). In developed countries with existing sewer systems, wastewater from households and industry is a major source of chemicals entering the aquatic environment despite the treatment processes taking place in the sewage treatment

plants (STPs). Pharmaceuticals, personal care products, biocides, industrial chemicals and/or detergents are found most often in municipal STP effluents (Kasprzyk-Hordern *et al.*, 2009). However, chemicals do not only enter surface water via point sources, such as STPs, but rather via diverse diffuse entry paths. Such entries stem from agricultural fields, traffic lanes or infrastructure issues because of rain, and consequently pollute surface waters with compounds such as plant protection products and biocides (Metz and Ingold, 2014).

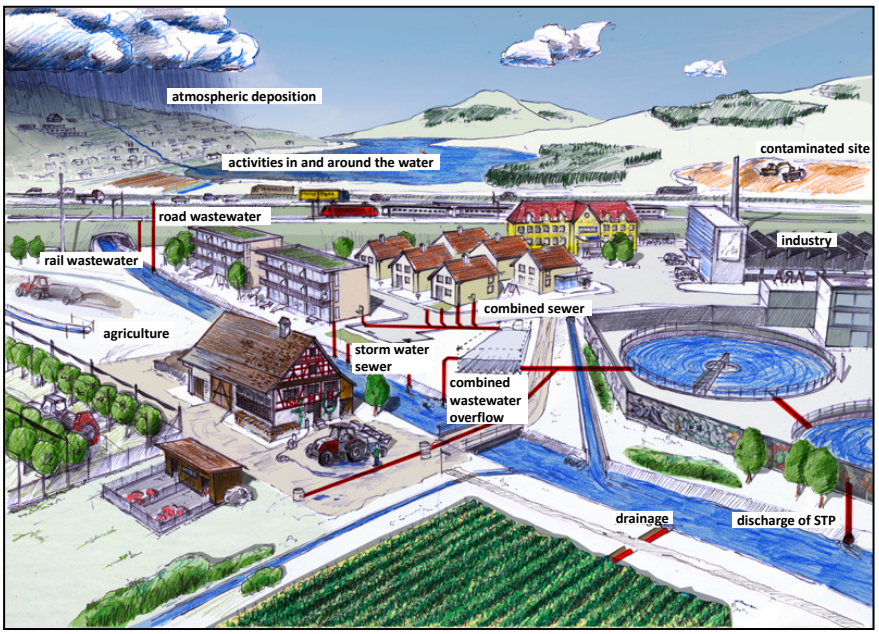


Fig. 1.2 Sources and routes of micropollutants in the aquatic environment. Red line represents point source entry paths. Adapted with permission from Eggen *et al.*, 2014. Copyright (2015) American Chemical Society.

As consequence of these varied sources and pathways, numerous compounds can be detected, mostly at trace concentrations in the $\mu g \cdot L^{-1}$ to $ng \cdot L^{-1}$ range, in freshwaters (Segura *et al.*, 2009, Loss *et al.*, 2009 and Herrera-López *et al.*, 2014) particularly in densely populated regions (Martínez-Bueno *et al.*, 2010 and Heeb *et al.*, 2012). Even at low concentrations, micropollutants can have adverse effects on aquatic life or affect drinking water resources (Eggen *et al.*, 2014). The widespread knowledge of these facts inevitably means that micropollutants are increasingly becoming a target for regulation.

At international level, the Stockholm Convention on Persistent Organic Pollutants (POPs), promoted by the United Nations Environmental Programme (UNEP), aims to reduce POPs worldwide by listing substances related to its persistence, bioaccumulation, potential for long-range environmental transport and toxicity. The initial list of 12 POPs has been amended in the last number of years, incorporating 11 new chemicals along with proposing new candidates for further research. Currently, it covers a series of pesticides, industrial chemicals and unintentionally produced chemicals such as polychlorinated dibenzo-*p*-dioxins and polychlorinated dibenzofurans (Stockholm Convention, 2015).

Considering environmental perspective with the aquatic environment focus, the big upturn was made in the European Union (EU) by establishing the Water Framework Directive 2000/60/EC, which aims to achieve a good ecological and chemical status of European water bodies and to prevent their further deterioration. In 2008, a list of 33 priority substances was established at Union level by the Directive 2008/105/EC. Environmental quality standards (EQS) were defined for these priority substances and for another 8 pollutants regulated by previous legislation, these being expressed as an annual average value and/or maximum allowable concentrations (Directive 2008/105/EC). Moreover, this list is reviewed and updated every 4 years and so recently it launched the Directive 2013/39/EU that updates the water framework policy. The Directive 2013/39/EU includes 12 new priority substances, three compounds included in the recommendation for the first watch list of substances as well as EQS for newly identified substances and revised EQS for substances already identified. Nowadays, the list of priority substances mainly covers pesticides, polyaromatic hydrocarbon, industrial compounds, solvents and metals. Nevertheless, there is a group of substances currently not included in routine monitoring programs at EU level, despite their presence in the environment, and they are obvious candidates for future regulations as long as research proves their toxic effects and/or their widespread occurrence. These substances compose a class of micropollutants denominated as emerging pollutants (Slobodnik and Dulio, 2008), which include pharmaceuticals and personal care products, surfactants, and biocides, amongst others.

1.1.2. Aquatic toxicity assessment

The scientific community has been worried about the fate and toxicity of emerging substances because they are pseudo-persistent compounds (*i.e.*, their high transformation/removal rates are compensated by their continuous entry into environment), multiple stressors (*i.e.*, these occur in mixture) and the fact that many of them (such as biocides or pharmaceuticals) are biologically active compounds (Daugthon, 2002 and Barceló and Petrović, 2007). Therefore, evaluation of the toxicity of these potentially harmful compounds is crucial in hazard assessment. In spite the fact that establishing cause-ecotoxicological effect relationships of these micropollutants in the environment is extremely challenging, there are some studies reporting worrisome number of environmental impacts. Ramirez *et al*. (2009) describe the accumulation of pharmaceuticals and personal care products such as galaxolide, tonalide, diphenhydramine, fluoxentine or carbamazepine in fish residing in STP effluent-dominated water bodies. This fact suggests a special human health concern as a consequence of the potential for bioaccumulation and biomagnifications of these micropollutants through the food chain. Emerging pollutants can also generate endocrine disruption by disturbing reproduction, stimulating hormones or causing feminisation in fish, among others (Bolong *et al.*, 2009). For instance, Alsev *et al.* (2005) demonstrated that the preservative butylparaben when tested on juvenile rainbow trout shows an estrogenic activity. Other scientists have described that antidepressants like fluoxentine may disrupt frog maturation (Foster *et al.*, 2010). However, one of the well-known effects in the aquatic environment is antibiotic resistance (Marti and Balcázar, 2013). Antibiotic resistance has become an increasing concern with reports of high antibiotic resistance frequencies and detection of antibiotic resistance genes in aquatic environment, especially in STP effluent (Schwartz *et al.*, 2003, Rizzo *et al.*, 2014 and Rodríguez-Mozaz *et al.*, 2015). In fact, antibiotic resistance is one of the most critical human healthcare challenges, as the selection of resistance strains eventually compromises the effectiveness of antimicrobial therapy (Rodríguez-Rojas *et al.*, 2012 and Ashbolt *et al.*, 2013) and influences the ecological function of water ecosystem (Bouki *et al.*, 2013).

Although in specific cases even single emerging substances have been proven to cause environmental harm, several reviews have concluded that clear toxic effects of studied micropollutants are only to be expected at concentrations above environmentally realistic levels (Santos *et al.*, 2010, Brausch and Rand, 2011, Brausch *et al.*, 2012 and Vasquez *et al.*, 2014). Nonetheless, most of the available studies have been based on laboratory exposures that estimate the toxicity of a single compound on a single species by measuring the response as physiological or population-based parameters (Geiszinger *et al.*, 2009).

Single-species tests are effective tools that facilitate reproducible toxicity testing with high precision and throughput, but they cannot reflect the interactions between species (Geiszinger *et al.*, 2009). Such interactions can only be included in toxicity estimates when biological communities composed of many different species are used. The use of natural microbial communities, directly collected from the water body of concern and exposed to emerging substances under controlled conditions, has improved the ecological relevance of laboratory toxicity tests (Sabater *et al.*, 2007 and Proia *et al.*, 2013). On the other hand, aquatic ecosystems are exposed to various multi-component mixtures, whose joint toxicity is typically higher than each its component alone (Kortenkamp *et al.*, 2009). The focus on a substance-by-substance assessment in most studies for aquatic toxicity evaluation therefore runs the risk of underestimating the actual toxic pressure that an ecosystem is exposed to. And even though the concentrations of individual pollutants might be low, combined effects have been shown to occur even when the compounds are present in concentrations below their respective toxicity threshold (Backhaus *et al.*, 2000a,b and Fent *et al.*, 2006) (Fig. 1.3).

Mixture toxicity assessments can either be retrospective or prospective, *i.e.* either the hazard of a current exposure situation is determined or the effect of an expected exposure is predicted. Different approaches are employed depending on the aims of the study, commonly there are divided into whole mixture or component-based approaches (Backhaus *et al.*, 2008). Whole mixture approach directly tests the mixture of interest in order to provide an experimental estimation of its level of hazard. Since it would be an endless task to experimentally determine the toxicity of all relevant

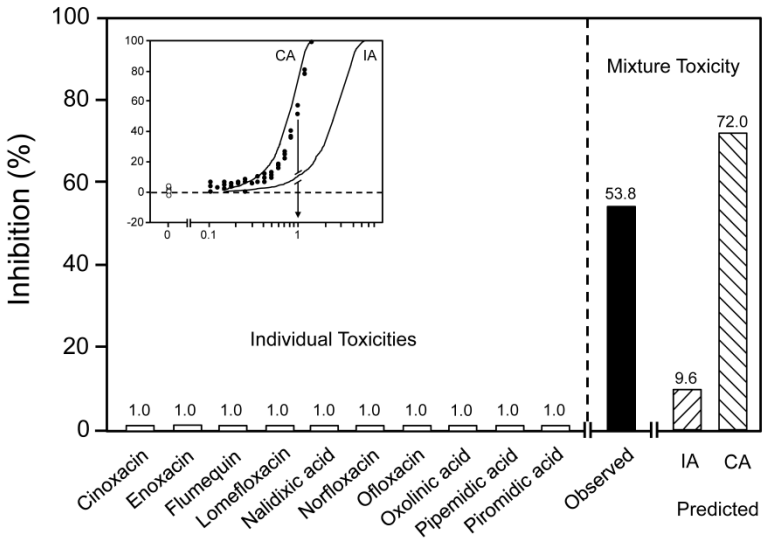


Fig. 1.3 Observed and predicted mixture toxicity of a ten-component mixture of quinolone antibiotics in a chronic bioluminescence-inhibition assay with the bacterium *Vibrio fischeri*. Whole mixture approach is represented by observed mixture and component-based approach is represented by predicted mixture using the concepts of Concentration Action (CA) and Independent Action (IA). Adopted with permission from Backhaus *et al.* 2000a. Copyright (2015) Elsevier.

mixtures, predictive approaches have been proposed instead. The mathematical concepts of Concentration Addition (CA) and Independent Action (IA) predict the toxicity of a mixture based on the individual toxicities of the mixture components (Backhaus *et al.*, 2003, Altenburger *et al.*, 2004 and Kortenkamp *et al.*, 2009), describing two mutually exclusive reference situations of additivity. CA is based on the assumption that all components in the mixture behave as if they are simple dilutions of one another (Loewe and Muischnek, 1926 and Loewe, 1953), which is often taken to means that CA describes the joint action of compounds with an identical mechanism of action (Backhaus *et al.*, 2000a and Fent *et al.*, 2006). In contrast to CA, the alternative concept of IA assumes that the resulting combined effect can be calculated from the effects caused by the individual mixture components (Bliss, 1939), which is often taken to mean that IA describes the joint action of compounds with a dissimilar mechanism of action (Backhaus *et al.*, 2000b and Faust *et al.*, 2003). Table 1.1 summarizes the principal features and equations of CA and IA models, together with other commonly used models to analyze mixture toxicity in ecotoxicology (Fernández-Piñas *et al.*, 2014). It is important to point out that a prerequisite for the predictive concepts to be valid is that

the mixture components do not interact. The consequence of such interactions can either be an increase in toxicity (usually referred to as synergism) or a decrease (antagonism). Although real mixtures cannot be expected to be composed of either only similarly or dissimilarly acting compounds, reviews of data have shown that the toxicity of mixtures can usually be predicted by the CA model and that antagonistic and synergistic interactions only occur in a few cases (Belden *et al.*, 2007, Kortenkamp *et al.*, 2009, Cedergreen, 2014 and Backhaus, 2014).

Table 1.1 Mainly used models to analyze mixture toxicity in ecotoxicology (based on Fernández-Piñas *et al.*, 2014).

Model	Additivity definition	Generalized equation	References
CA[a]	Loewe	$\sum_{i=1}^{n} \frac{c_i}{EC_{x_i}} = 1$ $EC_{x_{mix}} = \left(\sum_{i=1}^{n} \frac{p_i}{EC_{x_i}}\right)^{-1}$	Backhaus *et al.* (2000a) Fent *et al.* (2006)
CI[b]	Loewe	$\sum_{i=1}^{n} \frac{D_i}{D_{x_i}} = CI_x$ $EC_{x_{mix}} = \left(\sum_{i=1}^{n} \frac{p_i}{EC_{x_i} \cdot CI_x}\right)^{-1}$	Chou (2006) González-Pleiter *et al.* (2013)
TUs[c]	Loewe	$TU_{mix} = \sum_{i=1}^{n} TU_i$	Junghans *et al.* (2006) Backhaus and Karlsson (2014)
IA[d]	Bliss	$E(c_{mix}) = 1 - \prod_{i=1}^{n} [1 - E(c_i)]$	Backhaus *et al.* (2000b) Faust *et al.* (2003)

[a] Concentration Addition. In the generalized equation c_i is the concentration of the ith component in a mixture that is expected to cause x% effect, and EC_{x_i} gives the concentration at which the compound i alone causes the same x% effect. From a mathematical perspective CA hence simply represents the weighted harmonic mean of the individual EC_{x_i} values, with the weights just being the fraction p_i of the mixture components.

[b] Combination Index. In the generalized equation D_i is the dose [concentration] of compound i in a mixture that is expected to cause x% effect and D_{x_i} gives the dose [concentration] at which the compound i alone causes the same x% effect. CI is the Combination Index, where CI <1 indicates synergism, CI = 1 indicates additivity, and CI >1 indicates antagonism. As can be seen CI model is equivalent to CA model, in fact a simplification of the CI model where CI is fixed as CI = 1 (additivity).

[c] Toxic Units. In the generalized equation $TU_i = (c_i/EC_{x_i})$. This approximation is equivalent to that of CA.

[d] Independent Action. In the generalized equation $E(c_i)$ is the effect of compound i if applied alone at concentration c_i, the concentration at which it is present in the mixture.

1.1.3. Environmental risk assessment

The assessment of whether or not a particular emerging substance poses a potential environmental risk is performed by comparing the predicted (PEC) or measured environmental concentration (MEC) with the predicted no effect concentration ($PNEC$), which is derived from effect data ($NOEC$ and/or EC_{50}) obtained in the most sensitive test available (van Leeuwen and Vermeire, 2007). As a result of the knowledge on mixture toxicity obtained from studies over the years (Altenburger and Greco, 2009, Kortenkamp *et al.*, 2009, ECETOC, 2011 and SCHER, 2011), a conceptual framework for the environmental risk assessment of chemical mixtures has been proposed by Backhaus and Faust (2012). The approach is based on an approximation of the CA concept. In the first step, a risk quotient is based on the sum of (PEC or MEC)/ $PNEC$ ratios of the detected compounds ($RQ_{MEC/PNEC}$), as was suggested by Calamari and Vighi (1992). $RQ_{MEC/PNEC}$ is a pragmatic first approach as existing $PNEC$ values, which have already undergone regulatory assessment, can be used directly, without the need to go back to the underlying studies with the various organisms (Peterssen *et al.*, 2015). The shortcoming of this approach is that $PNECs$ for the different compounds might be derived by different taxa. Despite the fact that $RQ_{MEC/PNEC}$ violates a fundamental assumption of CA (*i.e.*, all individual toxicity data refer to the same biological endpoint and organism), it might serve as a justifiable first-tier approximation of a conceptually more sound CA-based mixture toxicity assessment (Backhaus and Faust, 2012). If the resulting $RQ_{MEC/PNEC}$ is equal to or above 1, there is considered to be a potential risk and the mixture toxicity should then be assessed separately for each taxa or species group. In a second step, sum of toxic units for each organism group ($STU = \sum(MEC/EC_x)$) results in taxa-specific risk quotients. Following the standard environmental risk assessment practice the overall risk for the environment is then based on the most sensitive taxa, termed RQ_{STU} ($RQ_{STU} = STU \cdot AF$, in which AF is the assessment factor) (Backhaus and Faust, 2012). As a risk to the environment is indicated if the RQ_{STU} is equal or above 1, the assignment of an appropriate AF may be crucial for the final assessment of cumulative risk (Petersen *et al.*, 2015).

Despite the presence of hundreds of emerging substances in environmental mixtures, experimental findings suggest that the overall risk may often be governed by just a few compounds (Price *et al.*, 2012 and Backhaus and Karlsson, 2014). The EU therefore considers the development of methodologies for the identification of such "drivers of mixture toxicity" as a research priority (European Commission, 2012). Backhaus and Karlsson (2014) have proposed the analysis of the toxic unit distribution of the compounds in a mixture as a tool to prioritise and rank the ecotoxicological importance of emerging substances in a complex mixture. Fig. 1.4 shows the results of RQ_{STU} distribution of emerging substances detected in the STP effluent of Ryaverket (Gothenburg, Sweden) (Backhaus and Karlsson, 2014). It can be clearly seen that ofloxacin alone is responsible for more than 50% of the expectable joint toxicity towards algae and that the first five compounds, four of them antibiotics, explain more than 90% of RQ_{STU}. The contribution of more than half the detected emerging substances is negligible for all intents and purposes, even under the assumption of concentration-additive mixture behaviour. The "top n" approach is useful for subsequent steps of the assessment process, especially if appropriate risk management or mitigation measures need to be identified (Backhaus and Faust, 2012 and Altenburger *et al.*, 2015).

Finally, it is important to take into account that, in order to ensure adequate protection of the whole aquatic ecosystem, the validity of the additivity principle should be confirmed for levels of biological organisation higher than populations, such as communities (SCHER, 2011). Ecotoxicological risk assessment is routinely conducted with data from single-species tests using organisms from major trophic levels. It is assumed that by protecting the most sensitive trophic level all other organism groups are protected as well and that protecting the structure of an ecosystem also protects ecosystem functions (van Leeuwen and Vermeire, 2007). Different considerations must be made for the effects at community level that depend on the complex interactions amongst different populations and can hardly be predicted only on the basis of single-species tests as it has been described above. Risk assessment analysis of emerging substance mixtures should be extended from single species testing to higher levels of biological complexity, such as communities, in order to provide ecologically more

realistic data as well as to fill gaps in knowledge about their level of environmental hazard.

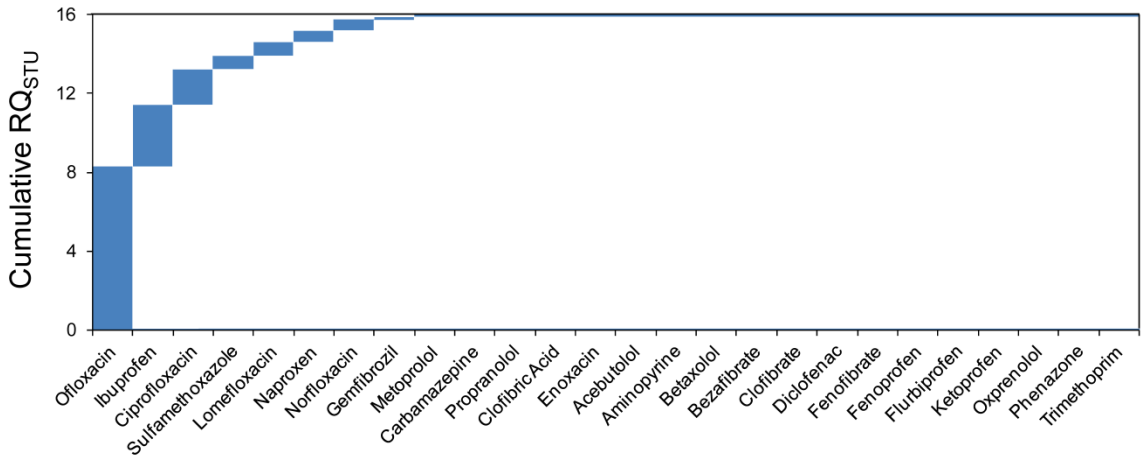


Fig. 1.4 Distribution of Risk Quotients based on the sum of toxic units (RQ_{STU}) for algal toxicity in a mixture of emerging pollutants (n = 25) monitored in Ryaverket STP effluent (Gothenburg, Sweden) For further details see Backhaus and Karlsson (2014).

1.1.4. Removal of emerging substances in conventional STPs

The presence of emerging pollutants in the aquatic environment and by consequence, their related environmental risk is mainly a consequence of their limited removal in conventional STPs (Schwarzenbach *et al.*, 2006). Existing treatment plants were not designed to eliminate substances of this kind, but to reduce the input of solids, organic matter and nutrients. Despite the fact that there are substantial differences in the technologies used for wastewater treatment and in the level of treatment achieved in different countries and even within a single country, wastewater treatment has a common set of objectives (Eggen *et al.*, 2014):

- to improve the water quality within the receiving water (*i.e.*, removing degradable organic compounds in order to minimize oxygen depletion in receiving waters),
- to remove the nutrients nitrogen and phosphorus that are responsible for the eutrophication of aquatic ecosystems and
- to improve the hygienic conditions of receiving waters by functioning as a barrier for fecal bacteria and pathogens.

By achieving these objectives, STPs have been adapted in a step-up-step approach in response to tightening of the discharge quality regulations such as Directive 91/271/EEC. Fig. 1.5 describes these changes by showing the most common types of activated sludge (Ternes *et al.*, 2004). As a result, conventional treatment plants are protective of recreational and bathing water, tackle major threats to aquatic biodiversity and to ecosystem function by preventing for example oxygen depletion, and, furthermore, reduce the requirements for drinking water treatment when water supply intakes are downstream of STPs (Eggen *et al.*, 2014). Nevertheless, the increasing use of chemicals along with growing populations and increasing urbanization pose new challenges to wastewater treatment that are not able to be remedied by conventional STPs.

Indeed, raw STP influents are usually a mixture of domestic and industrial discharges, in which pollutants subject to be removed include not only organic compounds such as lipids, proteins, and carbohydrates, which occur at order of

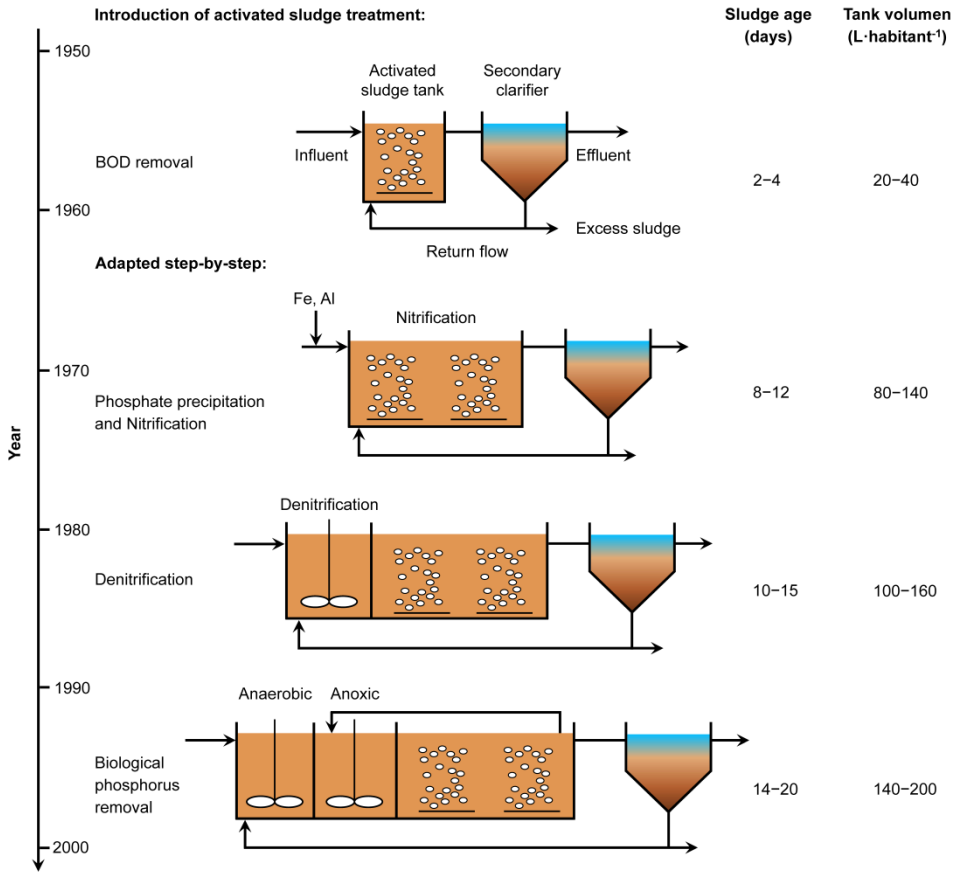


Fig. 1.5 Schematic presentation of historical development of activated sludge treatment in Europe. Adopted with permission from Ternes *et al.* 2004. Copyright (2015) American Chemical Society.

mg·L^{-1}, but also micropollutants, which occur at a concentration in the range of 0.001–100 µg·L^{-1} (Verlicchi *et al.*, 2012). In spite of this, observed removal efficiency of emerging pollutants vary widely for the different compounds, as well as for the same substance, due both to the different chemical and physical characteristics of contaminants and to the operational conditions of the STP (Buttiglieri and Knepper, 2008 and Verlicchi *et al.*, 2013); approximately half of micropollutants load is eliminated either by sorption to sludge or by degradation (Lou *et al.*, 2014). In fact, Rosal *et al.* (2010) showed that the removal of emerging pollutants in STP correlates well with their hydrophobicity when measured in terms of logarithm of apparent octanol-water partition coefficient (Fig. 1.6).

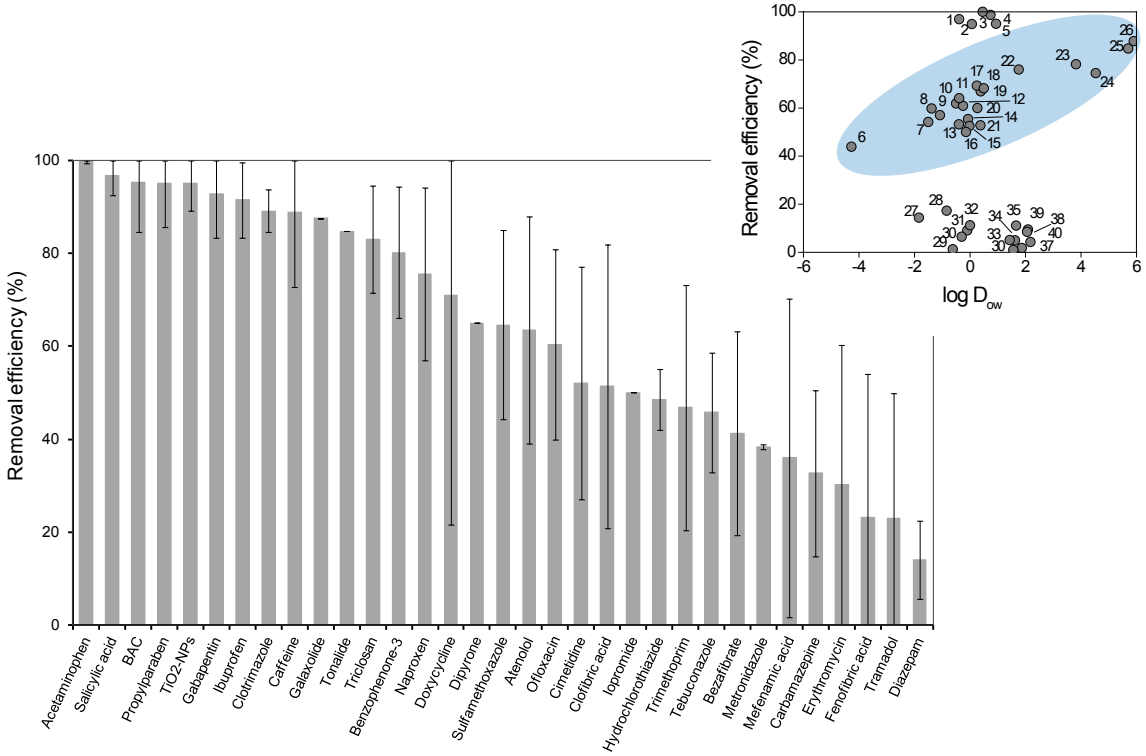


Fig. 1.6 Mean removal efficiencies from the liquid phase for the selected compounds in conventional STPs. Main plot: Error bars represent the standard deviations of the data. Inset plot: (1) paraxanthine, (2) caffeine, (3) acetaminophen, (4) nicotine, (5) ibuprofen, (6) ketorolac, (7) clofibric acid, (8) furosemide, (9) ciprofloxacin, (10) fluoxethine, (11) ofloxacin, (12) naproxen, (13) hydrochlorothiazide, (14) 4-amino-antipyrine, (15) metronidazole, (16) N-acetyl-4-amino-antipiryne, (17) codeine, (18) N-formyl-4-amino-antipiryne, (19) 4-methylaminoantipyrine, (20) ranitidine, (21) antipyrine, (22) gemfibrozil, (23) benzophenone-3, (24) triclosan, (25) tonalide, (26) galaxolide, (27) atenolol, (28) sulfamethoxazole, (29) fenofibric acid, (30) metoprolol, (31) bezafibrate, (32) ketoprofen, (33) trimethoprim, (34) diclofenac, (35) indomethacine, (36) propanolol, (37) mefenamic acid, (38) omeprazole, (39) carbamazepine, (40) erythromycin. Adopted with permission from Rosal *et al.* 2010. Copyright (2015) Elsevier.

The amount of compounds that are not sorbed to the sludge and persist over the retention time in the plant still remain in the effluent and are continuously discharged to the receiving water bodies. In general, the occurrence of emerging substances in STP effluents were a one to two order of magnitude lower than those in influent, ranging from 0.001 to 1 $\mu g \cdot L^{-1}$ (Clara *et al.*, 2007, Kasprzyk-Hordern *et al.*, 2009, Rosal *et al.*, 2010, Verlicchi *et al.*, 2012, Lou *et al.*, 2014 and Lazareva and Keller, 2014). However, scientific literature has reported that some compounds are discharged at relatively high concentrations. For instance, four analgesics (tramadol, dipyrone, ibuprofen and naproxen), a contrast media (iopromide), a beta-blocker (atenolol), a diuretic (hydrochlorothiazide), a lipid regulator (fenofibric acid), three psychiatric drugs (diazepam, gabapentin and carbamazepine), a receptor antagonistic (cimetidine), a synthetic musk (galaxolide), an antimicrobial agent (triclosan), and a stimulant (caffeine) were detected in the highest average concentrations higher than 1 $\mu g \cdot L^{-1}$ in biologically treated effluent (Deblonde *et al.*, 2011, Verlicchi *et al.*, 2012 and Lou *et al.*, 2014).

1.1.5. Ozonation for upgrading conventional STPs

As a consequence of the fact that the current STPs are unable to act as a reliable barrier towards some emerging substances, discussion has focused on upgrading the overall treatment process with additional treatment steps in order to enhance the removal efficiencies of micropollutants and mitigate the potential risks associated with their continuous release into the environment (Jones *et al.*, 2007 and Joss *et al.*, 2008). Several advanced treatment technologies have been evaluated for this purpose in recent years, including membrane filtration such as nanofiltration and reverse osmosis (Snyder *et al.*, 2007 and Yoon *et al.*, 2007), powdered activated carbon adsorption (PAC) (Westerhoff *et al.*, 2005 and Nowotny *et al.*, 2007) and chemical oxidation using ozone or advanced oxidation processes (Huber *et al.*, 2005, Kim *et al.*, 2007, Esplugas *et al.*, 2007 and Zimmerman *et al.*, 2011). Nowadays, two main technologies with the potential for large-scale applications in terms of efficiency, cost and energy requirements have been identified: adsorption of micropollutant onto PAC or oxidation with ozone (Table 1.2) (Joss *et al.*, 2008 and Margot *et al.*, 2013).

Table 1.2 Cost and energy needs for construction and operation of wastewater tertiary treatments (including post sand filtration) for an average removal of 80% of 65 micropollutants according to Margot *et al.*, 2013.

Process		Ozonation	PAC
Dosage		5.7 mg $O_3 \cdot L^{-1}$	15 mg PAC$\cdot L^{-1}$
Capacity (average flow)	[$L \cdot s^{-1}$]	60	15
Electricity consumption	[$KWh \cdot m^{-3}$]	0.117	0.080
Operating costs	[€$\cdot m^{-3}$]	0.043	0.054
Investment costs	[€$\cdot m^{-3}$]	0.133	0.107
Total costs (excluding VAT)	[€$\cdot m^{-3}$]	0.176	0.161

PAC allows removal via adsorption to its high specific surface area for a broad spectrum of micropollutants, especially hydrophobic and positively charged compounds whereas, highly polar contaminants need higher doses or can only be removed partly (Snyder *et al.*, 2007, Nowonty *et al.*, 2007 and Margot *et al.*, 2013). Moreover, as the organic matter present in wastewater can compete for adsorption sites, larger amounts of activated carbon are required (Bolong *et al.*, 2009 and Margot *et al.*, 2013). On the other hand, ozonation is considered to be an attractive technology because it has been demonstrated to be efficient for the degradation of a broad range of micropollutants at a rate of over 80%, many of which have potential environmental and public health risks (Huber *et al.*, 2005, Hollender *et al.*, 2009, Zimmerman *et al.*, 2011 and Michael *et al.*, 2013a). A further advantage of the ozonation is the disinfection potential, which is able to deactivate antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs), preventing the dissemination of antibiotic resistance (Dodd, 2012).

Nonetheless, the integration of the ozonation process as an additional treatment step at conventional STPs cannot only be performed as tertiary treatment with the aim of degrading biological persistent compounds from secondary effluent. As shown in Fig. 1.7, there are two potential points for applying ozonation process to upgrade conventional STPs in order to enhance the removal efficiencies of emerging pollutants: pre- and post-biological treatment (Ikehata *et al.*, 2006 and Ried *et al.*, 2009). The predominant aim of ozonation pre-treatment stage is the partial oxidation of toxic and/or non-biodegradable emerging pollutants in order to be able to then use the conventional biological treatment process for the reduction of biodegradable oxidation products (Oller *et al.*, 2011 and Guieysse and Norvill, 2014).

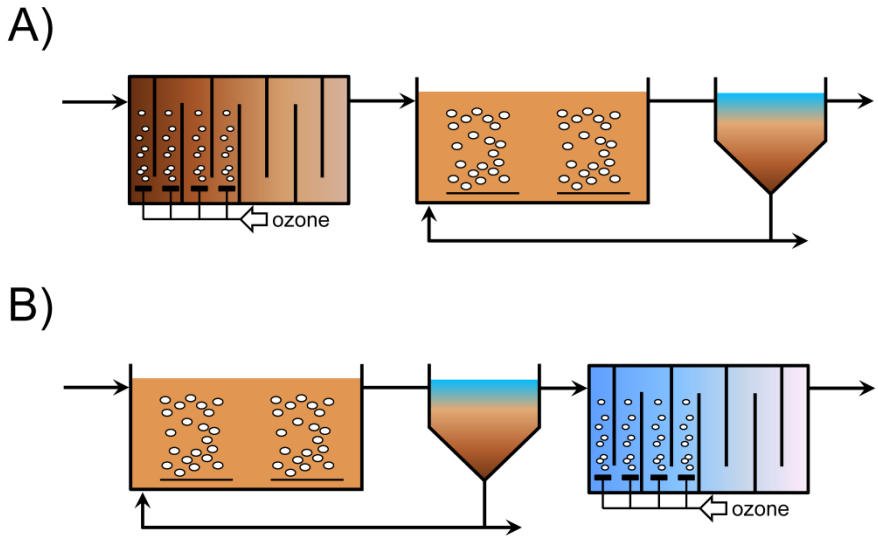


Fig. 1.7 Possible points for applying ozonation in a conventional STP in order to increase removal efficiencies of emerging substances. Ozonation as pre-treatment stage (A) and polishing step (B) of activated sludge process.

During ozonation process, organic compounds either directly react with ozone in specific reactions, or they are decomposed by hydroxyl radical-mediated reactions. The ozone molecule, consisting of three oxygen atoms, exists as a hybrid of four possible resonance structures (Fig. 1.8), which is attributed its high reactivity (Beltrán, 2004). Ozone reacts readily with electron-rich functional groups, such as olefins, aliphatic amines, activated aromatic systems and sulfur-containing compounds by electrophilic addition reactions leading to mainly oxygen atom transfer and electron transfer (Hübner *et al.*, 2015), but not towards aromatic rings with amide or carboxyl groups (Demeestere *et al.*, 2014). Due to its selectivity second-order rate constants for the reaction of organic compounds with ozone cover a range of more than nine orders of magnitude (von Sonntag and von Gunten, 2012). In aqueous solution, the ozone molecule is unstable, and decomposes into secondary oxidant species such as hydroxyl radicals through several initiation reactions via an autocatalytic chain reaction (Beltrán, 2004). Hydroxyl radical is the strongest oxidant that can be applied in water and in contrast to ozone, it acts less selectively. Second-order rate constants for hydroxyl radicals are hence generally higher and cover a range of only about three orders of magnitude (von Sonntag and von Gunten, 2012). The decomposition of ozone in wastewater is enhanced compared to its rate in pure water as a consequence of the direct reactions of ozone

with specific reactive moieties of dissolved organic matter (DOM) (Buffle *et al.*, 2006). An increasing ozone decomposition rate was observed with an increasing pH level (von Sonntag and von Gunten, 2012).

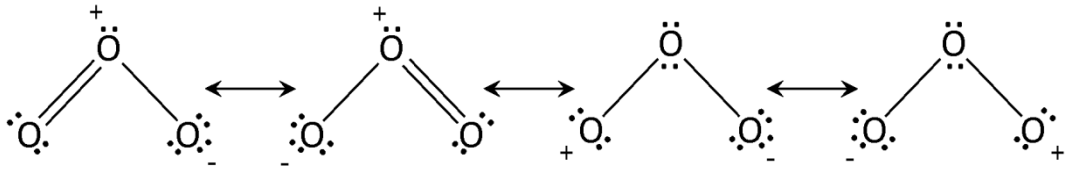


Fig. 1.8 Hybrid resonance structures for the molecule of ozone in aqueous solution.

Altogether, the efficiency of an ozonation process with regard to emerging pollutant oxidation widely depends on several issues. These are the ozone dose, the ozone stability, the hydroxyl radical yield and the second-order rate constants for the reaction of target compounds with ozone and hydroxyl radicals (Nöthe *et al.*, 2009, Katsoyiannis *et al.*, 2011 and Antoniou *et al.*, 2013). Type and concentration of DOM, pH and alkalinity in turn influence the ozone stability considerably. Ozone is more stable at lower DOM concentrations, at lower pH values (protonated DOM slows down ozone decay) and at a higher alkalinity (carbonate is a hydroxyl radical scavenger and inhibits ozone decomposition) (von Sonntag and von Gunten, 2012). The amount of ozone required in the process depends on various parameters and since approximately 70% of the total energy costs in ozone process are for ozone generation (Gottschalk *et al.*, 2010), improving our understanding and level of control over the reaction system should be priority. It is should be meticulously studied in order to optimize ozone usage and consequently, the cost efficiency of the process.

1.1.6. Transformation products and aquatic toxicity

Despite the combined action of molecular ozone and hydroxyl radicals leading to a significant oxidation of emerging pollutants both with and without functional groups reactive towards ozone, the disappearance of the original parent compounds does not imply that the treatment was suitable (Radjenović *et al.*, 2009). Indeed, oxidation does not usually lead to a full mineralization but to the transformation of micropollutants, resulting in the formation of transformation products (TPs) (von Sonntag and von

Gunten, 2012 and Lee *et al.*, 2014). There is a growing concern on whether TPs retains or drop the biological effects of the parent compounds (Lee *et al.*, 2008, Dodd *et al.*, 2009 and Mestankova *et al.*, 2012), or whether or not new and undesired biological effects are developed (Li *et al.*, 2008, Dodd *et al.*, 2010 and Gómez-Ramos, *et al.*, 2011). In addition to the formation of TPs, other organic by-products such as carboxylic acids, aldehydes or ketones are formed from the oxidative breakdown of complex DOM and emerging substances. These compounds are usually readily biodegradable and constitute a considerable fraction of assimilable organic carbon (AOC) (Hammes *et al.*, 2006), which causes a proliferation of microbes. This proliferation then decreases the river water quality downstream from the discharge point of ozonated wastewater (Zimmermman *et al.*, 2011).

Given the limited extent that mineralization of emerging pollutants occurs during ozonation along with the potential environmental hazard of TPs, their identification and quantification, as well as elucidation of their main degradation pathways, are necessary for the safe application of such process for wastewater treatment (Radjenović *et al.*, 2009). The identification of unknown TPs is not an easy task and very often requires the combined use of several analytical techniques and strategies such as mass spectrometry (MS) coupled with either liquid or gas chromatography (LC or GC) (Fatta-Kassinos *et al.*, 2011). The use of LC-MS, combined with a new generation of MS systems, has great advantages for the analysis of polar compounds (Petrović *et al.*, 2010). They allow for a more sensitive analysis and provide abundant structural information for elucidating unknown structures. Triple quadruple (QqQ) or linear ion trap (QqLIT) analysers involve TPs elucidation on the basis of structural information gained in tandem MS/MS experiments, whereas the measurement of an accurate mass and the subsequent determination of the empirical formula provided by time-of-flight (TOF) or quadrupole time-of-flight (QqTOF) instruments are a very valuable information source when assigning structures (Gros *et al.*, 2012). All these techniques have been widely applied to the identification of metabolites and TPs generated by different water treatments (Radjenović *et al.*, 2009, Fatta-Kassinos *et al.*, 2011 and Haddad *et al.*, 2015).

However, specific analytical determination may confront certain limitations; TPs detection is laborious and difficult to accomplish (Aguayo *et al.*, 2004 and Lee *et al.*, 2014) and in addition, potential interaction effects between the components of a mixture (*i.e.*, synergistic or antagonistic) and the bioavailability of the compounds cannot be predicted by the performance of single chemical measurements. Thus, extensive aquatic toxicity evaluations are required to provide a holistic direct estimation of the hazard of a given ozonated wastewater, which is essential for the optimization of ozonation process (Petala *et al.*, 2008 and Escher and Fenner, 2011).

The selection of the appropriate bioassays is crucial for the success of hazard assessment (Fig. 1.9). In many cases toxicity evaluation has been exclusively based on single-species tests, which are performed using a select species according to international standard protocols produced by the Organisation for Economic Cooperation and Development (OECD) and the International Organisation for Standardisation (ISO) (Dantas *et al.*, 2007, Li *et al.*, 2008, Dantas *et al.*, 2008, Beltrán *et al.*, 2008, Khan *et al.*, 2010 and El Najjar *et al.*, 2013). Nevertheless, due to the different compounds that might be present in any complex water sample a battery of complementary standardized biotests that cover different molecular receptors, physiological pathways, organism groups and levels of biological complexity might be the best option for any hazard assessment (Escher and Fenner, 2011). Moreover, the selected bioassays should represent the main organism groups presented in the system whose aim is protect it. For instance, a biotest battery could be composed by a bacterium (*Pseudomonas putida*), a protozoan (*Tetrahymena thermophila*), an alga (*Pseudokirchneriella subcapitata*), a crustacean (*Daphnia magna*) and a fish (*Danio rerio*), which allows for the combination of prokaryotes and eukaryotes as well as representing different functional groups present in the freshwater ecosystem.

Single-species tests are fast, simple to perform, cost-effective and reliable. However, they have significant shortcomings such as not taking into account the interaction among species, and they often use genetically homogeneous populations of standard species that are not indigenous to the receiving water body and the tests are often conducted under experimental conditions very different from the receiving water

body of concern. Just as in the case of risk assessment of emerging pollutants, ecotoxicological assessment of treated wastewater should also be completed using indigenous communities that are present in the receiving water body, in order to provide a more realistic indication of the toxic effects of ozonated wastewater on exposed biological systems (Selivanovskaya *et al.*, 2004 and Proia *et al.*, 2013).

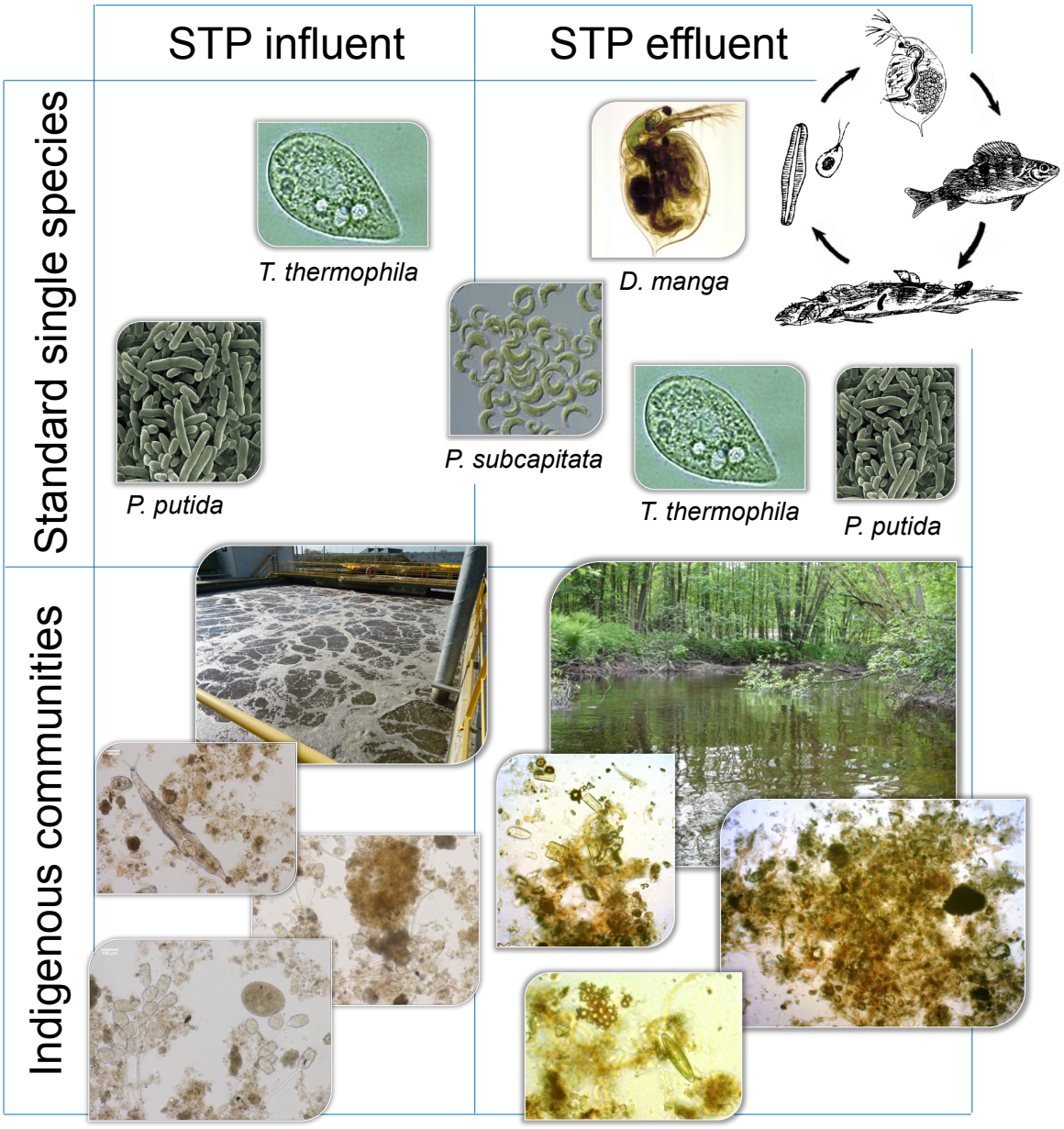


Fig. 1.9 Overview of possible bioassays used in aquatic toxicity assessment of ozone treated wastewater, whose recipient water body is an activated sludge tank of an STP or the freshwater environment. Inset plot: Simplified aquatic food chain consisting of primary producers (various species of algae), herbivores (daphnids), carnivores (fish) and decomposers (bacteria and fungi).

1.2. Aims and outline

The overarching aim of the present study was to assess the potential environmental risks of emerging substances as well as to evaluate the alternatives for applying ozonation for upgrading conventional STPs in order to minimize the discharge of these micropollutants to the receiving water bodies. While screening for hundreds of emerging substances was worthwhile and relevant in the context of this study, it would generally be too costly for monitoring purposes. Based on consumption data, occurrence in wastewaters and the aquatic environment and their inherent antimicrobial properties, two groups of emerging substances were selected for integration into future assessment efforts: personal care product preservatives and antibiotics. The main aim involved the following specific objectives:

1. To evaluate the aquatic toxicity of emerging substances towards single species and indigenous biological communities of the aquatic compartments.
2. To assess the potential risk of emerging substances to the process performance of activated sludge and to the surface water ecosystem, and to identify the risk drivers of the mixture.
3. To study the efficiency of continuous ozonation in removing the risk drivers' emerging pollutants, evaluating water matrix effects in synthetic and real wastewater and determining the optimum ozone dose.
4. To elucidate the transformation products that result from continuous ozonation in order to propose any possible degradation pathways of the risk-drivers' emerging pollutants.
5. To study the efficiency of continuous catalytic ozonation in the increasing the mineralization of emerging pollutants, evaluating water matrix effects in synthetic and real wastewater as well as determining the optimum ozone and catalyst dose.
6. To assess the aquatic toxicity of ozone treated wastewater on single species and biological communities using indigenous microorganisms of the aquatic compartments.

These objectives are further developed through chapters 2–7, each of which corresponds to a self-standing unit organized in two parts according to the two studied groups of emerging substances (personal care product preservatives and antibiotics) and the potential points for applying ozonation in a conventional STP (pre-treatment stage or polishing step of activated sludge process) in order to minimize their release into the aquatic environment (Fig. 1.10). These chapters match with papers published or submitted to peer-reviewed journals prior to PhD. defense.

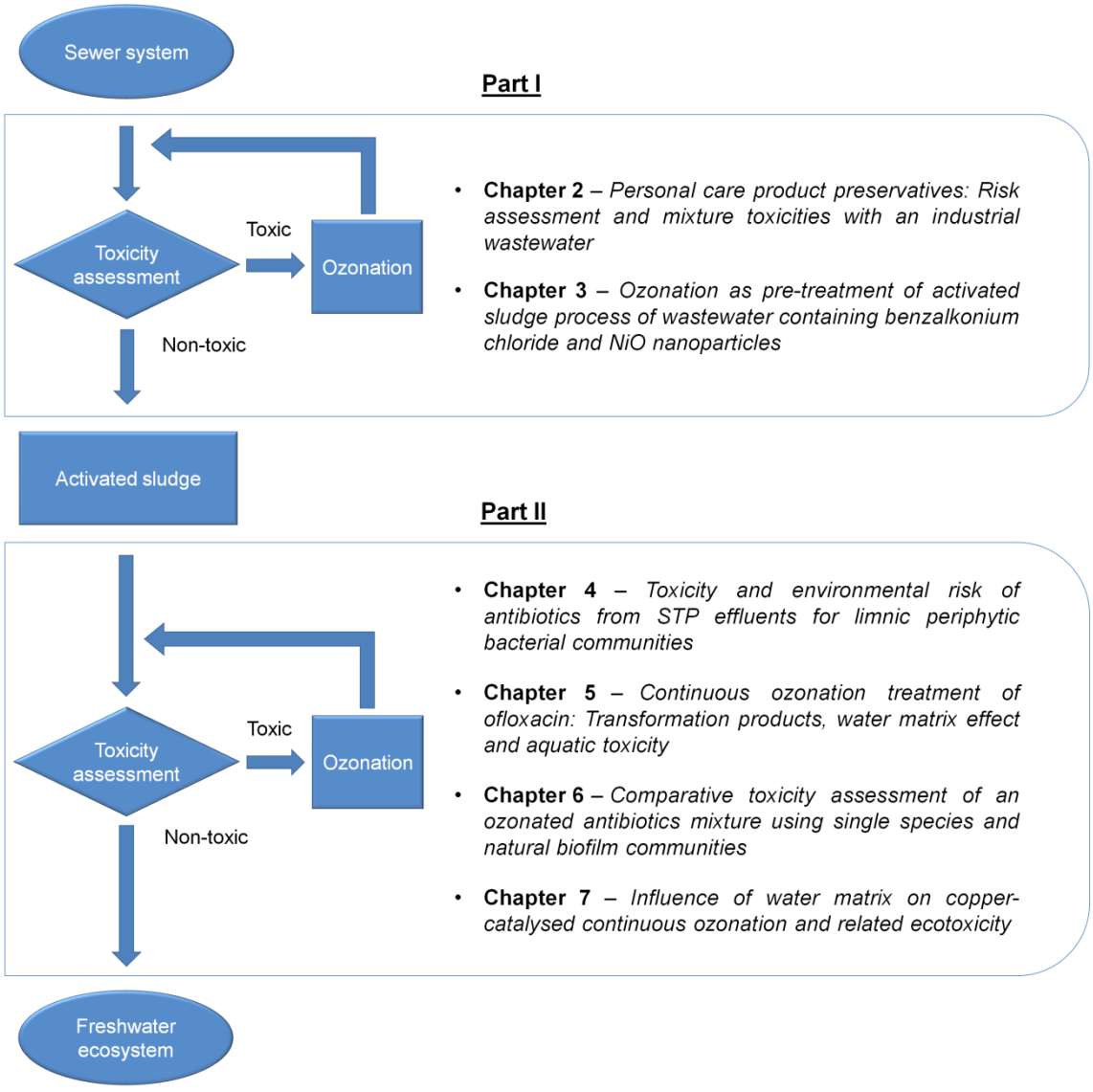


Fig. 1.10 The outline of the current study, which represents the self-standing units around emerging pollutants and the potential points for applying ozonation in a conventional STP to their degradation. Part I: personal care product preservatives and pre-treatment stage of activated sludge process (chapter 2 and 3); Part II: antibiotics and polishing step of activated sludge process (chapter 4–7).

A brief description of the following chapters is presented:

Chapter 2 – *Personal care product preservatives: Risk assessment and mixture toxicities with an industrial wastewater (Water Research* 72 *(2015) 174–185)* – studies the aquatic toxicity of eight frequently used preservatives (iodopropynyl butylcarbamate, bronopol, diazolidinyl urea, benzalkonium chloride, zinc pyrithione, propylparaben, triclosan and a mixture of methylchloroisothiazolinone and methylisothiazolinone) using two levels of biological complexity: a biotest battery composed of single-species test of bacteria (*V. fischeri* and *P. putida*) and protozoan (*T. thermophila*), and a whole biological community assay using activated sludge microorganisms. On the basis of this toxicity data it is then assessed whether the tested preservatives might pose a risk to activated sludge process, and furthermore the nature of interactions between the preservatives and a complex industrial wastewater using CA or IA concept is studied.

Chapter 3 – *Ozonation as pre-treatment of activated sludge process of a wastewater containing benzalkonium chloride and NiO nanoparticles* (*accepted in Chemical Engineering Journal)* – describes the ozonation of benzalkonium chloride and NiO nanoparticles in a synthetic water matrix and an STP influent. It includes the identification of transformation products and the degradation pathways of benzalkonium chloride. Toxicity assessment of treated wastewater are performed with a battery of bioassay composed of single-species tests of bacteria (*V. fischeri* and *P. putida*) and protozoan (*T. thermophila*), and an activated sludge assay.

Chapter 4 – *Toxicity and environmental risk of antibiotics from STP effluents for limnic periphytic bacterial communities (submitted to Environmental Pollution)* – assesses the chronic toxicities of six antibiotics (doxycycline, erythromycin, metronidazole, ofloxacin, sulfamethoxazole and trimethoprim) on natural bacterial communities. The joint toxicities of the six antibiotics, mixed in proportion to their occurrence in the effluents from two European STPs (Ryaverket STP, Sweden; West-Alcalá, Spain), are studied in order to determine whether the wastewater effluents might impact on the receiving freshwater ecosystem. On basis of toxicity data, it is then

assessed whether the single antibiotics and their mixture might pose a risk to the aquatic ecosystems, and finally identifies the major driver of the mixture toxicity.

Chapter 5 – *Continuous ozonation treatment of ofloxacin: Transformation products, water matrix effect and aquatic toxicity (Journal of Hazardous Materials 292 (2015) 34–43)* – describes the removal of ofloxacin in a synthetic water matrix and an STP effluent. It includes the identification of the transformation products and degradation pathways of ofloxacin, and an aquatic toxicity assessment of ozone treated wastewater is carried out with two antibiotic target- (*V. fischeri* and *P. putida*) and two antibiotic non-target-organisms(*T. thermophila* and *P. subcapitata*).

Chapter 6 – *Comparative toxicity assessment of an ozonated antibiotic mixture using single species and natural biofilm communities (submitted to Science of the Total Environment)* – studies the removal of a mixture of six antibiotics in an STP effluent. A study of the aquatic toxicity of ozonated wastewater is performed using two levels of biological complexity: single-species tests (*P. putida* and *P. subcapitata*) and natural biofilm community assay, in which are assessed the effects on heterotrophic and phototrophic part of the limnic periphyton. The predictive power of a component-based approach, primarily based on the CA concept, is also studied with the aim of providing reliable estimates of the aquatic toxicity of treated STP effluents.

Chapter 7 – *Influence of water matrix on copper-catalysed continuous ozonation and related ecotoxicity (Applied Catalysis B: Environmental 163 (2015) 233–240)* – explores the effect of the water matrix using synthetic water and STP effluent on the non-catalytic and copper-catalysed continuous ozonation of a mixture of reaction intermediates and ozone-refractory compounds: formic, acetic, oxalic and maleic acids. The aquatic toxicity of treated wastewater is carried out using a biotest battery composed of single species of bacteria (*V. fischeri* and *P. putida*), protozoan (*T. thermophila*), alga (*P. subcapitata*) and crustacean (*D. magna*).

In **Chapter 8**, the general conclusions and outlooks are presented.

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PART I

- Preservatives
- Ozonation as pre-treatment stage
- Activated sludge

Chapter 2

Personal care product preservatives: Risk assessment and mixture toxicities with an industrial wastewater

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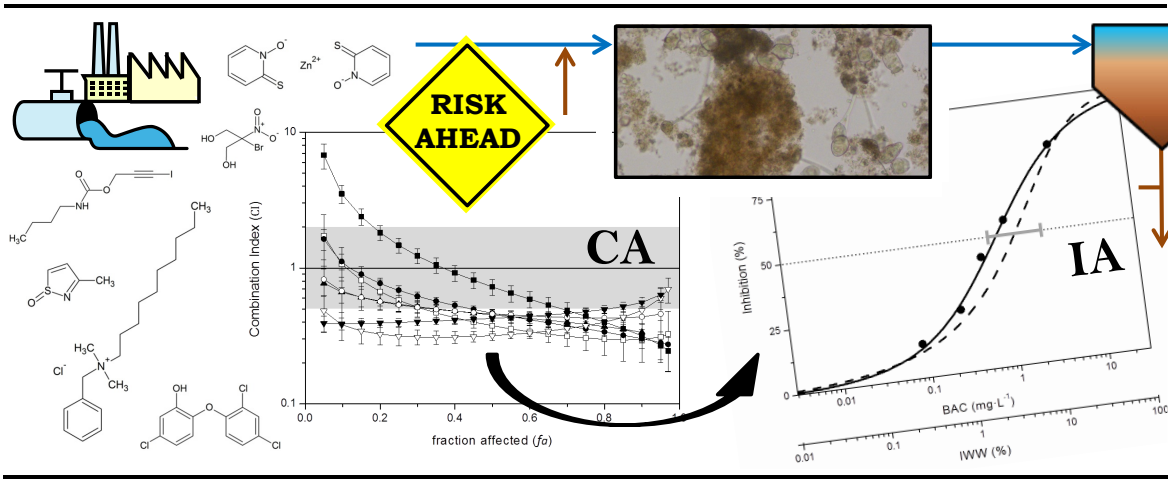
Graphical Abstract



PERSONAL CARE PRODUCT PRESERVATIVES: RISK ASSESSMENT AND MIXTURE TOXICITIES WITH AN INDUSTRIAL WASTEWATER

Abstract

The aquatic toxicity of eight preservatives frequently used in personal care products (PCPs) (iodopropynyl butylcarbamate, bronopol, diazolidinyl urea, benzalkonium chloride, zinc pyrithione, propylparaben, triclosan and a mixture of methylchloroisothiazolinone and methylisothiazolinone) was assessed by means of two different approaches: a battery of bioassays composed of single-species tests of bacteria (*Vibrio fischeri* and *Pseudomonas putida*) and protozoa (*Tetrahymena thermophila*), and a whole biological community resazurin-based assay using activated sludge. The tested preservatives showed considerable toxicity in the studied bioassays, but with a marked difference in potency. In fact, all biocides except propylparaben and diazolidinyl urea had EC_{50} values lower than 1 mg·L^{-1} in at least one assay. Risk quotients for zinc pyrithione, benzalkonium chloride, iodopropynyl butylcarbamate and triclosan as well as the mixture of the studied preservatives exceeded 1, indicating a potential risk for the process performance and efficiency of municipal sewage treatment plants. These four single biocides explained more than 95% of the preservative mixture risk in all bioassays. Each individual preservative was also tested in combination with an industrial wastewater (IWW) from a cosmetics manufacturing facility. The toxicity assessment was performed on binary mixtures (preservative+IWW) and carried out using the median-effect principle, which is a special case of the concept of Concentration Addition (CA). Almost 70% of all experiments resulted in EC_{50} values within a factor of 2 of the values predicted by the median-effect principle (CI values between 0.5 and 2). The rest of the mixtures whose toxicity was mispredicted by CA were assessed with the alternative concept of Independent Action (IA), which showed higher predictive power for the biological community assay. Therefore, the concept used to accurately predict the toxicity of mixtures of a preservative with a complex industrial wastewater depends on degree of biological complexity.

1. Introduction

The activated sludge process is widely used in sewage treatment plants (STPs), based on the development of a heterogeneous community composed of bacteria, protozoa, fungi and rotifers in an aeration tank. The activity and population of these organisms are crucial for proper system operation, and the presence of toxic substances in the influent may result in the depletion of the biomass activity and a lower performance of the STP (Dalzell *et al.*, 2002 and Ricco *et al.*, 2004).

STPs often receive industrial wastewater discharges, which are partially treated or even untreated. In fact, the failure of the effective operation of sewage works is usually attributed to the presence of certain pollutants of industrial origin that are toxic to the activated sludge organisms (Soupilas *et al.*, 2008). Therefore, the continuous monitoring of potential toxic influent is essential in order to ensure effluent quality, reduce operating costs and increase reliability. Conventional chemical analyses have been found inadequate to ensure that the influent is not negatively influencing STP performance (Soupilas *et al.*, 2008). The use of bioassays provides a holistic approach that allows the toxicity assessment of all components in any given complex mixture. The evaluation of industrial effluent toxicity should include a battery of bioassays composed of representative species of different trophic levels present in the activated sludge. However, although single-species tests are fast, simple to perform, cost-effective and reliable, they have significant shortcomings such as not taking into account the interaction among species, the use of species that are not indigenous to the activated sludge with genetically homogeneous populations and the fact that tests are usually conducted under experimental conditions very different from an aeration tank (Selivanovskaya *et al.*, 2004). Thus, toxicity assessment should be monitored using indigenous microbial population from an activated sludge process under conditions of forced aeration in order to provide a more accurate indication on the effects of STP influents on biological systems. These effects can be followed by changes in metabolic activity of the activated sludge population using a resazurin-based assay, which has been shown to be a reliable and cost-effective manner to monitor the performance of STP (McNicholl *et al.*, 2007).

An industrial sector of special concern for STP management is the cosmetics industry, which generates wastewater with an elevated concentration of biocides including many preservatives used in cosmetic formulations to avoid the development of microorganisms in the final product (Chapman, 2003 and Russell, 2003). As a consequence of their biological activity, preservatives are of particular interest as they can potentially affect and harm activated sludge biomass. In addition to industrial wastewater, the regular usage of pharmaceuticals and personal care products (PPCPs) also contributes to the discharge of large quantities of unalterated preservatives (Ternes *et al.*, 2004). Preservatives have been detected in concentrations of up to $mg \cdot L^{-1}$ and $\mu g \cdot L^{-1}$ in industrial effluents and STP influents, respectively (Kümmerer *et al.*, 1997, Woldegiorgis *et al.*, 2007, Norstrom *et al.*, 2008, Kasprzyk-Hordern *et al.*, 2009, Kumar *et al.*, 2010 and Poberznik *et al.*, 2011). However, in comparison with other PPCPs such as antibiotics, relatively little is known about occurrence and toxicity of preservatives (Brausch and Rand, 2011). Even less attention has been paid to their risk towards microorganisms on activated sludge, which determines whether a particular preservative or mixture has the potential to cause harmful effects in order to protect the process performance and efficiency of an STP (van Leeuwen and Vermeire, 2007).

The co-occurrence of preservatives with other components of industrial wastewater is another case for concern due to the potential interactive effects, such as synergistic or antagonistic toxicity, that may occur from complex mixtures (Kolpin *et al.*, 2002) in the STP influents. Therefore, it is essential to study the interactions of a preservative with industrial wastewater in order to determine the hazard that preservative spillage in cosmetics industry effluents could cause on activated sludge microorganisms. Since it would be an endless task to experimentally determine the toxicity of all relevant mixtures, predictive approaches based on the mathematical concepts of Concentration Addition (CA) and Independent Action (IA) have been proposed (Backhaus *et al.*, 2003, Altenburger *et al.*, 2004 and Kortenkamp *et al.*, 2009). Both predict the toxicity of a mixture based on the individual toxicity of the mixture components. Several reviews have shown that CA provides a reliable and frequently

used tool for predicting and assessing the ecotoxicity of multi-component mixtures (Belden *et al.*, 2007, Kortenkamp *et al.*, 2009 and Coors and Frische, 2011).

The study aims to assess the aquatic toxicities of eight preservatives using two different approaches: a battery of bioassays composed of single-species tests of bacteria (*Vibrio fischeri* and *Pseudomonas putida*) and protozoa (*Tetrahymena thermophila*), and a whole biological community assay from activated sludge process. On the basis of toxicity data, it is then assessed whether the tested preservatives might pose a risk to activated sludge process, and the nature of interactions between the preservatives and a complex industrial wastewater using CA or IA concept is studied.

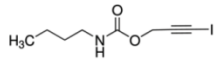
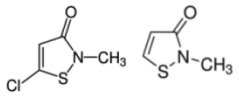
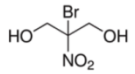
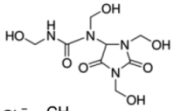
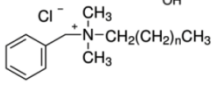
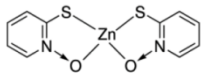
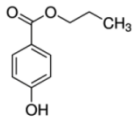
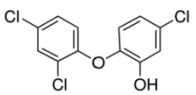
2. Materials and methods

2.1. Preservatives

The preservatives used in this study belong to different classes and were selected based on their potential aquatic toxicity, their volume of consumption and their occurrence in STP influents (Table 2.1). The following eight compounds were selected: iodopropynyl butylcarbamate (IPBC), bronopol (BNP), diazolidinyl urea (DIU), zinc pyrithione (ZPT), propylparaben (PPB) and triclosan (TCS) purchased from Sigma-Aldrich; benzalkonium chloride (BAC) purchased from Fluka, and a technical mixture of methylchloroisothiazolinone and methylisothiazolinone (CMI/MI) from Dow Chemical. The purity was IPBC ≥97%, BNP ≥98%, DIU ≥95%, TCS >97%, PPB ≥99%, ZPT ~95%, BAC ≥95% (consisting of homologues of different alkyl chain lengths, mainly C12 60% and C14 40%), and CMI/MI 1.5%, which are the active ingredients of a commercial biocide Kathon™ CG (CMI 1.15%, MI 0.35%, magnesium salts 23% and water to 100%).

The selected preservatives have mechanisms of action belong to the two broad categories proposed by Chapman (2003). IPBC, CMI/MI, BNP and DIU are electrophilic agents. These biocides react covalently with cellular nucleophiles to inactivate enzymes and there is evidence that they initiate the formation of intracellular free radicals which contribute to their lethal action. BAC, PPB and ZPT are membrane active preservatives.

Table 2.1 Physical and chemical characteristics, total consumption and occurrence in STP influents of the studied preservatives.

Trade name (Abbreviation)	Group	CAS number	Structural formula	Molecular weight ($g \cdot mol^{-1}$)	$\log K_{OW}$	Water solubility ($mg \cdot L^{-1}$)[a]	Consumption ($Tons \cdot year^{-1}$)[a]	Occurrence in STP influent ($\mu g \cdot L^{-1}$)
Iodopropynyl butylcarbamate (IPBC)	Halogenated compounds (carbamate)	55406-53-6		281.09	2.81[a]	156	–	130[e]
Methylchloroishothiazolinone Methylisothiozolinone (CMI/MI)	Isothiazolinones	55965-84-9		149.60 115.15	-0.34[b] -0.83[b]	>1 000	–	0.64[f] 0.69[f]
Bronopol (BNP)	Nitro compounds	52-51-7		199.99	0.22[a]	>1 000	100–1 000	ND (LOD=0.1)[g]
Diazolidinyl urea (DIU)	Imidazolidines	78491-02-8		278.22	-7.49[b]	>1 000	100–1 000	–
Benzalkonium chloride (BAC)	Quaternary ammonium compounds	63449-41-2		340.00 368.05	0.59[c] 1.67[c]	>1 000	1 000–10 000	170[h] 110[h]
Zn pyrithione (ZPT)	Metal organic compounds	13463-41-7		317.70	0.97[d]	4.93	1 000–10 000	17[d]
Propylparaben (PPB)	Parabens	94-13-3		180.20	3.04[a]	500	100–1 000	8.29[i]
Triclosan (TCS)	Halogenated compounds (phenolic)	3380-34-5		289.54	4.76[a]	10	1 000–10 000	86.2[j]

ND: not detected; LOD: limit of detection

[a] ECHA (2014); [b] EPI Suite™ program KOWWIN™ v. 1.68 (estimate); [c] Tezel (2009); [d] Woldegiorgis *et al.* (2007); [e] Norstrom *et al.* (2008); [f] Rafoth *et al.* (2007); [g] Dye *et al.* (2007); [h] Clara *et al.*(2007); [i] Kasprzyk-Hordern *et al.* (2009); [j] Kumar *et al.* (2010).

BAC is a lytic agent that destabilizes membranes leading to rapid cell lysis, whereas PPB and ZPT are protonophore causing leakage of intracellular constituents. TCS is also membrane-active, but studies have also indicated that its growth-inhibitory properties against bacteria arise from its blocking lipid biosynthesis by specially inhibiting NADH-dependent enoyl-acyl carrier protein reductase, FabI (Russell, 2003). Unlike antibiotics, preservatives as biocides are multi-targeted antimicrobial agents. Several of the damaging effects reported to occur in the most widely studied organisms, bacteria, may also take place to varying degrees in other organisms. Nevertheless, there is considerable variation in the response of different microorganisms to biocides (Russell, 2003).

The stock solutions and the dilution series of each preservative were freshly prepared in ultrapure water obtained from a Millipore Milli-Q with a resistivity of at least 18 MΩ·cm at 25ºC. The stability of preservatives under bioassay conditions was examined at the beginning and at the end of the exposure time according to OECD Guidance (OECD, 2008). The concentrations of CMI/MI, PPB, IPBC, TCS, BAC and ZPT remained 80–120% of nominal, therefore, the effect concentrations was expressed relative to nominal concentrations in accordance with OECD Guidance. The concentrations of DIU and BNP did not remain within 80–120% of nominal as a result of their highly instability in aqueous solutions, which together with their low biodegradability have been previously reported (Madsen *et al.*, 2001 and ECHA, 2014). However, the toxicity of their degradation by-products has been shown to be comparable or higher than that of their parent compounds (Madsen *et al.*, 2001 and Cui *et al.*, 2011) consequently, nominal concentrations were used in these cases as well.

2.2. Industrial wastewater

Wastewater was obtained from a cosmetics manufacturing facility located in Madrid (Spain) before further treatments. The physico-chemical characteristics of untreated IWW are shown in Table 2.2. Analytical determinations were carried out using standard methods (APHA *et al.*, 1998). Trace metal concentrations were determined by Agilent 7700x ICP-MS. Industrial effluent samples gave high values for COD, total

surfactants, total phenols and low for BOD_5/COD, indicating that IWW was largely loaded by non-biodegradable organic matter. Low concentration of AOX and heavy metals, which Zn represents near 95%, were detected. Wastewater samples were filtered using 0.45 µm glass-fibre filters and their pH adjusted to 7.0 ± 0.2 before conducting toxicity bioassays.

Table 2.2 Main physico-chemical parameters of the cosmetics industry wastewater.

pH	4.1	Total surfactants ($mg \cdot L^{-1}$)	288
Conductivity ($\mu S \cdot cm^{-1}$)	1473	Anionic surfactants ($mg \cdot L^{-1}$)	179
TSS ($mg \cdot L^{-1}$)	167	Cationic surfactants ($mg \cdot L^{-1}$)	0.32
COD ($mg \cdot L^{-1}$)	21 280	Non-ionic surfactants ($mg \cdot L^{-1}$)	109
BOD_5 ($mg \cdot L^{-1}$)	77	AOX ($mg\ Cl \cdot L^{-1}$)	0.26
Chloride ($mg \cdot L^{-1}$)	206	Total phenols ($mg \cdot L^{-1}$)	13.6
Fluoride ($mg \cdot L^{-1}$)	ND	Arsenic ($\mu g \cdot L^{-1}$)	7.93
Sulphate ($mg \cdot L^{-1}$)	39.8	Cadmium ($\mu g \cdot L^{-1}$)	0.30
Sulfide ($mg \cdot L^{-1}$)	0.37	Chromium ($\mu g \cdot L^{-1}$)	7.62
Sodium ($mg \cdot L^{-1}$)	359	Nickel ($\mu g \cdot L^{-1}$)	14.5
Potassium ($mg \cdot L^{-1}$)	19.3	Mercury ($\mu g \cdot L^{-1}$)	0.16
Magnesium ($mg \cdot L^{-1}$)	9.0	Lead ($\mu g \cdot L^{-1}$)	14.1
Calcium ($mg \cdot L^{-1}$)	66.5	Selenium ($\mu g \cdot L^{-1}$)	0.71
Total phosphorous ($mg\ P \cdot L^{-1}$)	4.89	Copper ($\mu g \cdot L^{-1}$)	16.8
Total nitrogen ($mg\ N \cdot L^{-1}$)	26.4	Zinc ($\mu g \cdot L^{-1}$)	841

ND: not detected

2.3. Procedures for aquatic toxicity tests

The aquatic toxicities of the aforementioned compounds were assessed using a battery of bioassays composed of single-species tests of the two main groups present in the activated sludge, namely the bacteria *V. fischeri* and *P. putida* and the protozoa *T. thermophila*, as well as an activated sludge biological community assay.

V. fischeri acute test measures the decrease in bioluminescence induced in cell metabolism. The bioassay was carried out according to ISO 11348-3 standard protocol (ISO, 2007) using the BioFix®Lumi test (*V. fischeri*, NRRL-B 11177 from Macherey-Nagel, Germany). The test was carried out in 96-well white polypropylene microplate. 100 µL of test solution (2% w/v NaCl and pH 7.0 ± 0.5) was transferred into each well, which was supplemented with 100 µL of bacterial suspension. Light was measured at 15 ± 1ºC after 30 min by means of a Fluoroskan Ascent FL microplate luminometer (Thermo Scientific).

P. putida test determines the inhibitory effect of a substance on the bacteria (*P. putida*, NCIB 9494 from CECT, Spain) by means of cell growth inhibition. The bioassay was performed according to ISO 10712 guideline (ISO, 1995). Bacterial cultures were exposed to test solutions at 23 ± 1ºC for 16 h in glass incubation vials which were constantly shaken in darkness. The cell growth was determined by optical density (λ 600 nm) in 96-well clear microplate (200 µL test suspension per well) using a Rayto RT-2100C microplate reader. Growth inhibition assay with the ciliate protozoan *T. thermophila* was performed according to the Standard Operational Procedure Guideline of Protoxkit F™ (1998). The test is based on the turnover of substrate into ciliate biomass. Substrate was purchased from MicroBioTest Inc. (Belgium) whereas *T. thermophila* (SB 210) was kindly supplied by D. Cassidy-Hanley (Tetrahymena Stock Center, Cornell University, USA). Ciliates were incubated with water samples and food suspension in test vessels at 30 ± 1ºC for 24 h in darkness. Growth inhibition was determined on the basis of turbidity changes (OD at λ 440 nm). $ZnSO_4 \cdot 7H_2O$ for *V. fischeri* (EC_{50} between 17 and 22 mg·L^{-1}), 3,5-dichlorophenol for *P. putida* (EC_{50} between 10 and 30 mg·L^{-1}) and $K_2Cr_2O_7$ for *T. thermophila* (EC_{50} between 15 and 24 mg·L^{-1}) were used as reference substances in order to check each test procedure.

Activated sludge bioassay was carried out by evaluating the effect of water samples on activated sludge metabolic activity using the resazurin (7-hydroxy-3H-phenoxazin-3-one-10-oxide) method under the experimental conditions described in OECD Method 209 (OECD, 2010). Briefly, resazurin, blue and non-fluorescent in its oxidized stated, is reduced by metabolically active microorganisms to a pink fluorescent derivative (resorufin) by means of a dehydrogenase enzyme (McNicholl *et al.*, 2007). Fresh activated sludge was collected from the aeration tank of an STP located in Guadalajara (Spain). Activated sludge was characterized by determining physico-chemical parameters (Table 2.3). Inoculum (3.0 g·L^{-1} of MLSS) supplemented with synthetic sewage feed was exposed to tested water samples at 20 ± 2ºC for 3 h in glass vials which were constantly shaken (200 rpm) in darkness under conditions of forced aeration (0.5–1.0 L·min^{-1}) (OECD, 2010). After exposure, biomass metabolic activity was measured in 96-well black polypropylene microplate by adding 200 µL test suspension

and 20 µL of resazurin (100 mg·L^{-1}) to each well. Resazurin reduction was measured after 20 min incubation using a Fluoroskan Ascent FL microplate fluorometer (Excitation 542 nm, Emission 592 nm). The suitability of each batch of activated sludge biomass was determined using $CuSO_4 \cdot 5H_2O$ as reference substance (EC_{50} between 15 and 35 mg·L^{-1}).

Table 2.3 MLSS of sludge matrix used in resazurin-based activated sludge test.

TSS (mg·L^{-1})	6 590
VSS (mg·L^{-1})	4 710
VSS/TSS	0.71
V_{30} (mL·L^{-1})	795
SVI (mL·g^{-1})	119
pH	8.27

2.4. Experimental design

Solutions of preservatives were tested singly and in binary mixtures with the industrial wastewater (preservative+IWW). The compounds were mixed relative to their potency (according to their EC_{50} values). Five to seven dilutions of each toxicant and combination, control and a reference substance were tested in three independent experiments with duplicate samples as described elsewhere (Rodea-Palomares *et al.*, 2010).

2.5. Data treatment for determining individual and mixture toxicities

The description of the concentration-response curve for each substance and mixtures were estimated using the median-effect equation based on the mass-action law (Chou and Talalay, 1984):

$$\frac{f_a}{f_u} = \left(\frac{D}{D_m}\right)^m \tag{2.1}$$

where D is the dose [concentration], D_m is the dose [concentration] for 50% (EC_{50}), f_a is the fraction affected by dose [concentration] D (*e.g.*, 0.75 if growth is inhibited by 75%), f_u is the fraction unaffected (*i.e.*, $f_u = 1 - f_a$) and m is the coefficient of the

sigmoidicity of the concentration-response curve: m = 1, >1, and <1 indicate hyperbolic, sigmoidal and flat sigmoidal concentration-response curve, respectively (Chou, 2006). Therefore, the method takes into account both potency (D_m) and shape (m) parameters. Eq. (2.1) may be rearranged as follows:

$$D = D_m \left(\frac{f_a}{1 - f_a}\right)^{1/m} \tag{2.2}$$

The D_m and m values for each individual compound or mixture were determined by the median-effect plot: $x = \log(D)$ versus $y = \log(f_a/f_u)$ which is based on the logarithmic form of Eq. (2.1). In the median-effect plot, m is the slope and $D_m = 10^{-(y-intercept)/m}$. The conformity of the data to the median-effect principle can be readily assessed by the linear correlation coefficient (r) of the fitting to Eq. (2.2) (Chou, 2006).

These parameters were then used to calculate doses [concentrations] of individual compounds and their mixtures required to produce various effect levels according to Eq. (2.1). For each effect levels, Combination Index (CI) values were then calculated according to the general Combination Index equation for n-chemical combination at x% inhibition (Chou, 2006):

$${}^{n}(CI)_x = \sum_{j=1}^{n} \frac{(D)_j}{(D_x)_j} = \sum_{j=1}^{n} \frac{(D_x)_{1-n}\left\{\frac{[D]_j}{\sum_1^n [D]}\right\}}{(D_m)_j \left\{\frac{(f_{a_x})_j}{[1-(f_{a_x})_j]}\right\}^{\frac{1}{mj}}} \tag{2.3}$$

where ${}^{n}(CI)_x$ is the Combination Index for n chemicals at x% inhibition (*e.g.*, growth inhibition); $(D_x)_{1-n}$ is the sum of the dose [concentration] of n chemicals that exerts x% inhibition in combination, $\{[D_j] / \sum_1^n [D]\}$ is the proportionality of the dose [concentration] of each n chemicals that exerts x% inhibition in combination, and $(D_j)\{(f_{ax})_j / [1-(f_{ax})_j]\}^{1/mj}$.

Combination Index is a special case of the more general concept of Concentration Addition (CA) (Backhaus, 2014), which is based on the assumption that all components in the mixture behave as if they are simple dilutions of one another, which is often taken to mean that CA describes the joint action of compounds with an identical mechanism of action (Kortenkamp *et al.*, 2009). For a mixture of n components, the CA concept can be mathematically expressed as:

$$\sum_{i=1}^{n} \frac{c_i}{EC_{x_i}} = 1 \tag{2.4}$$

where c_i denotes the concentration of compound i in a mixture that is expected to cause x% effect, and EC_{x_i} gives the concentration at which the compound i alone causes the same x% effect. If a mixture is accurately predicted by CA then the sum of fraction c_i/EC_{x_i} equals 1, in the same way as in Combination Index CI = 1. Thus, two-fold deviation was applied as a threshold to denote compliance between the observed and the predicted mixture toxicity by median-effect principle (*i.e.*, CI values between 0.5 and 2). Toxicity mixtures mispredicted by CA (*i.e.*, CI values out of range 0.5–2) were assessed with the alternative concept of Independent Action (IA).

IA assumes that the resulting combined effect can be calculated from the effects caused by the individual mixture components, which is often taken to mean that IA describes the joint action of compounds with a dissimilar mechanism of action (Kortenkamp *et al.*, 2009). The expected mixture effect can hence be calculated according to the joint probability of statistically independent events as:

$$E(c_{mix}) = 1 - \prod_{i=1}^{n} [1 - E(c_i)] \tag{2.5}$$

where $E(c_{mix})$ is the total expected effect of the mixture, n is the number of mixture components and $E(c_i)$ is the effect that the ith component would cause if applied singly in concentration c_i.

2.6. Risk assessment

In order to estimate and assess the potential risk that preservatives could cause on activated sludge microorganisms, risk quotients (RQs) for microbial activity in municipal STP are calculated for the worst case scenario, the maximum preservative concentrations measured in STP influents.

The toxic units (TUs, $EnvConc/EC_{50}$) of single preservatives were first calculated for each bioassay. Multiplying TUs by the assessment factor (AF), 10 for single-species tests (highly relevant *P. putida* and *T. thermophila*, and with limited relevance for STP process *V. fischeri*) and 100 for the activated sludge bioassay (ECHA, 2008), were calculated RQ for each single preservative. On this basis, the expected joint risk of the preservative mixture is then estimated using the strategy for the compound-based risk assessment of chemical mixtures (Backhaus and Faust, 2012), which is primarily based on the mixture toxicity concept of CA. In fact, the sum of toxic units (STU) was calculated in a first step for each bioassay. The final RQ for the mixture then equals the STU of the most sensitive bioassay (single-species and activated sludge tests) multiplied by the corresponding AF (ECHA, 2008). RQ higher than 1 suggests that preservative risk would be inadequately controlled for the microorganisms present in an STP.

The application of CA to the preservative mixtures violates a main assumption: similar mode or mechanism of action. Hence, the maximum error that occurs by ignoring IA can be estimated as follows (Junghans *et al.*, 2006):

$$\frac{EC_{50}^{IA}}{EC_{50}^{CA}} \leq \frac{\sum_{i=1}^{n} \frac{c_i}{EC_{50_i}}}{\underset{i \in (1 \ldots n)}{max} \left(\frac{c_i}{EC_{50_i}} \right)} \tag{2.6}$$

Under these circumstances a maximum possible ratio by which CA may predict a higher mixture toxicity than IA equals the number of mixture components (n) (Faust, 1999). Given the uncertainty of the hazard and exposure estimates of the individual preservatives, a possible maximum error of less than 2 might be considered acceptable (Backhaus and Karlsson, 2014).

3. Results and discussion

3.1. Toxicity of single preservatives

All tested preservatives showed considerable toxicity in the studied bioassays, but with a marked difference in potency. Table 2.4 provides EC_{50} values together with the shape parameter m used for curve fitting to the observed data by means of the median-effect equation. Linear regression correlation coefficients of the median-effect plot were >0.95 in all cases (data not shown), indicating the agreement of the experimental data with the mass-action law. In order to show the quality of both observed data and curve fitting, concentration-response curves for preservatives in the studied bioassays are represented in Fig. 2.1.

The single-species tests were highly sensitive to selected preservatives without significant differences to previously published data (Table 2.5). All biocides, except PPB and DIU, displayed EC_{50} values lower than 1 mg·L^{-1} in at least one assay. This fact is in line with their classification as hazardous to the aquatic environment according to Regulation (EC) No. 1272/2008, which harmonises the provisions and criteria for the classification and labelling of substances, mixtures and certain specific articles within the European Union (EU Parliament and the Council, 2008). In fact, IPBC, CMI/MI, BNP, BAC, ZPT and TCS have already been classified into the acute aquatic hazard category as *very toxic to aquatic life* ($H400$, EC_{50} ≤1 mg·L^{-1} for algae, crustacean or fish), while the toxicities of DIU and PPB are characterized as *conclusive but not sufficient for classification* (ECHA, 2014). It should be noticed that ZPT and CMI/MI were the most toxic studied preservatives, showing EC_{50} values <1 mg·L^{-1} for the three organisms. The data also indicated the relative non-sensitivity of *P. putida* to preservatives that present a phenol moiety: TCS and PPB. High-level intrinsic resistance to TCS and PPB of *Pseudomonas* due to use degradative enzymes has been previously shown by Russell (1991) and Schweizer (2001), in agreement with the results presented in this study.

In general, the same toxicity pattern displayed in single-species tests was observed in the activated sludge assay. ZPT, CMI/MI and BAC are powerful biocides as demonstrated by their EC_{50} values lower than 4 mg·L^{-1}, whereas low toxicity values

Table 2.4 Concentration-response parameter values of the studied preservatives for each bioassay (mean ± 95% confidence interval).

Preservative	*V. fischeri*		*P. putida*		*T. thermophila*		Activated sludge	
	EC_{50} (mg·L^{-1})	m	EC_{50} (mg·L^{-1})	m	EC_{50} (mg·L^{-1})	m	EC_{50} (mg·L^{-1})	m
IPBC	3.87 ± 0.29	1.12 ± 0.17	105 ± 16	1.65 ± 0.18	0.119 ± 0.021	3.52 ± 0.21	25.8 ± 5.2	0.851 ± 0.033
CMI/MI	0.063 ± 0.004	1.34 ± 0.15	0.509 ± 0.065	5.29 ± 0.09	0.195 ± 0.033	3.85 ± 0.18	3.04 ± 0.66	0.710 ± 0.040
BNP	0.171 ± 0.012	1.38 ± 0.09	1.25 ± 0.18	3.47 ± 0.21	4.66 ± 0.56	3.08 ± 0.21	12.1 ± 2.3	0.604 ± 0.051
DIU	51.4 ± 1.9	1.25 ± 0.03	171 ± 12	5.72 ± 0.13	37.0 ± 4.1	5.61 ± 0.08	335 ± 33	0.618 ± 0.073
BAC	0.259 ± 0.08	1.50 ± 0.12	8.40 ± 0.52	4.27 ± 0.18	4.28 ± 0.43	2.33 ± 0.12	3.43 ± 0.58	0.620 ± 0.055
ZPT	0.072 ± 0.012	1.12 ± 0.11	0.128 ± 0.020	2.95 ± 0.11	0.039 ± 0.007	5.01 ± 0.10	1.84 ± 0.26	0.754 ± 0.047
PPB	5.57 ± 0.18	1.03 ± 0.02	–	–	10.0 ± 1.4	3.99 ± 0.11	414 ± 36	0.944 ± 0.075
TCS	0.228 ± 0.034	3.79 ± 0.18	–	–	1.33 ± 0.16	3.08 ± 0.19	13.6 ± 0.56 [a]	0.328 ± 0.029

[a] estimation of EC_{50} value outside the concentration range. Maximum inhibition of 44% was recorded at 6.0 mg·L^{-1}.

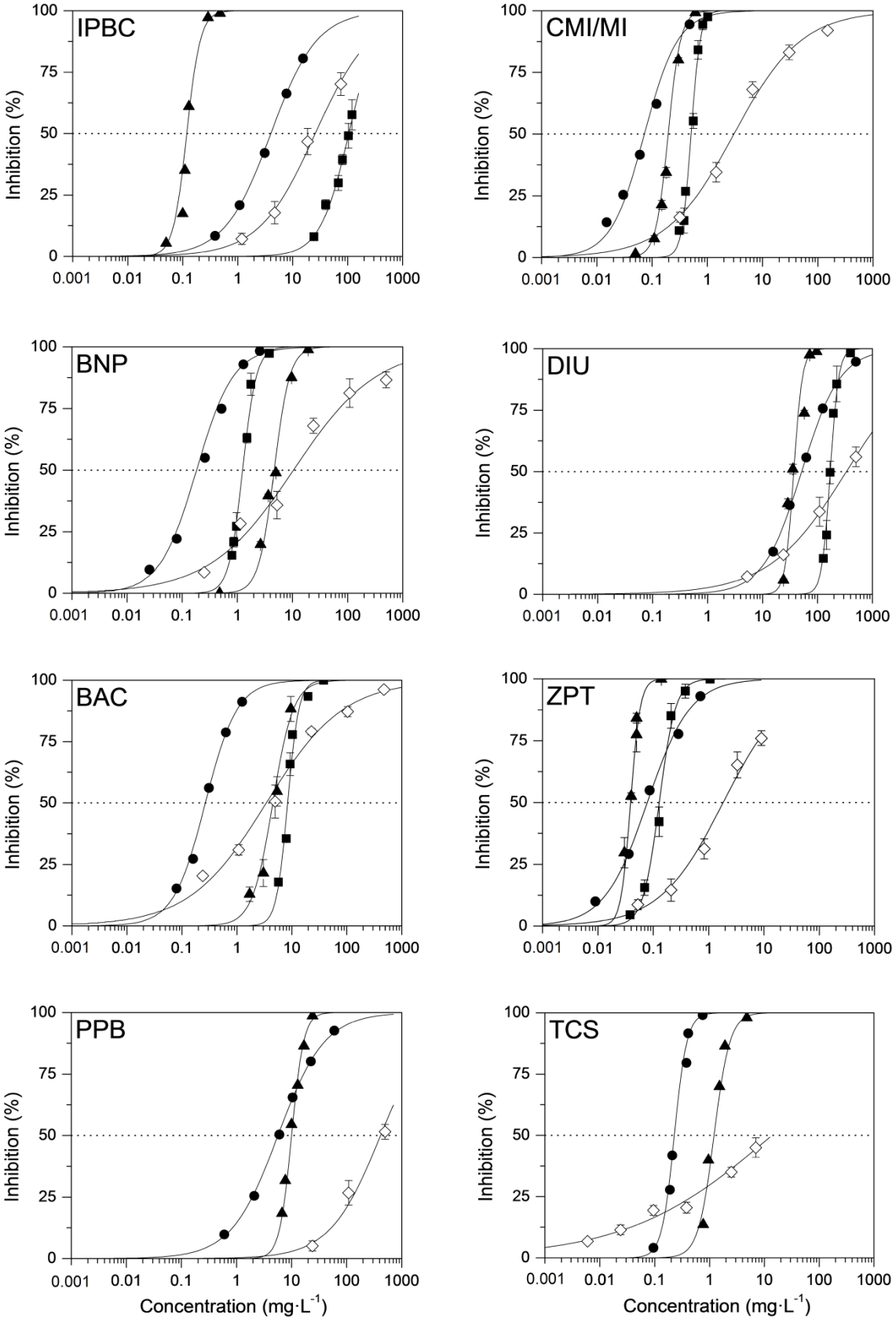


Fig. 2.1 Concentration-response curves of studied preservatives for the bioassays: *V. fischeri* (●), *P. putida* (■), *T. thermophila* (▲) and activated sludge test (◊) (mean ± 95% confidence interval). Solid lines give the median-effect equation fit.

were found for DIU and PPB. It is worth mentioning that the aquatic toxicity of preservatives for activated sludge based on oxygen consumption showed EC_{50} values in line or slightly higher than those obtained in this study despite the different endpoint used (Table 2.5). When comparing the toxicity of studied preservatives in biological community assay with single-species tests, it becomes evident that the compounds show a comparatively lower toxicity towards activated sludge. The higher tolerance observed should be expected, considering the heterogeneity and more variable growth

Table 2.5 Aquatic toxicity from previously published studies on the selected preservatives to the studied bioassays.

Preservative	Bioassay	EC_{50} (mg·L^{-1})	Reference
IPBC	*Vibrio fischeri*	8.5	Zhou *et al.*, 2006
	Pseudomonas putida	91	ECHA, 2014
	Activated sludge	44	ECHA, 2014
CMI/MI	*Vibrio fischeri*	0.072	Williams and Jacobson, 1999
BNP	*Vibrio fischeri*	19.2	Cui *et al.*, 2011
	Pseudomonas putida	2.33	ECHA, 2014
	Activated sludge	43	ECHA, 2014
DIU	Activated sludge	567	ECHA, 2014
BAC	*Vibrio fischeri*	0.5	Sütterlin *et al.*, 2008
	Vibrio fischeri	0.24–0.42	Nalecz-Jawecki *et al.*, 2003
	Vibrio fischeri	0.14–0.27	Tezel, 2009
	Pseudomonas putida	6.0	Sütterlin *et al.*, 2008
	Tetrahymena thermophila	4.37–5.30	Nalecz-Jawecki *et al.*, 2003
	Tetrahymena thermophila	2.94	Kreuzinger *et al.*, 2008
	Activated sludge	7.75	ECHA, 2014
	Activated sludge	10	Kümmerer *et al.*, 2004
	Activated sludge	22	Zhang *et al.*, 2011
ZPT	*Vibrio fischeri*	0.08	Zhou *et al.*, 2006
	Pseudomonas putida	0.22	ECHA, 2014
	Activated sludge	1.84	ECHA, 2014
	Activated sludge	2.4	ECHA, 2014
PPB	*Vibrio fischeri*	0.26	Terasaki *et al.*, 2008
	Vibrio fischeri	2.6	Bazin *et al.*, 2010
	Pseudomonas ATCC 9027	>180	Eklund, 1980
	Tetrahymena thermophila	9.7	Bazin *et al.*, 2010
TCS	*Vibrio fischeri*	0.22	Farré *et al.*, 2008
	Vibrio fischeri	0.28	Stasinakis *et al.*, 2008
	Tetrahymena pyriformis	0.58	Rudzok *et al.*, 2011
	Activated sludge	20	Orvos *et al.*, 2002

environment of the microorganisms used (Ricco *et al.*, 2004), as well as the numerous mechanisms of resistance to biocides of activated sludge biomass as consequence of floc structure (Russell, 2003 and Henriques and Love, 2007). The *V. fischeri* test was the most sensitive bioassay to the studied preservatives in line with previously published results from other authors (Dalzell *et al.*, 2002 and Ricco *et al.*, 2004). Nevertheless, the use of the *V. fischeri* test alone to assess effluent discharges to the sewer may lead to an overestimation of the toxicity effects on the biomass operating in the STP.

3.2. Preservative risk assessment

First, the potential risk for activated sludge microorganisms from the individual preservatives assuming a worst case scenario for a municipal STP is briefly assessed. Table 2.6 shows the maximum preservative concentrations detected in STP influents, the toxic units (TUs) calculated from toxicity data for the set of four bioassays (Table 2.4) and the risk quotients (RQs). RQs were calculated for each single preservative using the TUs and the corresponding assessment factor (10 for single-species tests and 100 for activated sludge bioassay according to ECHA, 2008). IPBC, BAC, ZPT and TCS exceed the threshold value of 1 for the protection of the activated sludge process. In all cases, RQs were based on toxicity data from the most sensitive bioassays: *V. fischeri* and *T. thermophila*. However, it is worth pointing out that the *V. fischeri* test has a limited relevance for the risk assessment of STP microorganisms (ECHA, 2008).

The data show that ZPT seems to be a risky preservative for both bacteria and ciliate protozoa (RQ >1 in all single-species tests), while IPBC, BAC and TCS might suppose a specific risk for one microorganism. Current published literature for STP influent concentrations is fairly extensive for some preservatives (*i.e.*, TCS, PPB and BAC) but relatively little information is available for others (*e.g.*, ZPT, IPBC, BNP, DIU) (Brausch and Rand, 2011). This fact constrains the calculation of their potential risk for activated sludge organisms as risk does not exist if exposure to a harmful substance or situation does not or will not occur (van Leeuwen and Vermeire, 2007).

Table 2.6 Risk quotient (RQ) of the studied preservatives assuming a worst case scenario (maximum concentration detected in STP influents) to microorganisms in an STP aeration tank. RQ higher than one are emphasized in bold.

Preservative	Occurrence in STP influent ($\mu g \cdot L^{-1}$)	RQ	*V. fischeri*		*P. putida*		*T. thermophila*		Activated sludge	
			TUs	RQ	TUs	RQ	TUs	RQ	TUs	RQ
IPBC	130[a]	**11**	0.034	0.34	0.001	0.01	1.092	**11**	0.005	0.50
CMI/MI	1.33[b]	0.210	0.021	0.21	0.003	0.03	0.007	0.07	0.000	0.04
BNP	ND (LOD=0.1)[c]	0.00	0.000	0.00	0.000	0.00	0.000	0.00	0.000	0.00
DIU	–	0.00	0.000	0.00	0.000	0.00	0.000	0.00	0.000	0.00
BAC	280[d]	**11**	1.081	**11**	0.033	0.33	0.065	0.65	0.082	**8.2**
ZPT	17[e]	**4.4**	0.236	**2.4**	0.133	**1.3**	0.436	**4.4**	0.009	0.92
PPB	8.29[f]	0.01	0.001	0.01	–	–	0.001	0.01	0.000	0.00
TCS	86.2[g]	**3.8**	0.378	**3.8**	–	–	0.065	0.65	0.006	0.63
MIXTURE		**18**	1.751	**18**	0.170	**1.7**	1.666	**17**	0.102	**10**

ND: not detected

LOD: limit of detection

[a] Norstrom *et al.* (2008)

[b] Rafoth *et al.* (2007)

[c] Dye *et al.* (2007).

[d] Clara *et al.* (2007)

[e] Woldegiorgis *et al.* (2007)

[f] Kasprzyk-Horden *et al.* (2009)

[g] Kumar *et al.* (2010)

On the basis of individual preservative, TUs the expected joint risk of their mixture is then estimated and assessed, summing up the toxic units (STUs) for each bioassay according to the strategy for the compound-based risk assessment of chemical mixture proposed by Backhaus and Faust (2012). Thus, using the STUs from the four biotests and considering their assessment factor (ECHA, 2008), the results showed final RQs for single species of 1.7 for *P. putida*, 17 for *T. thermophila* and 18 for *V. fischeri* , and 10 for the activated sludge assay. That is, for all bioassays the preservative mixture poses a potential risk for the activated sludge process. However, this strategy is primarily based on the concept of Concentration Addition (CA), which can be criticized for violating its main assumption when applied to the mixture of preservatives presents in this paper: similar mode or mechanism of action of the substances of the mixture. The maximum error that occurred by simply ignoring the competing concept of Independent Action (IA) was estimated by means of the ratio between the $EC_{50}s$ predicted by CA and IA as indicated in Eq. (2.6) (Junghans *et al.*, 2006). The results show maximum errors of 1.6 for *V. fischeri*, 1.3 for *P. putida*, 1.5 for *T. thermophila* and 1.3 for activated sludge; all of them lower than 2, the value considered acceptable according to Backhaus and Karlsson (2014). This fact shows that CA can predict toxicity mixtures of dissimilarly acting substances with reasonable accuracy. Indeed, empirical evidence suggests that CA predicts with a tendency to slightly overestimate the mixture toxicity of dissimilarly acting compounds (Backhaus *et al.*, 2010).

The distribution of the relative TUs is shown in Fig. 2.2 for the used bioassays. The plot clearly shows the uneven distribution of the toxic units in the mixture. BAC, ZPT and TCS contribute more than 95% to the overall STUs in all cases except for *T. thermophila*, for which IPBC explains a 66% of the total mixture toxicity. In fact, these four preservatives contribute most to the overall STUs, while the rest of the compounds has only a negligible contribution. It is also important to note that the main ecotoxicity risk driver depends on the bioassay: IPBC for *T. thermophila*, ZPT for *P. putida* and BAC for *V. fischeri* and activated sludge. In the same way, Backhaus and Karlsson (2014) have previously shown that the most risky compound for pharmaceuticals in STP effluents depends on the species group under consideration.

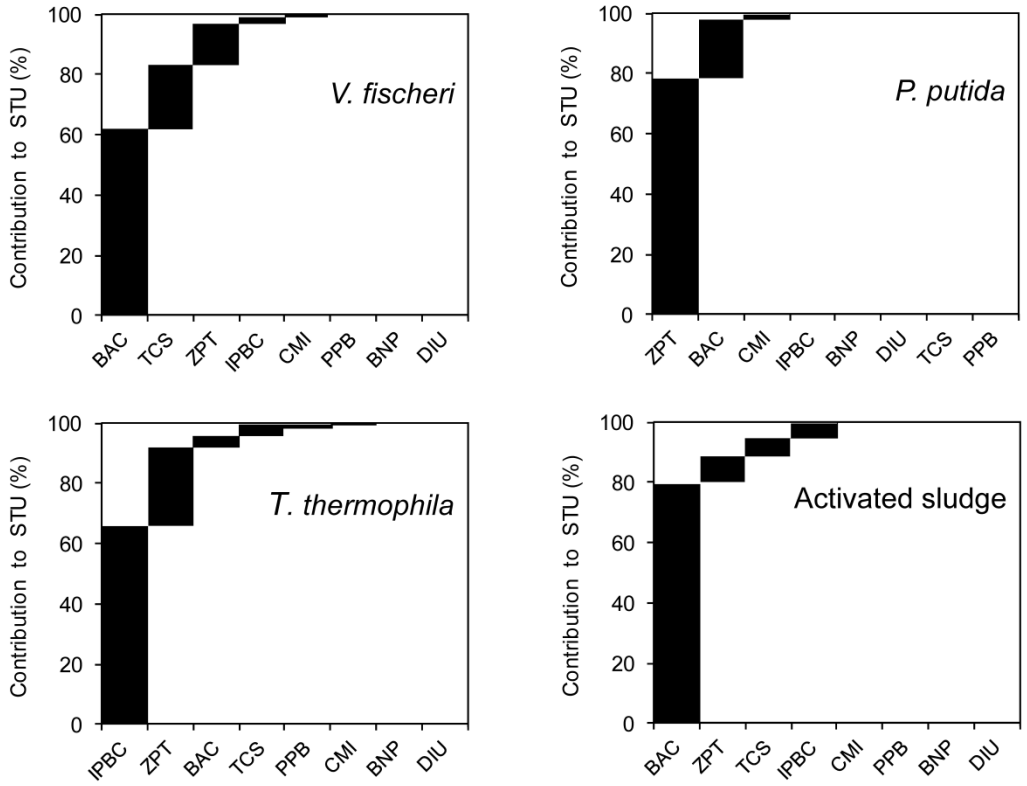


Fig. 2.2 Distribution of Toxic Units of the studied preservatives present in the worst case scenario (maximum measured concentration in STP influents) for each bioassay.

3.3. Interaction of preservatives with industrial wastewater

3.3.1. Toxicity of industrial wastewater

Industrial wastewater (IWW) is studied by a whole-mixture approach, which is based on the direct ecotoxicological assessment of a given effluent (Fig. 2.3). This approach allows analyzing the real industrial effluent as if it was a single chemical (Backhaus *et al.*, 2010). The tested IWW was highly toxic to *V. fischeri* and *T. thermophila* with EC_{50} of 0.051 and 0.318%, respectively. High toxicity of cosmetics industry effluents for single species has been previously reported (Perdigón-Melón *et al.*, 2010, Pliego *et al.*, 2012 and de Melo *et al.*, 2013), in which the elevated amount of toxicants present (surfactants, phenol derivatives, dyes, preservatives, etc.) and the possible mixture effects result in high toxicities. Particularly, de Melo *et al.* (2013) determined that surfactants were the main source of toxicity in a cosmetics industry effluent, whose concentrations in the presently studied wastewater were 288 mg·L^{-1} (Table 2.2). The

occurrence of 0.84 mg·L^{-1} of Zn could also contribute to its toxicity due to the low EC_{50} values reported for this metal: 0.41–4.6 mg·L^{-1} for *V. fischeri* (Dalzell *et al.*, 2002 and Teodorovic *et al.*, 2009) and 3.6–6.7 mg·L^{-1} for *T. thermophila* (Gallego *et al.*, 2007 and Mortimer *et al.*, 2010). On the contrary, low toxicity was detected for *P. putida* (EC_{50} = 64.4%), a fact that may be due to its different metabolic pathways, including the ability of this microorganism to degrade organic pollutants and solvents (Hafner, 2004). The activated sludge assay was not especially sensitive to the tested IWW either, showing a 50% inhibition at 11.8%.

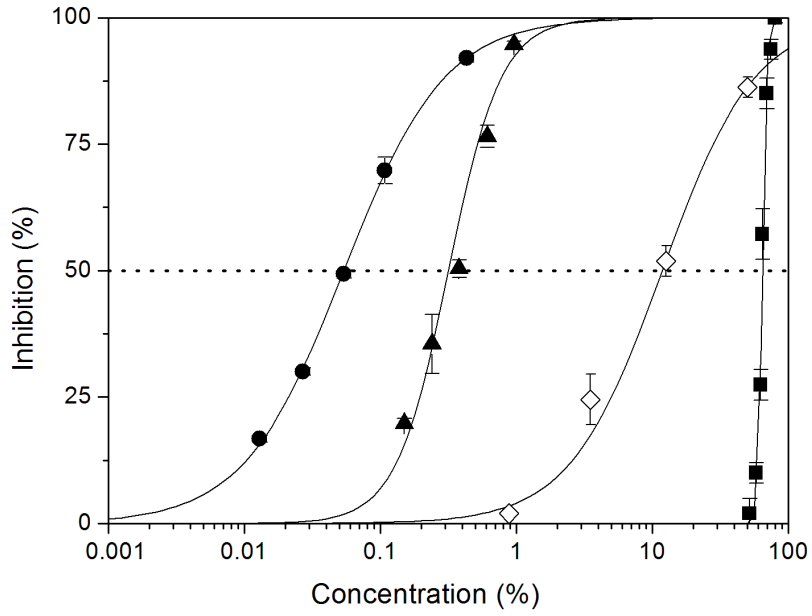


Fig. 2.3 Concentration-response curves of the studied industrial wastewater for the bioassays: *V. fischeri* (●), *P. putida* (■), *T. thermophila* (▲) and activated sludge test (◊) (mean ± 95% confidence interval). Solid lines give the median-effect equation fit.

3.3.2. Toxicity of binary mixtures: preservative and industrial wastewater

The EC_{50} (D_m) and m values from IWW and single preservatives, and their binary combinations (preservative+IWW) were used to quantify the predictive accuracy of median-effect law by Combination Index (CI) equation (Chou, 2006). The ratio between observed and predicted mixture toxicities was expressed as CI. A two-fold deviation has been applied as a threshold to denote compliance between observed and predicted mixture toxicity in the present study in accordance with previous studies (Belden *et al.*, 2007 and Coors and Frische, 2011).

Fig. 2.4 shows f_a-CI plots of binary mixtures for single-species and activated sludge tests. Twenty out of twenty-nine combinations that evaluated the median-effect principle observed effective concentration within a factor of 2 of predicted values (CI values between 0.5 and 2) on the f_a = 0.5 (further data Table 2.7), where the inflexibility of the median-effect principle matters least (Backhaus, 2014). Hence, the combination effects of a given preservative in a complex industrial wastewater for almost 70% of mixtures could be approximated well by the median-effect principle, a special case of the more general concept of CA (Backhaus, 2014). Specifically, 86% of the studied binary combinations could be accurately predicted by CA concept for single-species tests. These results are in line with the review on the predictive power of CA for pesticide mixtures performed by Belden *et al.* (2007), which demonstrated that in the majority of experiments (80% and more), mixture toxicity predictions based on CA deviated from the observed mixture toxicity by less than factor 2.

Nevertheless, the CA model was not able to correctly describe the mixture toxicity of nine of the combinations. In particular, BAC+IWW, ZPT+IWW, PPB+IWW toxicities for *V. fischeri* were overestimated with CI values higher than 2, while binary combinations of IPBC, CMI/MI, BNP, BAC, ZPT and PPB with IWW for activated sludge were underestimated, yielding CI values between 32 and 0.47 at f_a = 0.5. Toxicity mixtures mispredicted by CA were assessed with the alternative concept of IA. Fig. 2.5 displays the comparison for *V. fischeri* and activated sludge assays of CA- and IA-prediction with experimental data from the previously mentioned binary mixtures. CA and IA predict similar toxicities despite their mutually exclusive concepts, especially at low effects levels. This finding can be explained as a consequence of the similar shape and slope of the concentration-response curves of individual biocides (see Figs. 2.1 and 2.2) and do not involve any mechanistic implication (Backhaus *et al.*, 2004).

For *V. fischeri* test, IA and CA did not differ in predicting mixture toxicities at EC_{50} level, contrary to what is expected in most situations in which CA is considered the conservative model (Kortenkamp *et al.*, 2009). This fact is due to the steepness of the concentration-response curves of single substances, which is known to be the major

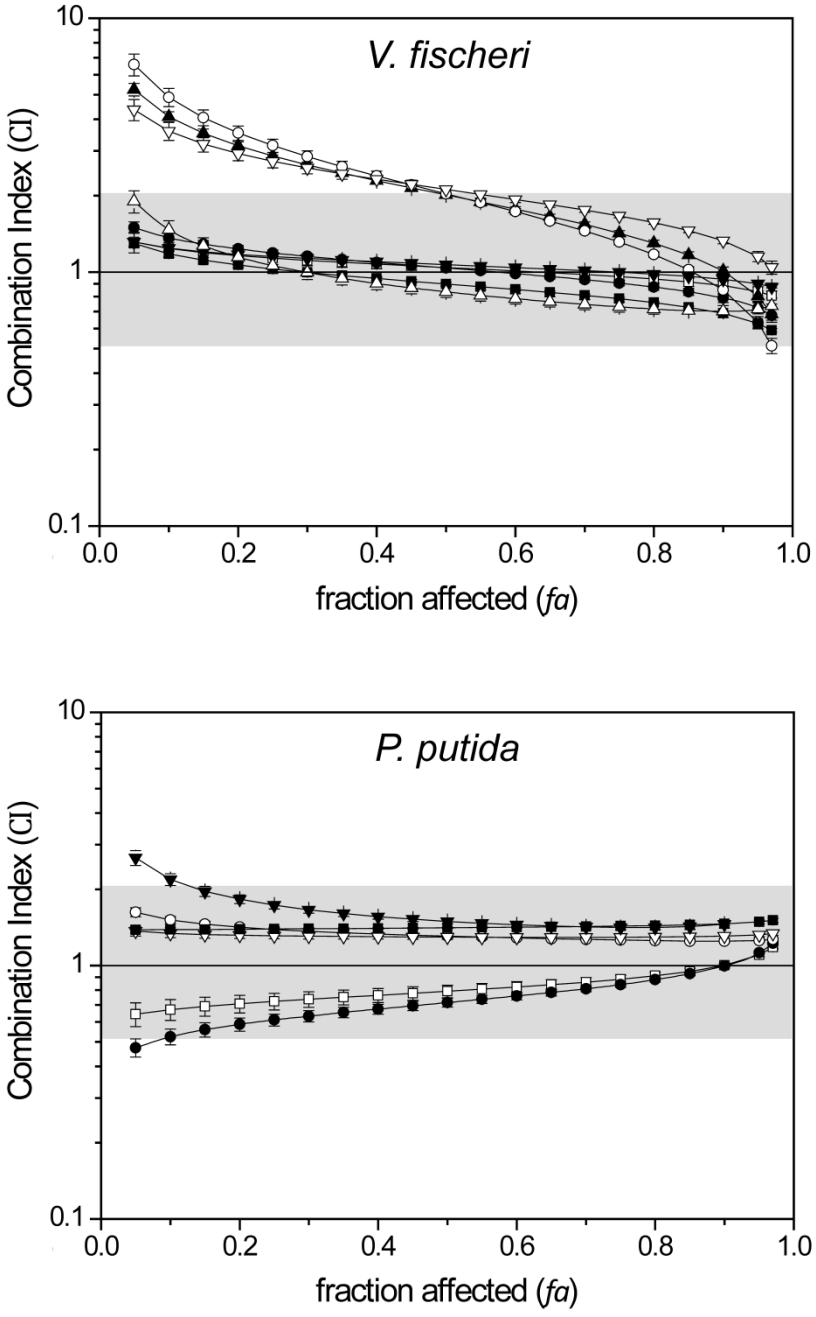


Fig. 2.4 Combination Index plot (f_a-CI plot) for the used bioassays of binary mixtures (preservative+IWW): IPBC+IWW (▼), CMI/MI+IWW (●), BNP+IWW(□), DIU+IWW(■), BAC+IWW(▽), ZPT+IWW (○), PPB+IWW (▲), TCS+IWW (Δ). CI values are plotted as function of the fractional inhibition of bioluminescence/growth (f_a) by computer simulation (CompuSyn). CI between 0.5 and 2 indicate compliance between observed and predicted mixture toxicity by median-effect principle with CA. The vertical bars indicate 95% confidence intervals for CI values based on SDA (Sequential Deletion Analysis) (Chou and Martin, 2005) (continued on next page).

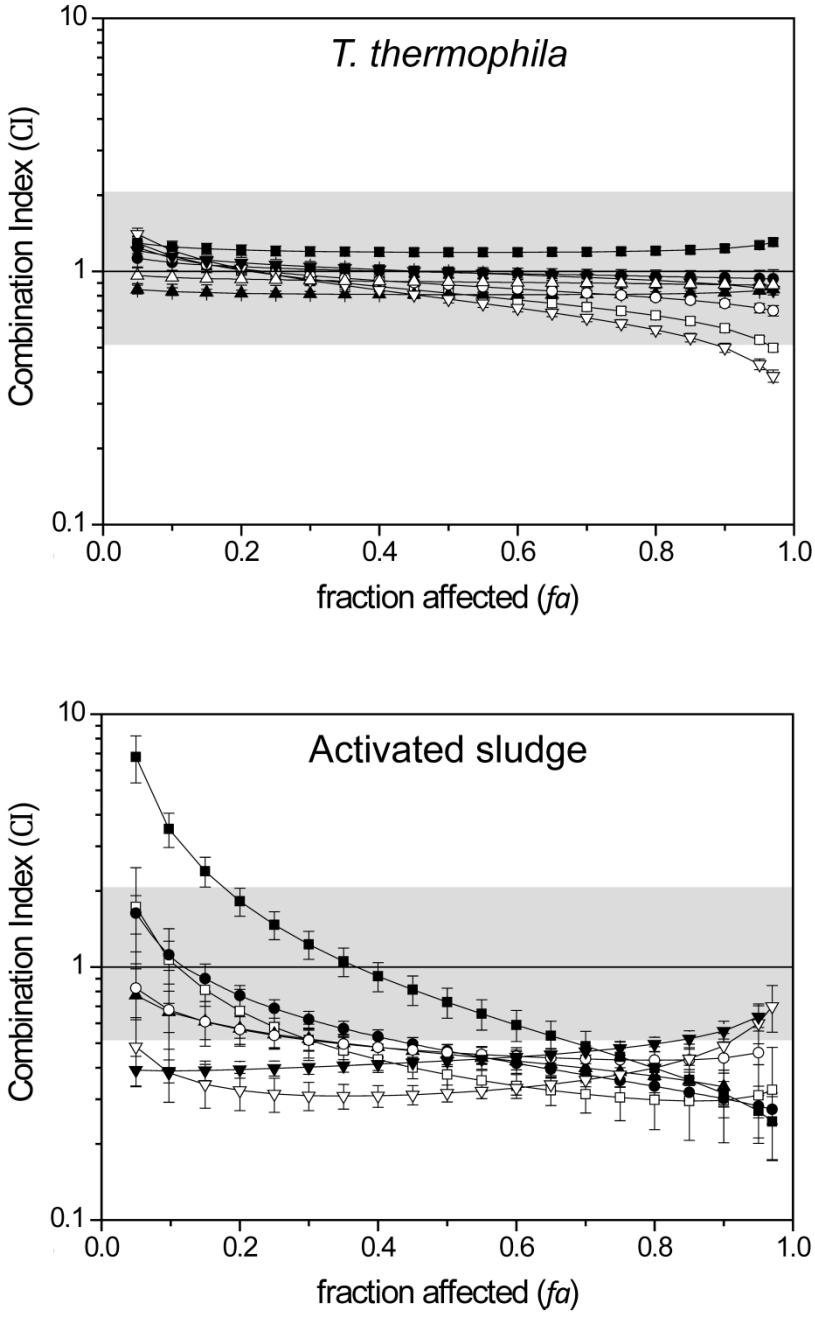


Fig. 2.4 Combination Index plot (f_a-CI plot) for the used bioassays of binary mixtures (preservative+IWW): IPBC+IWW (▼), CMI/MI+IWW (●), BNP+IWW (□), DIU+IWW(■), BAC+IWW(▽), ZPT+IWW (○), PPB+IWW (▲), TCS+IWW (Δ). CI values are plotted as function of the fractional inhibition of bioluminescence/growth (f_a) by computer simulation (CompuSyn). CI between 0.5 and 2 indicate compliance between observed and predicted mixture toxicity by median-effect principle with CA. The vertical bars indicate 95% confidence intervals for CI values based on SDA (Sequential Deletion Analysis) (Chou and Martin, 2005).

Table 2.7 Observed and predicted mixture toxicity under Concentration Addition (CA) and Independent Action (IA) concepts of binary combinations (preservative+IWW) for the studied bioassays. Deviation between observed and predicted mixture toxicities higher than two-fold is emphasized in bold. EC_{50} values of preservatives and IWW are expressed as $mg \cdot L^{-1}$ and %, respectively.

Mixture	*V. fischeri*			*P. putida*		*T. thermophila*		Activated sludge		
	Observed	CA	IA	Observed	CA	Observed	CA	Observed	CA	IA
IPBC+IWW	2.08 + 0.029	1.97 + 0.027	–	78.3 + 48.1	52.4 + 32.2	0.057 + 0.162	0.058 + 0.163	5.50 + 2.52	**12.9 + 5.90**	11.0 + 5.03
CMI/MI+IWW	0.031 + 0.027	0.034 + 0.029	–	0.182 + 23.1	0.253 + 32.3	0.097 + 0.158	0.098 + 0.159	0.710 + 2.75	**1.52 + 5.91**	1.24 + 4.82
BNP+IWW	0.097 + 0.028	0.093 + 0.027	–	0.494 + 25.5	0.624 + 32.2	1.95 + 0.127	2.38 + 0.156	2.28 + 2.22	**12.9 + 5.91**	4.55 + 4.44
DIU+IWW	20.1 + 0.021	25.8 + 0.027	–	121 + 45.3	85.5 + 32.2	23.2 + 0.179	19.5 + 0.150	122 + 4.29	168 + 5.91	126 + 4.45
BAC+IWW	0.294 + 0.057	**0.136 + 0.026**	**0.140 + 0.027**	5.43 + 41.8	4.19 + 32.3	1.64 + 0.125	2.11 + 0.161	0.802 + 2.76	**6.05 + 5.90**	1.30 + 4.46
ZPT+IWW	0.077 + 0.057	**0.038 + 0.028**	**0.035 + 0.026**	0.084 + 42.1	0.064 + 32.2	0.017 + 0.143	0.019 + 0.162	0.423 + 2.71	**0.921 + 5.90**	0.749 + 4.80
PPB+IWW	5.67 + 0.055	**2.81 + 0.027**	**2.51 + 0.024**	–	–	3.95 + 0.132	4.88 + 0.163	94 + 2.67	**207 + 5.90**	186 + 5.29
TCS+IWW	0.086 + 0.025	0.103 + 0.030	–	–	–	0.578 + 0.151	0.635 + 0.166	–	–	–

factor determining the relation between the EC_{50} values predicted by CA and IA (Boedeker *et al.*, 1993 and Drescher and Boedeker, 1995). Backhaus *et al.* (2004) showed that both concepts predict equal mixture toxicity with ratio EC_{50}/EC_{05} = 13.5, which corresponds to a median-effect parameter m = 1.13. If the steepness for the concentration-response curve of the mixture components is lower, CA predicts a lower EC_{50} for the mixture than IA and *vice versa* (Brosche and Backhaus, 2010). Specifically, m values of IWW (1.18), BAC (1.50), ZPT (1.12) and PPB (1.03) were in the interval 1.03–1.50. Thus, both CA and IA were not able to predict mixture toxicities for the binary combinations of BAC, ZPT and PPB with IWW for *V. fischeri* test. Observed mixture toxicities were slightly less toxic than those predicted by both models, displaying an antagonistic effect. The antagonism of these mixtures might be explained considering that BAC, ZPT and PPB are membrane active agents that can be inactivated by surfactants (Rieger and Rhein, 1997). In the case of BAC, whose mechanism of action is based on the interaction of bipolar quaternary ammonium compound with the bacterial phospholipid bilayer, the occurrence of anionic surfactants in IWW (179 mg·L^{-1}) may lead to ion pair formation, losing the bipolar structure and BAC bioactivity. Sütterlin *et al.* (2008) observed the same behaviour for *V. fischeri* exposed to mixtures of BAC and different anionic surfactants. In a similar way, the antagonism between PPB and an anionic surfactant (perfluorooctane sulfonic acid) has been reported to *Anabaena* sp. CPB4337 (Rodea-Palomares *et al.*, 2012). PPB can also be inactivated by non-ionic surfactants (Denyer, 1995), which were present at a significant concentration in the studied IWW (109 mg·L^{-1}).

For activated sludge, it is interesting to note that IA predicts a higher toxicity than CA at EC_{50} values (Table 2.7). As it has already explained, this fact is due to the low steepness of the concentration-response curves of the single substances. In fact, m values of individual compounds are substantially smaller than the critical threshold of 1.13 (m <1.13 equivalent to EC_{50}/EC_{05} >13.5, Backhaus *et al.*, 2004). Similar results were found for the prediction of mixture toxicity of antibiotic combinations on bacterial communities from artificial (STP in Christensen *et al.*, 2006) and natural environment (lake in Brosche and Backhaus, 2010). In this study, higher predictive power was

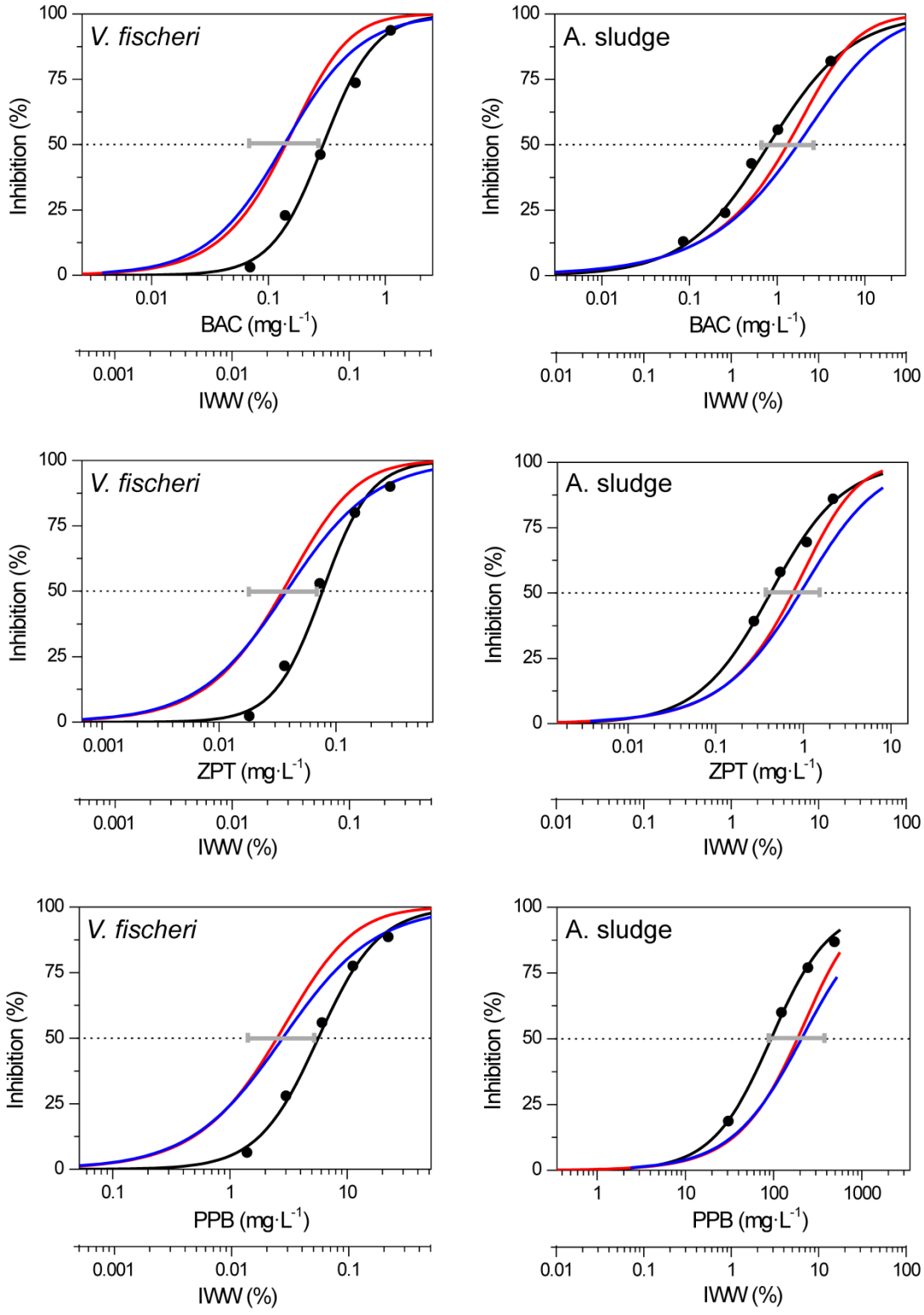


Fig. 2.5 Predicted and observed concentration-response curve of BAC+IWW (A,B), ZPT+IWW (C,D), PPB+IWW (E,F), IPBC+IWW (G), CMI/MI+IWW (H), BNP+IWW (I) and DIU+IWW (J) mixtures. Solid lines give CA (blue) and IA (red) prediction and median-effect equation fit (black). Two-fold deviation as a threshold to denote compliance between observed and predicted mixture toxicity is represented with a grey line (continued on next page).

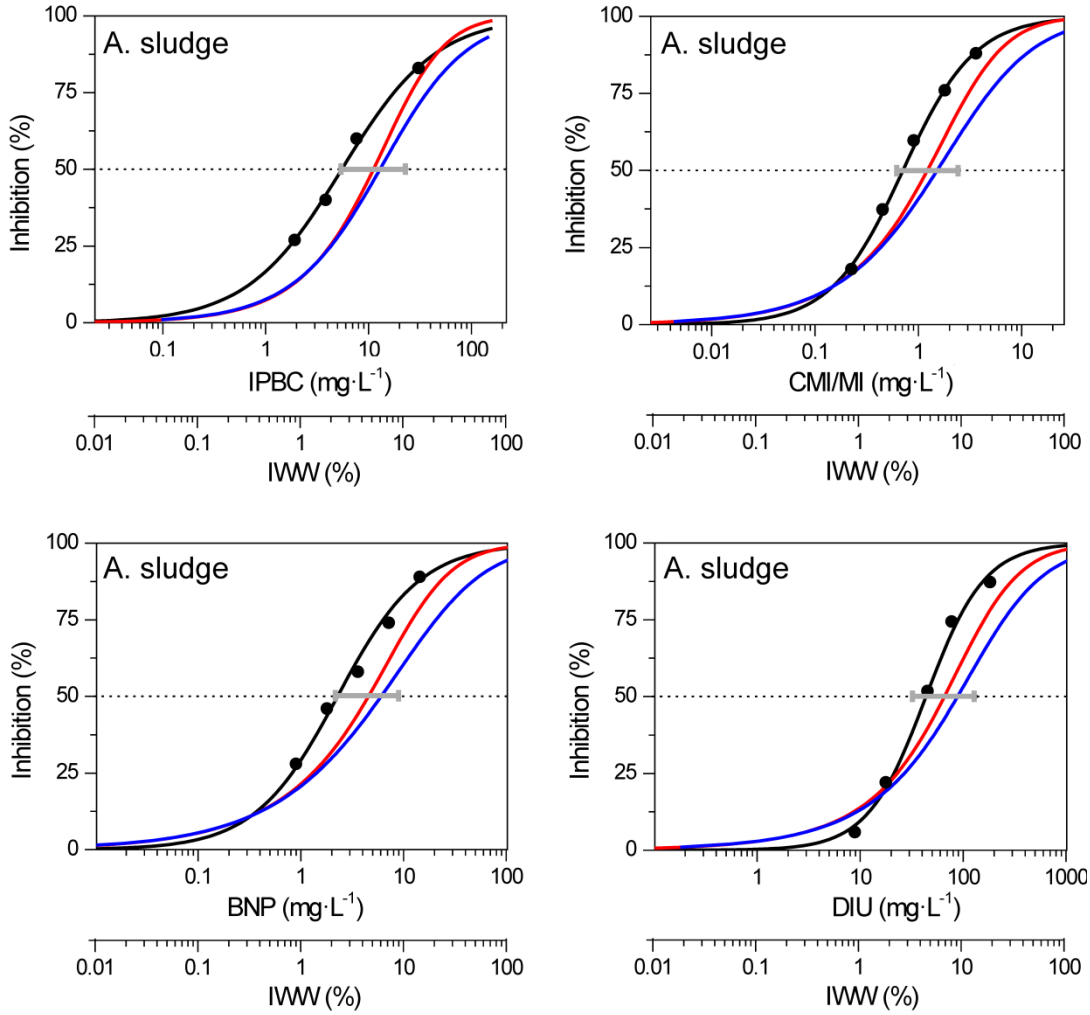


Fig. 2.5 Predicted and observed concentration-response curve of BAC+IWW (A,B), ZPT+IWW (C,D), PPB+IWW (E,F), IPBC+IWW (G), CMI/MI+IWW (H), BNP+IWW (I) and DIU+IWW (J) mixtures. Solid lines give CA (blue) and IA (red) prediction and median-effect equation fit (black). Two-fold deviation as a threshold to denote compliance between observed and predicted mixture toxicity is represented with a grey line.

illustrated for IA than CA concept for the combination effects of preservatives in a complex industrial wastewater on a biological community. This was the case independent of whether or not these mixtures had been well approximated to CA such as DIU+IWW (Fig. 2.5J). This fact could be explained by the complexity of IWW on a biological community. Activated sludge biomass is composed by flocs that are biological aggregates containing several levels of organization and porous structures in which cells are embedded in a polymer matrix (Henriques and Love, 2007). Reduced access of biocides to microbial cells because of the chemical interactions between extracellular

polymeric substances and antimicrobial molecules is one of the proposed resistance mechanisms (Russell, 2003 and Henriques and Love, 2007). However, the higher concentration of monovalent cations relative to the concentrations of divalent cations and the occurrence of poor biodegradable surfactants in the IWW (Table 2.2) could cause the disruption of exopolymer bridging thus, microorganisms would not adhere to each other and loose one floc resistance mechanism (Jenkins *et al.*, 2003).

Therefore, the application of CA as the first predictive approach to mixture whose toxicity is actually better described by IA would hence lead to a slight overestimation of the mixture toxicity, and it was therefore suggested to apply CA as a somewhat conservative default approach for the predictive assessment of mixture toxicity in general (Backhaus and Faust, 2012). However, the toxicity of preservatives with a complex industrial wastewater towards intricate microbial communities might be an exception from this pattern, as the vast differences in sensitivity of the involved species seems to lead to extraordinary flat concentration-response curves.

4. Conclusions

The studied preservatives (iodopropynyl butylcarbamate, bronopol, diazolidinyl urea, benzalkonium chloride, zinc pyrithione, propylparaben, triclosan and a mixture of methylchloroisothiazolinone and methylisothiazolinone) showed considerable aquatic toxicity in single-species tests and biological community activated sludge assay, but with a marked difference in potency. In fact, benzalkonium chloride, zinc pyrithione, iodopropynyl butylcarbamate and triclosan as well as the mixture of the tested preservatives displayed a potential risk to municipal STP performance. Among them, benzalkonium chloride is shown as the most problematic compound as is the risk driver of the mixture of preservatives in the activated sludge assay.

The degree of biological complexity of the used bioassays influences on the more suitable concept to predict the joint toxicity of the tested compounds with a cosmetic industry wastewater. In spite of the mixture toxicity in single-species tests can be accurately predicted by Concentration Addition (CA), in the biological community

activated sludge assay the prediction power is lower, and the alternative concept of Independent Action (IA) should be considered.

These results highlight that the toxic effects of preservatives towards activated sludge need to be carefully evaluated and that the potential risk management options should be studied. Special attention may be placed on benzalkonium chloride, on which should be assessed whether there is a need to perform mitigation measures such as source control by targeted restrictions or wastewater pre-treatment before activated sludge process.

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Chapter 3

Ozonation as pre-treatment of activated sludge process of wastewater containing benzalkonium choloride and NiO nanoparticles

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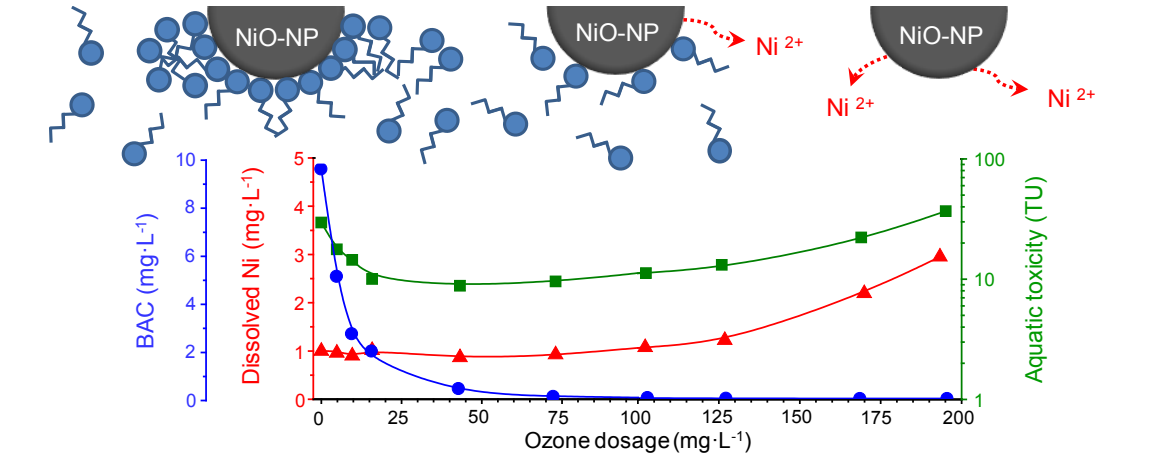
Chemical Engineering (accepted)

Graphical Abstract



OZONATION AS PRE-TREATMENT OF ACTIVATED SLUDGE PROCESS OF WASTEWATER CONTAINING BENZALKONIUM CHLORIDE AND NiO NANOPARTICLES

Abstract

The continuous ozonation of benzalkonium chloride (BAC) and nickel oxide nanoparticles (NiO-NPs) has been performed in a synthetic water matrix and in a sewage treatment plant influent. This study aims to assess ozonation as pre-treatment of an activated sludge process, with emphasis on the toxicity of treated water. BAC was completely removed in synthetic matrix independently of the presence of NiO-NPs, although the ozone dose was influenced by NPs co-occurrence. The extent of mineralization was limited and a number of intermediate transformation products (TPs) appeared, twelve of which could be identified. The degradation pathway was shown to initiate both on the hydrophobic (alkyl chain) and hydrophilic (benzyl and ammonium moiety) region of the surfactant. The reactions on the hydrophilic region were affected by the presence of NiO-NPs as a consequence of the adsorption of BAC onto NP surface via the aromatic group. Water matrix strongly influenced BAC depletion. The aquatic toxicity of treated mixtures was assessed using a battery of bioassays composed of single-species tests (the bacteria *Vibrio fischeri* and *Pseudomonas putida* and the protozoan *Tetrahymena thermophila*), as well as on activated sludge using a resazurin-based assay. Although, BAC showed considerable aquatic toxicity in all bioassays, ozonation decreased the toxic effects of treated water samples at ozone dosages below those required for total BAC depletion. Further treatment would not be justified, neither for a significant increase in BAC abatement nor concerning the toxicity of treated wastewater, which increased as a result of nickel leaching from the NPs.

1. Introduction

Quaternary ammonium compounds (QACs) are an important class of industrial chemicals extensively used in domestic and industrial applications such as detergents, emulsifiers, fabric softeners, disinfectants, corrosion inhibitors and processing aids (Ismail *et al.*, 2010). As a result, about 75% of QAC consumed end up in wastewater treatment systems (Zhang *et al.*, 2011). In fact, their concentration in hospital wastewater, sewage treatment plant (STP) influents and effluents, and sewage sludge has been reported as 0.05–6.03 $mg \cdot L^{-1}$, 25–300 $\mu g \cdot L^{-1}$, 0.3–3.6 $\mu g \cdot L^{-1}$ and 22–103 $mg \cdot kg^{-1}$, respectively (Kümmerer, 1997, Clara *et al.*, 2007 and Martínez-Carballo *et al.*, 2007a,b). Because of their chemical properties, QACs rapidly and strongly sorb on suspended solids (Zhang *et al.*, 2011). Sorption on (bio)solids combined with the persistence of QAC, result in their accumulation on the biomass and their transfer to anaerobic digesters as part of the primary and waste activated sludge. Among QACs, benzalkonium chloride (BAC) has been shown to pose a potential risk for the activated sludge (Carbajo *et al.*, 2015a). It is toxic to aquatic organisms at environmental relevant concentration and is classified as *very toxic to aquatic life* according to Regulation (EC) No. 1272/2008 (ECHA, 2015). Furthermore, it has been reported that the widespread use of biocides such as BAC could select for antibiotic-resistant bacteria (Kümmerer, 2009).

On the other hand, the increasing use of engineered nanoparticles (NPs) in industrial and household applications will very likely lead to the release of such materials into sewage collection systems (Nowack and Bucheli, 2007 and Keller and Lazareva, 2014). Once in the STP, the majority of NPs are captured through adhesion into the sludge and removed from the water stream (Limbach *et al.*, 2008, Kiser *et al.*, 2009 and Westerhoff *et al.*, 2011). However, the presence of surfactants in STP influents, such as QACs, has been shown to hinder the removal of NPs from water as a result of the modification of their surface and interfacial properties (Limbach *et al.*, 2008, Brar *et al.*, 2010 and Kiser *et al.*, 2010). QACs adsorption on NP surfaces reduces the tendency of NPs to agglomerate and stick to the sludge. Consequently, QAC+NPs remain suspended in the water stream through the STP, are able to affect the microorganisms in secondary

treatment processes, increase the turbidity, foul membranes and influence the efficiency of tertiary disinfection processes (Brar *et al.*, 2010). Besides, QAC+NP eventually may leave STP, with NPs acting as a delivery vehicle for QAC into aquatic environments.

The use of ozonation as chemical pre-treatment followed by a biological process has been shown to be a suitable technology for the removal of pollutants which cause toxic effects on microorganisms (Ikehata and El-Din, 2004 and Oller *et al.*, 2011). Ozonation has several advantages over conventional chemical oxidation processes using potassium permanganate or chloride, including higher oxidation potential, absence of potentially carcinogenic chlorinated by-products, and short life time of the oxidant, which would be toxic to microorganisms in subsequent biological treatments (Beltrán *et al.*, 2000 and Guieysse and Norvill, 2014). BAC degradation has not been thoroughly studied, with most studies performed in semi-batch conditions and synthetic water matrix and focused on the degradation and/or mineralization of the target pollutant (Dantas *et al.*, 2009), and not on the biological effects of oxidation by-products. Ozonation as chemical pre-treatment should remove toxic pollutants without producing transformation products (TPs), which could cause an adverse effect on the microorganisms present in the subsequent biological treatment (Oller *et al.*, 2011 and Guieysse and Norvill, 2014). Continuous treatment is a closer approximation to full-scale systems than batch experiments and allows a better understanding of the fate of pollutants under oxidizing conditions, the influence of water matrix and the toxicity of treated wastewater (Huber *et al.*, 2005, Nöthe *et al.*, 2009 and Katsoyiannis *et al.*, 2011).

The study aims to assess the continuous ozonation of a wastewater contaminated with BAC and NiO-NPs intended as pre-treatment of an activated sludge process. The influence of water matrix was explored using real STP influent. The toxicity of ozone treated wastewater was monitored using a battery of bioassays, composed of single-species tests (the bacteria *Vibrio fischeri* and *Pseudomonas putida* and the protozoan *Tetrahymena thermophila*), and an activated sludge resazurin-based assay.

2. Materials and methods

2.1. Materials

Benzalkonium chloride (BAC; ≥95%, consisting of homologues of different alkyl chain lengths, mainly, ~60% C12 and ~40% C14) and nickel (II) oxide nanopowder (NiO-NPs; ≤50 nm particle size, 99.8% trace metals basis) were purchased from Fluka and Sigma-Aldrich, respectively. Raw water was prepared with an initial BAC concentration of 10 $mg \cdot L^{-1}$, with and without the addition of NiO-NPs (20 $mg \cdot L^{-1}$). A 1 000 $mg \cdot L^{-1}$ of NiO-NPs suspension in water was prepared by sonication for 15 min at 20 KHz and 200 $W \cdot L^{-1}$ (BioBlock Scientific, France). 500 mL of the concentrated suspension was added to 25 L of the water to be treated in order to achieve 20 $mg \cdot L^{-1}$. Water was then kept under agitation at 400 rpm with a two-arm propeller 3 h prior to the ozonation with the aim of reaching the adsorption equilibrium of BAC on NiO-NPs.

Two different matrices were used: a synthetic water matrix and a municipal STP influent. The synthetic matrix was prepared in ultrapure water (resistivity ≥18 MΩ·cm at 25ºC) with the required amount of sodium bicarbonate to equal the alkalinity and pH values of the STP influent. Raw wastewater was collected from the outlet of mechanical preliminary treatment and before biological treatment in the Carrión de los Céspedes Experimental Plant in Seville (Spain), which treats domestic wastewater and has a capacity of 2 500 population equivalent (Fahd *et al.*, 2007). Wastewater was autoclaved at 121ºC during 20 min before use. Details on wastewater characterization are shown in Table 3.1.

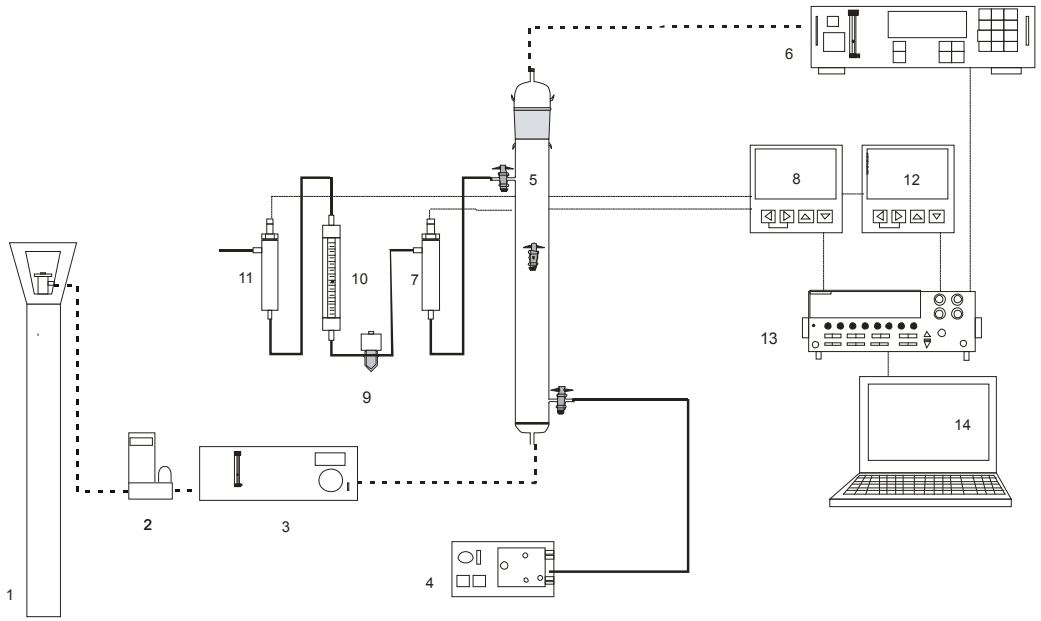
Table 3.1 Main physico-chemical parameters of STP influent.

pH	8.3	Na^+ ($mg \cdot L^{-1}$)	110	Cr ($\mu g \cdot L^{-1}$)	2.08
Conductivity ($\mu S \cdot cm^{-1}$)	1 234	NH_4^+ ($mg \cdot L^{-1}$)	53.8	Co ($\mu g \cdot L^{-1}$)	0.64
TSS ($mg \cdot L^{-1}$)	108	K^+ ($mg \cdot L^{-1}$)	26.2	Ni ($\mu g \cdot L^{-1}$)	4.04
VSS ($mg \cdot L^{-1}$)	93.1	Mg^{2+} ($mg \cdot L^{-1}$)	19.3	Cu ($\mu g \cdot L^{-1}$)	32.4
Turbidity (NTU)	145	Ca^{2+} ($mg \cdot L^{-1}$)	41.4	Zn ($\mu g \cdot L^{-1}$)	88.0
COD ($mg \cdot L^{-1}$)	121	Cl^- ($mg \cdot L^{-1}$)	127	As ($\mu g \cdot L^{-1}$)	1.66
DOC ($mg \cdot L^{-1}$)	61.2	NO_2^- ($mg \cdot L^{-1}$)	0.51	Se ($\mu g \cdot L^{-1}$)	ND
BOD_5 ($mg \cdot L^{-1}$)	30	NO_3^- ($mg \cdot L^{-1}$)	0.10	Cd ($\mu g \cdot L^{-1}$)	0.13
$SUVA_{254}$* ($L \cdot mg\ C^{-1}\ m^{-1}$)	0.91	PO_4^{3-} ($mg \cdot L^{-1}$)	14.9	Hg ($\mu g \cdot L^{-1}$)	ND
Alkalinity (mg $CaCO_3 \cdot L^{-1}$)	372	SO_4^{2-} ($mg \cdot L^{-1}$)	56.0	Pb ($\mu g \cdot L^{-1}$)	3.95

*Specific ultraviolet absorption at 254 nm; ND: not detected

2.2. Experimental procedure

The experiments were carried out in a cylindrical reactor made of Pyrex (internal diameter of 6.0 cm and working height of 51 cm) with a total working volume of 1.44 L, which operated in continuous co-current mode (Scheme 3.1). Water flow rate was 142 $mL \cdot min^{-1}$ (Gilmont rotameter) and gas flow rate was 390 $mL \cdot min^{-1}$ (Aalborg mass flow controller). The mixture of ozone and oxygen was produced by a corona discharge ozonator (Anseros ozone generator COM-AD-02) fed by high-purity oxygen. Gas mixture was introduced into the reactor by means of a sintered glass plate (porosity no. 3), which was used as gas diffuser at the bottom of the column. Inlet and outlet ozone gas concentrations (Anseros NDUV ozone GM-PRO analyzer), dissolved ozone (Mettler Toledo-Thomton dissolved ozone sensor), pH and temperature (EasyfermPlus VP 120 Hamilton pH sensor) were continuously monitored (Keithley 2700 Data Acquisition System) and recorded in a computer.



Scheme 3.1 Experimental set-up. (1) oxygen cylinder, (2) mass flow controller, (3) ozone generator, (4) peristaltic pump, (5) bubble column, (6) ozone gas analyzer, (7) dissolved ozone sensor or conductometric probe, (8) dissolved ozone or conductivity transmitter, (9) needle valve, (10) rotameter, (11) pH sensor, (12) pH transmitter, (13) data acquisition system, (14) computer. Water line is represented as solid line, gas line as dashed line and electrical wiring as dotted line.

The hydrodynamic behaviour of the ozone reactor was characterized using sodium chloride as a non-reactive tracer. A pulse-dose method was used wherein an instantaneous dose of concentrated sodium chloride solution (70 g·L^{-1}) was injected at the inlet of the reactor. The tracer concentration was determined with a conductometric probe (Alpha CON 190, Thermo Scientific) at the outlet of the bubble column and a computer recorded the signals from the conductivity after being captured every 5 seconds by the data acquisition system (Scheme 3.1).

Table 3.2 shows the results of the hydrodynamic parameters calculated from the data obtained in the tracer tests.

Table 3.2 Summary of tracer test operating conditions and results.

Mode	V_R (L)	υ_g (L·min^{-1})	υ_l (L·min^{-1})	τ (min)	$\bar{t}$ (min)	t_{10} (min)	$\bar{t}/\tau$	t_{10}/τ	n
Co-current	1.44	0.390	0.142	10.1	10.3	1.26	1.02	0.125	1.13

V_R reactor working volume
υ_g gas flow rate
υ_l liquid flow rate
τ hydraulic residence time
$\bar{t}$ mean residence time
t_{10} time for 10% of tracer to exit reactor
n equivalent number of continuously stirred tank reactors

Mean residence time ($\bar{t}$) of the reactor was 10.3 min, which is a value close to the theoretical hydraulic residence time (τ). This fact indicates that there are no dead zones within the bubble column because $\bar{t}/\tau$ was considered as an index reflecting the percentage of stagnant space in a reactor (Roustan *et al.*, 1996 and Chiang *et al.*, 1999). It has also been suggested that the t_{10}/τ (t_{10} is the time required for 10% of the total tracer mass to leave the reactor) is measurement of the degree of short-circuiting in the reactor (Langlais *et al.*, 1991). As seen in Table 3.2, t_{10}/τ value is slightly higher than the theoretical value for an ideal mixed flow reactor ($F(t) = 0.1;\ t_{10}/\tau = 0.105$), which indicates that there is no evidence of short-circuiting fluid within the bubble column (Chiang *et al.*, 1999).

The flow pattern of the ozone reactor was studied using the tanks-in-series model (Levenspiel, 1999), which assumes that the flow through the reactor can be characterise by a series of n equalized continuously stirred tank reactors (n-CSTRs). The number of reactors in series (n) calculated according to the procedure described in the literature (Burrows *et al.*, 1999 and Levenspiel, 1999) was 1.13. Fig. 3.1 shows CSTR theoretical, empirical (n-CSTR in which n = 1.13) and experimental $E(\theta)$ curves, which display marginal differences. The results indicated that the bubble column under the operational conditions can be approached to a perfect CSTR. It is generally accepted that short columns with intense gas phase hydrodynamics can be assimilated to a CSTR due to the bubble back mixing (Asenjo and Merchuck, 1995).

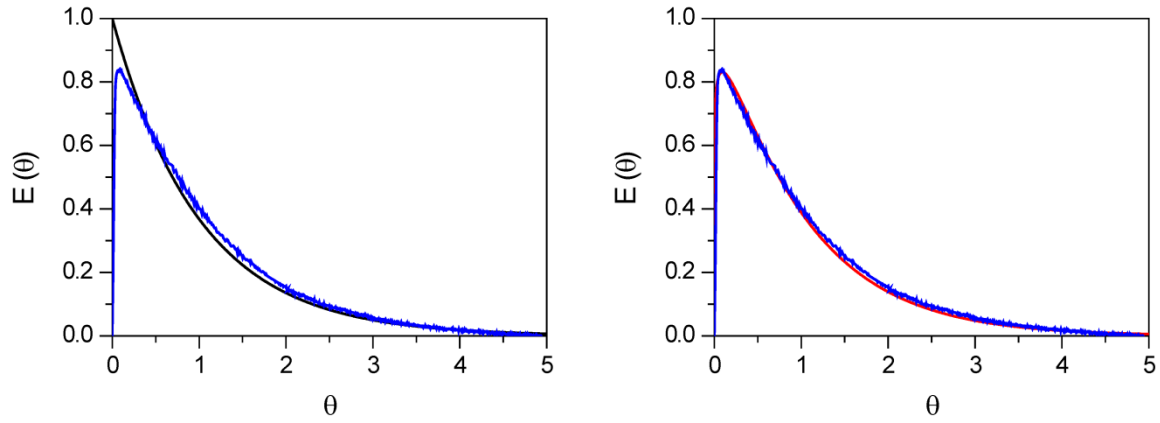


Fig. 3.1 $E(\theta)$ curve for CSTR theoretical (black line), 1.13-CSTRs (red line) and experimental (blue line).

During the ozonation runs, the inlet ozone dosage was stepwise increased from 5 to 300 milligrams of ozone per liter of wastewater (mg·L^{-1}). For the different ozone dosages, samples were withdrawn for analysis at the column outlet once the stationary state was reached. This was ensured by circulating four times the hydraulic retention volume after constant ozone concentration was obtained both in the liquid and gas phases at the column outlet. Assuming CSTR behaviour, the amount of ozone consumption at the stationary state can be obtained from the following mass balance:

$$Consumed\ O_3 = F_{O_3}^{gas,in} - F_{O_3}^{gas,out} - F_{O_3}^{liq,out} \quad (3.1)$$

in which F_{O_3} is the rate of ozone entering the system in the gas phase (gas, in) or leaving it either in the exhaust gases (gas, out) or dissolved in water (liq, out).

2.3. Analytical methods

BAC in 2.5 mL samples was extracted with a mixture of 1 mL of silver nitrate (100 mM), 1.5 mL of acetonitrile and 2.5 mL of ethyl acetate. BAC concentration was measured using an Agilent 1100 Series LC unit equipped with a reversed-phase C18 analytical column (Phenomenex Luna SCX, 250 × 4.6 mm, 5 µm) connected to a time-of-flight mass spectrometer (TOF/MS, Agilent Technologies). A gradient elution was applied using 5% acetonitrile in HPLC-grade water [A] and 5% HPLC-grade water in acetonitrile [B], both with 0.1% formic acid, as mobile phases at a flow rate of 1 mL·min^{-1} (gradient curve: 0–5 min, 20% [B]; 5–10 min, linear change from 20 to 40% [B]; 10–15 min, 40% [B]; 15–20 min, linear change from 40 to 60% [B]; 20–30 min, 60% [B]; 30–35 min, linear change from 60 to 100% [B]; 35–40 min, 100% [B]; post run-time, 40–55 min). The column was maintained at 30ºC. MS analysis was conducted by electrospray ionization (ESI) in positive mode at 70 eV fragmentation voltage with a mass scan range of *m/z* 100–1 000. The drying gas (nitrogen) flow was 13 L·min^{-1} at 350ºC, the nebulizer pressure was 50 psi, and the capillary voltage was 4 000 V. Described analytical method was adapted from Tezel (2009).

Raman spectra of BAC adsorbed onto NiO-NPs were recorded using a Thermo Scientific DXRxi Raman imaging microscope (Waltham, MA, USA). Measurements were collected using a 532 nm emitting laser, power 0.1 mW, frequency 0.5 Hz and confocal pinhole size of 50 µm. The microscope was set to 100x magnification. Spectral acquisition times were 2 seconds and 3 scans were averaged in order to improve the signal to noise ratio. The wet NiO-NPs collected by centrifuge were placed on quartz slides and allowed to dry before measurements. Control experiments were also performed using the pure NiO-NPs or background BAC solution deposited onto a quartz plate surface.

Water samples were analyzed for their nickel oxide and dissolved nickel content using an Agilent 7700x ICP-MS operating at 3 MHz in helium cell gas mode. NiO-NPs were quantified after digestion to ensure the full dissolution of nanoparticles. 2 mL liquid suspension were digested using 8 mL nitric acid (Sigma-Aldrich, suprapur) and 2 mL hydrogen peroxide heating up to 150ºC for 30 min in a microwave system (Ethos One, Milestone) and finally diluted to 25 mL with ultrapure water. To determine dissolved nickel, water samples were centrifuged (15 000 g for 30 min in Heraeus-Multifuge 3L-R centrifuge) and the supernatant was filtered through 0.2 µm filter. The size distribution of nanoparticles was obtained using dynamic light scattering (DLS, Malvern Zetasizer Nano ZS). ζ-potential was measured via electrophoretic light scattering combined with phase analysis light scattering in the same instrument equipped with a Malvern autotitrator MPT-2.

Cationic surfactant concentrations were determined spectrophotometrically with bromophenol blue method (Hach-Lange LCK 331). Dissolved Organic Carbon (DOC) analyses were performed on a Shimadzu TOC-V_{CSH} TOC analyzer. Carboxylic acids were measured by a Dionex DX120 IC. Oxalic acid concentration was determined by IonPac AS9-HC analytical column (4 × 250 mm) with ASRS-Ultra suppressor. Acetic and formic acids were measured using an IonPacICE analytical column (9 × 250 mm) with AMMS-ICE II suppressor. Inorganic ions were determined by means of a Metrohm 861 Advance Compact IC; Metrosep A Supp 7-250 and Metrosep C3 analytical columns were used in anion and cation analysis, respectively.

2.4. Procedures for aquatic toxicity tests

The aquatic toxicity of water samples was determined using a battery of bioassays, composed of single-species tests of two bacteria (*V. fischeri* and *P. putida*) and one protozoan (*T. thermophila*), which represent the microorganisms present in activated sludge, and a whole biological community assay using activated sludge.

V. fischeri acute test measures the decrease in bioluminescence induced in the cell metabolism. The bioassay was performed according to ISO 11348-3 standard

protocol (ISO, 2007) using the commercial BioFix®Lumi test (*V. fischeri*, NRRL-B 11177 from Macherey-Nagel, Germany). The test was carried out in 96-well white polypropylene microplate. 100 µL of test solution (2% w/v NaCl and pH 7.0 ± 0.5) was transferred into each well, which were supplemented with 100 µL of bacterial suspension. Light was measured at 15 ± 1ºC after 30 min by means of a Fluoroskan Ascent FL microplate luminometer (Thermo Scientific). *P. putida* test determines the inhibitory effect of a water sample on the bacteria (*P. putida*, NCIB 9494 from CECT, Spain) by means of cell growth inhibition. The bioassay was performed according to ISO 10712 guideline (ISO, 1995). Bacterial culture was exposed to test solutions at 23 ± 1ºC for 16 h in glass incubation vials which were constantly shaken in the dark. The cell growth was determined by optical density (λ 600 nm) in 96-well clear microplate (200 µL test suspension per well) using a Rayto RT-2100C microplate reader. Growth inhibition assay with the ciliate protozoa *T. thermophila* was performed according to the Standard Operational Procedure Guideline of Protoxkit F™ (1998). The test is based on the turnover of substrate into ciliate biomass. Substrate was purchased from MicroBioTest Inc. (Belgium) whereas *T. thermophila* (SB 210) was kindly supplied by D. Cassidy-Hanley (Tetrahymena Stock Center, Cornell University, USA). Ciliates were incubated with water samples and food suspension in test vessels at 30 ± 1ºC for 24 h in the dark. Growth inhibition was determined on the basis of turbidity changes (OD at λ 440 nm). $ZnSO_4 \cdot 7H_2O$ for *A. fischeri*, 3,5-dichlorophenol for *P. putida* and $K_2Cr_2O_7$ for *T. thermophila* were used as reference substances in order to check each test procedures.

The toxicity to activated sludge community was carried out by evaluating the effect of water samples on activated sludge metabolic activity using the resazurin (7-hydroxy-3H-phenoxazin-3one-10-oxide) method under the experimental conditions described in OECD Method 209 (OECD, 2010) (Carbajo *et al.*, 2015a). Briefly, resazurin, blue and non-fluorescent in its oxidized stated, is reduced in the presence of active organism cultures to a pink fluorescent derivative (resorufin) by means of a dehydrogenase enzyme. Fresh activated sludge was collected from the aeration tank of an STP located in Guadalajara (Spain). Activated sludge was characterized by determining physico-chemical parameters (Table 3.3). Inoculum (3.0 $g \cdot L^{-1}$ of MLSS)

supplemented with synthetic sewage feed was exposed to tested water samples at 20 ± 2ºC for 3 h in glass vials which were constantly shaken (200 rpm) in darkness under conditions of forced aeration (0.5–1.0 L·min^{-1}) (OECD, 2010). After exposure, biomass metabolic activity was measured in 96-well black polypropylene microplate by adding 200 µL test suspension and 20 µL of resazurin (100 mg·L^{-1}) to each well. Resazurin reduction was measured after 20 min incubation using a Fluoroskan Ascent FL microplate fluorometer (Excitation 542 nm, Emission 592 nm). The suitability of each batch of activated sludge biomass was determined using $CuSO_4 \cdot 5H_2O$ as reference substance (EC_{50} = 26 mg·L^{-1}).

Table 3.3 MLSS of sludge matrix used in resazurin-based activated sludge test

TSS (mg·L^{-1})	6 590
VSS (mg·L^{-1})	4 710
VSS/TSS	0.71
V_{30} (mL·L^{-1})	795
SVI (mL·g^{-1})	119
pH	8.27

Three independent experiments with duplicate samples were carried out to ensure reproducibility. All aquatic toxicity data are expressed as mean ± 95% confidence interval and data analysis were performed using a nonlinear-regression sigmoidal dose-response curve model provided in the GraphPad Prism 6.0 software (GraphPad software Inc., San Diego, USA).

2.5. Data treatment for aquatic toxicity assessment

The toxic-effects obtained were transformed into toxic units (TUs) following the procedure described by Persoone *et al.* (2003). TU is defined as the reciprocal of the wastewater dilution (expressed in percentage) need to achieve 50% inhibition (EC_{50}) (Sprague and Ramsay, 1965):

$$TU = \frac{100}{EC_{50}} \quad (3.2)$$

TUs of non-diluted samples whose effect percentage observed was higher than controls but below 50% (<1 TU) were estimated using the approach proposed in the literature (Persoone *et al.*, 2003) (*i.e.*, $TU = inh/50$, in which inh is the percentage of inhibition). On the basis of TU values, water samples were classified into *non-toxic* (<0.4 TU), *slightly toxic* (0.4–1 TU), *toxic* (1–10 TU) and *highly toxic* (>10 TU).

3. Results and discussion

3.1. Synthetic water matrix

The evolution of BAC, DOC, consumed ozone and dissolved ozone as a function of the amount of ozone supplied is represented in Fig. 3.2A. Fig. 3.2B shows the concentration of cationic surfactants and carboxylic acids (sum of oxalic, acetic and formic acids) during ozonation. Based on the evolution of consumed and dissolved ozone profiles, three zones can be observed throughout ozone dosages. In all of them, dissolved ozone was detected (≥0.01 mg·L^{-1}) due to the fact that ozone mass transfer rate was greater than that of ozone consumption. This suggests that BAC ozonation reactions are relatively slow (Carbajo *et al.*, 2015b). In zone 1, up to 54 mg·L^{-1}, consumed and dissolved ozone linearly increased with ozone dosage. In it, BAC decreased steeply with ozone exposure reaching a value as low as 0.4 mg·L^{-1}. Cationic surfactants declined in parallel to BAC. However, although the concentration of surfactants was significantly reduced, a large organic load remained as shown by DOC values, which slightly decreased. A remarkable increase was observed for carboxylic acids, whose concentration increased steadily with ozone exposure, which suggests a partial oxidation of BAC molecules. In zone 2, ozone consumption steadily increased up to a dosage of 168 mg·L^{-1}. This additional ozone input (from 54 to 168 mg·L^{-1}) was necessary to attain a final BAC concentration below 0.1 mg·L^{-1}. In this zone, the depletion of cationic surfactants continues while DOC stabilized at 6.5 mg·L^{-1}, even though the concentration of organic acids rose slightly. The increasing consumption of ozone indicated the presence of organic matter oxidized but not mineralized. Mineralization is not a single chemical process and represents a series of reactions that

are slow for highly oxidized molecules such as carboxylic acids (Petre *et al.*, 2015). The contribution of low molecular weight acids to total mineralization is an insignificant fraction of the overall rate, which is mainly produced in the ozonation of the high molecular weight compounds as in zone 1 (van Geluwe *et al.*, 2011). For dosages above 168 $mg \cdot L^{-1}$ (zone 3), ozone consumption remained constant and ozone concentration at the reactor outlet (gas and liquid) increased proportionally to ozone input. Extra ozone inlet in this zone was not used for reaction and merely increased dissolved ozone and exhaust gas ozone concentrations. Under these conditions, the upper operational limit of the system, the consumed ozone and the ozone dose were 32 $mg \cdot L^{-1}$ and 3.17 mg $O_3 \cdot (mg\ BAC)^{-1}$, respectively.

It is interesting to emphasize the steep BAC depletion at low ozone dosages because ozone reacts slowly with aromatic compounds with electron-withdrawing substituent, quaternary amines and aliphatic chains (von Sonntag and von Gunten, 2012). Hence, the degradation of BAC under the working conditions used in this work (pH 8.5), seems to be predominantly driven by the attack of hydroxyl radicals, whose rate constants are in the range of 10^9 to 10^{10} $M^{-1} \cdot s^{-1}$ for the moieties present in the BAC molecule (Adams and Kuzhikannil, 2000). The prevalence of indirect mechanism was confirmed by the strong inhibition of BAC abatement occurring in the run carried out using *t*-butanol (30 mM) as radical scavenger. Nonetheless, although the radical chain mechanism is predominant, the maximum mineralization was low, 12%. A third of the remaining DOC is explained by measured carboxylic acids: 1.7 $mg \cdot L^{-1}$ of oxalic and 4.4 $mg \cdot L^{-1}$ of acetic. These results are consistent with previous studies of the degradation of other surfactants by means of ozonation processes (Beltrán *et al.*, 2000 and Ikehata and El-Din, 2004).

3.2. NPs co-occurrence effect

The influence of NiO-NPs on BAC ozonation was studied using a synthetic water matrix. The detailed characterization of BAC and NiO-NPs co-occurrence was performed before ozonation runs (Fig. 3.3). Adsorption isotherm (carried out by measuring BAC

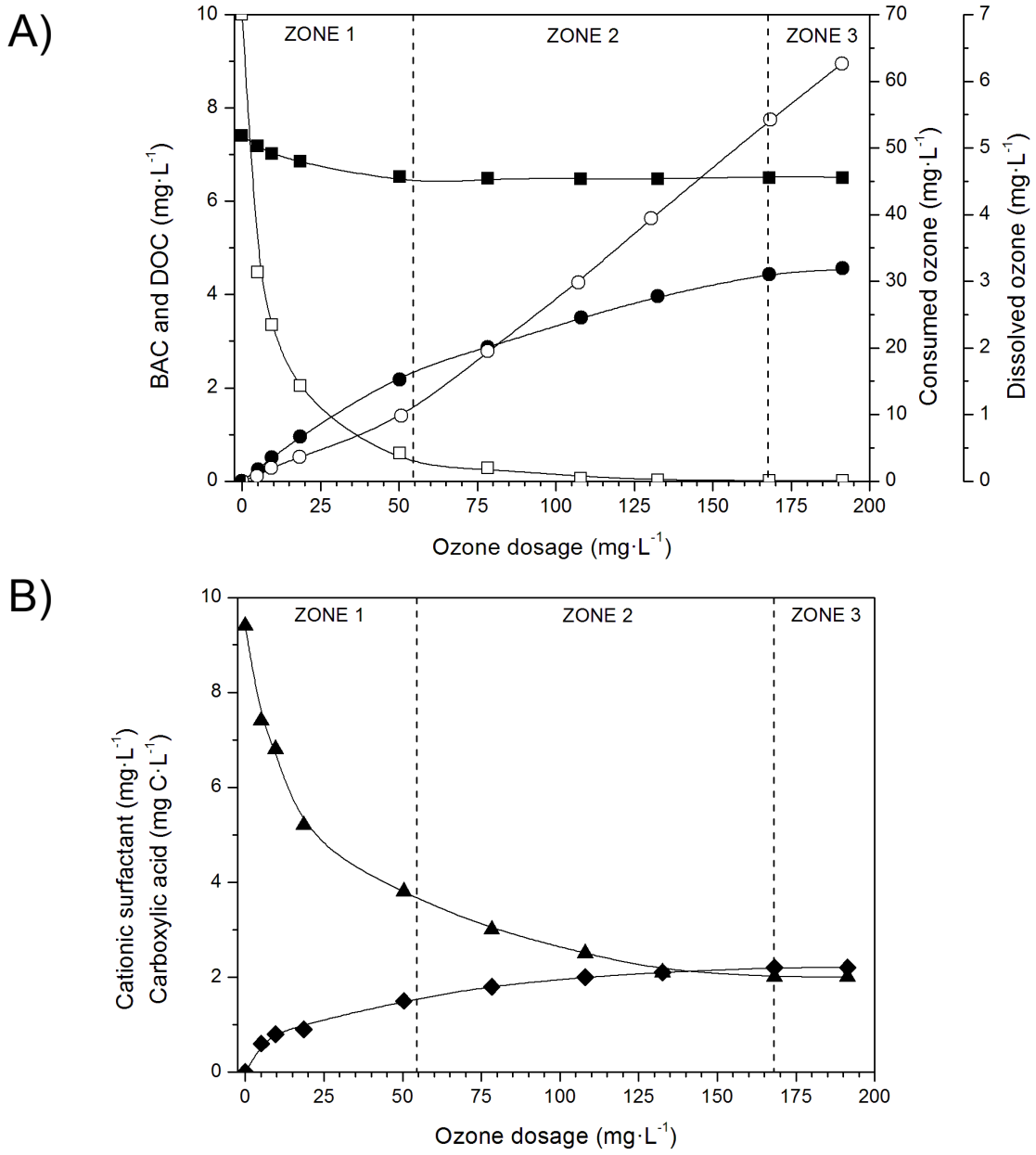


Fig. 3.2 Evolution of BAC (□), DOC (■), consumed (●) and dissolved ozone (○) (A) and cationic surfactants (▲) and carboxylic acids (sum of oxalic, acetic and formic acids, ♦) (B) for different ozone dosages in synthetic water matrix.

concentration without previous extraction) showed that the initial working conditions (BAC: 10 $mg \cdot L^{-1}$; NiO-NPs: 20 $mg \cdot L^{-1}$) correspond to a concentration of BAC in equilibrium of 7.5 $mg \cdot L^{-1}$ and consequently, 2.5 $mg \cdot L^{-1}$ were adsorbed onto 20 $mg \cdot L^{-1}$ of NiO-NPs. The high adsorption of BAC is most probably a consequence of the high surface area-to-volume ratio (BET specific surface area: 86 $m^2 \cdot g^{-1}$) and negative zeta potential (ζ-potential: -15 mV) of the nanomaterial. The potentiometric titration displayed that

increasing BAC concentration caused a stepwise rise in the ζ-potential of NiO-NPs from -15 to +15 mV as a consequence of potential surface charge neutralization by positively charged BAC ions and admicelles (Koppal *et al.*, 1995). The hydrodynamic diameter of NiO-NPs reflected considerable particle aggregation, but the size of the nanomaterial was influenced by BAC concentration. BAC as surfactant has the ability to enhance the dispersion of NPs as well as reduce the charge repulsion between NPs in suspension (Limbach *et al.*, 2008, Brar *et al.*, 2010 and Kiser *et al.*, 2010). NiO-NPs in the presence and absence of BAC were also examined by Raman spectroscopy (Fig. 3.3C), which is a sensitive technique able to provide direct evidence of molecular conformation or

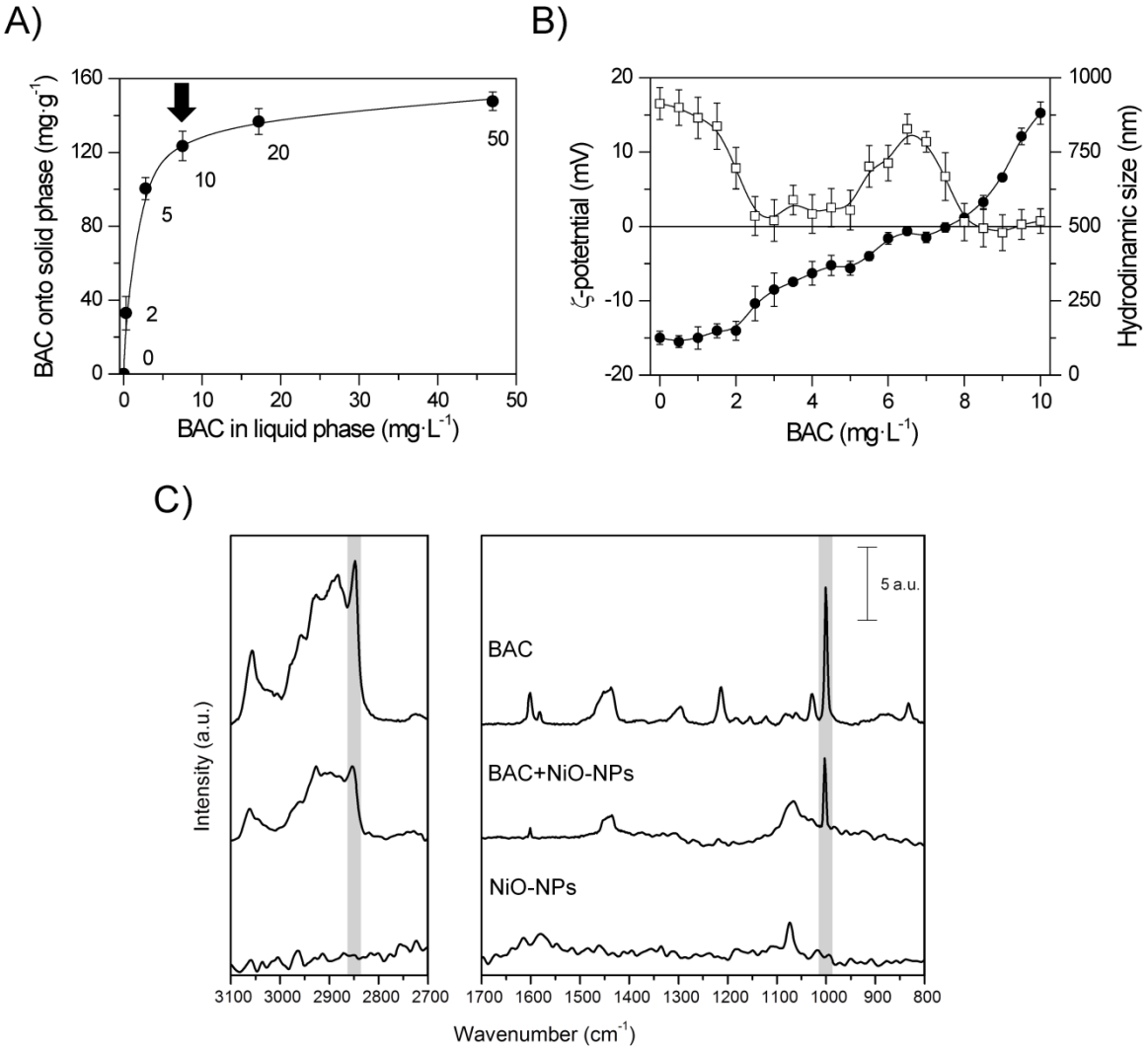


Fig. 3.3 Characterization of the co-occurrence of BAC and NiO-NPs: (A) Adsorption isotherm (numbers represent initial BAC concentration and the arrow indicates initial ozonation working conditions: BAC: 10 mg·L^{-1}, NiO-NPs: 20 mg·L^{-1}). (B) Potentiometric titration ζ-potential (●) and hydrodynamic diameter of NiO-NPs (□). (C) Raman spectra (grey lines represent wavenumbers 1 002 and 2 852 cm^{-1}).

interactions of adsorbed surfactants (Wang *et al.*, 2004). For the BAC+NiO-NPs mixture, signals with characteristic Raman bands such as those related to the alkyl chain ($\nu(CH_2)$ at 3 000–2 800 cm^{-1} and the $\delta(CH_2)$ at 1 449 cm^{-1}), to the aromatic group (ν(CH) at 3 100–3 000 cm^{-1} and ν(phenyl ring) at ~1 600, 1 448, 1 002 cm^{-1}) and to quaternary amine ($\nu(CH_3)$ at 2 852 cm^{-1}) were observed. From the comparison between the Raman spectra for pure BAC and the mixture of BAC+NiO-NPs, some conclusions about the adsorption configuration can be extracted. The change in relative intensity between bands in BAC+NiO-NPs mixture (2 852 cm^{-1}/1 002 cm^{-1} ~5/5) compared to pure BAC (2 852 cm^{-1}/1 002 cm^{-1} ~6/5), indicated that there is a preferential orientation of BAC on the surface of the NiO-NPs with respect to the random configuration in solution (pure BAC spectra). In the absence of specific interaction between BAC and NiO-NPs, the relative intensity of BAC bands in both spectra should remain equal. These observations suggest that molecules of BAC adsorb on NiO-NP surfaces via head group, aided by favourable electrostatic attraction. These data are in line with the conclusions found for the adsorption of cationic surfactants onto NPs by other authors (Wang *et al.*, 2004).

Fig. 3.4A represents the evolution of BAC, DOC and the profiles for consumed and dissolved ozone during the ozonation of BAC+NiO-NPs in synthetic water matrix. A similar behaviour to ozonation of BAC alone was observed. For lower ozone dosages (zone 1), BAC was significantly abated with ozone exposure up to 0.4 $mg \cdot L^{-1}$, while for input levels above 176 mg $O_3 \cdot L^{-1}$ (zone 3), almost total BAC depletion was reached and ozone consumption remained constant. As shown in Fig. 3.4A and B, the profile for DOC, cationic surfactants and carboxylic acids concentration as a function of ozone dosage was also similar to that found for BAC alone. The co-occurrence of NiO-NPs and BAC caused in general a higher ozone dosage and ozone consumption values than BAC ozonation in the absence of NiO-NPs, which suggests that NiO-NPs contribute to ozone consumption. The ozonation of NiO-NPs suspensions resulted in an ozone consumption of 27 $mg \cdot L^{-1}$ at the upper operational limit of the system (Fig. 3.5). Nevertheless, ozone decomposition by NiO-NPs does not seem to accelerate the production of hydroxyl radicals because BAC profile did not display significant differences with respect to BAC ozonation without NiO-NPs.

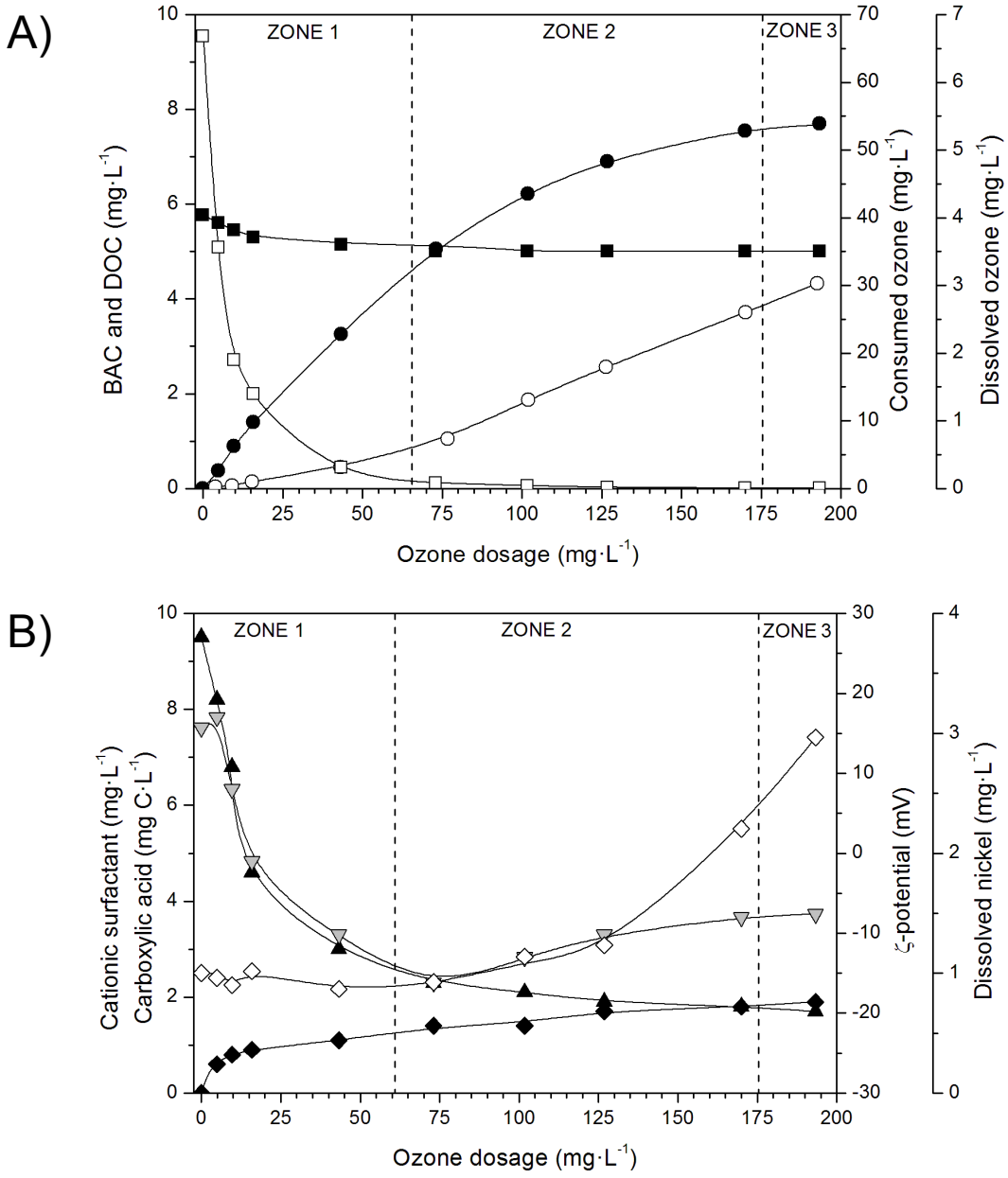


Fig. 3.4 Evolution of BAC (□), DOC (■), consumed (●) and dissolved ozone (○) (A) and cationic surfactants (▲), carboxylic acids (sum of oxalic, acetic and formic acids, ♦), ζ-potential (▽) and dissolved nickel (◊) (B) for different ozone dosages in synthetic water matrix (BAC+NiO-NPs).

The ζ-potential of BAC+NiO-NPs suspension sharply declined in parallel with cationic surfactant concentration from +17 mV at ozone dosage of 4.9 mg·L^{-1} to –17 mV at 106 mg O_3·L^{-1} (Fig. 3.4B). These values were similar to those obtained for the potentiometric titration of NiO-NPs with BAC (Fig. 3.3B), which is consistent with a total depletion of adsorbed BAC. ζ-potential increased slightly for higher ozone dosages throughout zone 2 reaching about -8.0 mV in zone 3. A significant increment in

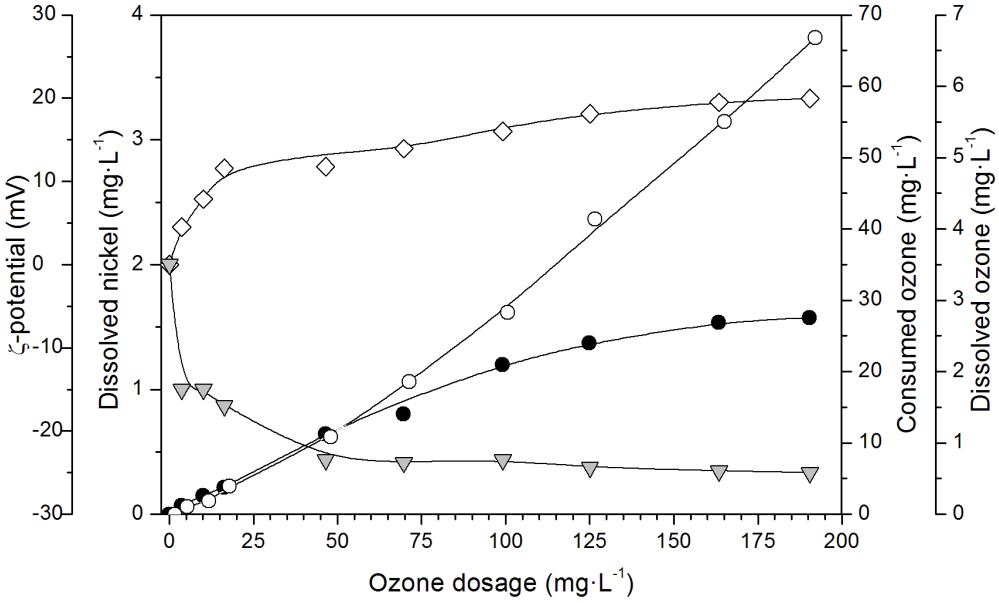


Fig. 3.5 Evolution of consumed (●) and dissolved ozone (○), ζ-potential (▽) and dissolved nickel (◊) for different ozone dosages in synthetic water matrix (NiO-NPs).

the amount of dissolved nickel from 1 to 3 $mg \cdot L^{-1}$ was also observed while increasing ozone exposure. The final nickel concentration was similar to that found in the ozonation of NiO-NPs alone (see Fig. 3.5). These facts suggest that adsorbed BAC acted as a coating agent, increasing the stability of NiO-NPs dispersion and preventing nickel ions passing to the solution (Mirsa *et al.*, 2012 and Garner *et al.*, 2014). Under these conditions, the amount of ozone consumed and the ozone dose were 54 $mg \cdot L^{-1}$ and 5.35 mg $O_3 \cdot (mg\ BAC)^{-1}$, respectively. The high values for both parameters with respect to the ozonation of BAC could be explained by the ozone consumption driven by NiO-NPs. The amount of ozone consumed by BAC+NiO-NPs at the operational limit was close to the sum of the ozone consumed by water matrix, 7 $mg \cdot L^{-1}$, NiO-NPs, 20 $mg \cdot L^{-1}$ (Fig. 3.5), and BAC abatement, 25 $mg \cdot L^{-1}$ (Fig. 3.2).

3.3. Elucidation of transformation products and degradation pathway

Twelve compounds were elucidated as TPs formed during the ozonation of BAC. Table 3.4 shows accurate mass measurements of BAC and its TPs and structures proposed for them. All of them with retention time lower than BAC, which indicates that transformation reactions lead to more polar molecules. The profile of TPs as a function

Table 3.4 Accurate mass measurement of BAC and its TPs as determined by LC-TOF/MS.

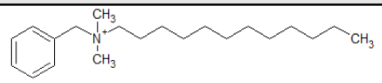
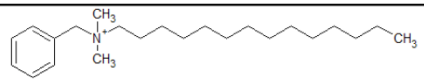
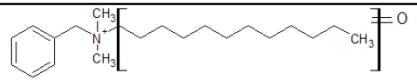
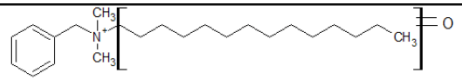
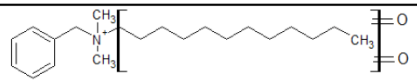
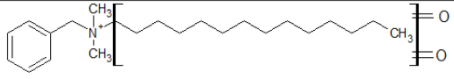
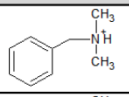
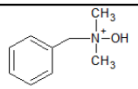
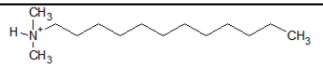
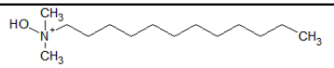
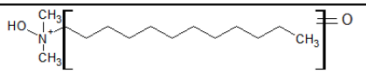
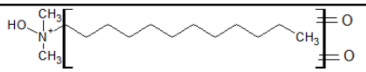
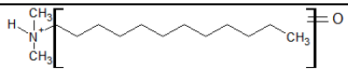
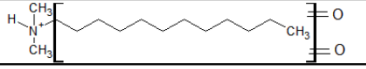
Compound	R_t (min)	Elemental formula	Mass (m/z)		Error		Proposed structure
			Theoretical	Experimental	ppm	DBE	
BAC-12	23.574	$C_{21}H_{38}N^+$	304.2999	304.2997	0.55	4	
BAC-14	27.125	$C_{23}H_{42}N^+$	332.3312	332.3310	0.56	4	
TP1	20.518	$C_{21}H_{36}NO^+$	318.2791	318.2786	1.60	5	
	18.631	$C_{21}H_{36}NO^+$	318.2791	318.2787	1.49	5	
	17.111	$C_{21}H_{36}NO^+$	318.2791	318.2787	1.25	5	
	16.159	$C_{21}H_{36}NO^+$	318.2791	318.2789	0.92	5	
	15.575	$C_{21}H_{36}NO^+$	318.2791	318.2788	1.02	5	
	15.198	$C_{21}H_{36}NO^+$	318.2791	318.2789	0.91	5	
	14.940	$C_{21}H_{36}NO^+$	318.2791	318.2787	1.30	5	
	14.598	$C_{21}H_{36}NO^+$	318.2791	318.2786	1.84	5	
TP2	23.140	$C_{23}H_{40}NO^+$	346.3104	346.3106	-0.55	5	
	22.422	$C_{23}H_{40}NO^+$	346.3104	346.3105	0.01	5	
	21.971	$C_{23}H_{40}NO^+$	346.3104	346.3103	0.48	5	
	21.570	$C_{23}H_{40}NO^+$	346.3104	346.3101	0.92	5	
	21.203	$C_{23}H_{40}NO^+$	346.3104	346.3098	1.78	5	
	20.947	$C_{23}H_{40}NO^+$	346.3104	346.3104	0.24	5	
	20.618	$C_{23}H_{40}NO^+$	346.3104	346.3100	1.36	5	
	20.409	$C_{23}H_{40}NO^+$	346.3104	346.3101	1.05	5	
	20.184	$C_{23}H_{40}NO^+$	346.3104	346.3101	1.04	5	
TP3	13.036	$C_{21}H_{34}NO_2^+$	332.2584	332.2582	0.76	6	
	12.518	$C_{21}H_{34}NO_2^+$	332.2584	332.2581	0.84	6	
	12.293	$C_{21}H_{34}NO_2^+$	332.2584	332.2582	0.58	6	
	12.101	$C_{21}H_{34}NO_2^+$	332.2584	332.2579	1.50	6	
	11.683	$C_{21}H_{34}NO_2^+$	332.2584	332.2582	-0.77	6	
	11.391	$C_{21}H_{34}NO_2^+$	332.2584	332.2583	0.42	6	
	11.207	$C_{21}H_{34}NO_2^+$	332.2584	332.2579	0.76	6	
TP4	13.645	$C_{23}H_{38}NO_2^+$	360.2897	360.2886	3.00	2	
	13.404	$C_{23}H_{38}NO_2^+$	360.2897	360.2889	2.32	2	

Table 3.4 Accurate mass measurement of BAC and its TPs as determined by LC-TOF/MS (continued)

Compound	R_t (min)	Elemental formula	Mass (m/z)		Error		Proposed structure
			Theoretical	Experimental	ppm	DBE	
TP5	2.757	$C_9H_{14}N^+$	136.1121	136.1117	2.73	4	
TP6	2.956	$C_9H_{14}NO^+$	152.1070	152.1070	0.21	4	
TP7	20.450	$C_{14}H_{32}N^+$	214.2534	214.2528	2.80	0	
TP8	21.092	$C_{14}H_{32}NO^+$	230.2478	230.2478	0.35	0	
TP9	14.719	$C_{14}H_{30}NO_2^+$	244.2271	244.2272	-0.45	1	
	13.901	$C_{14}H_{30}NO_2^+$	244.2271	244.2272	-0.54	1	
	13.340	$C_{14}H_{30}NO_2^+$	244.2271	244.2271	-0.12	1	
	12.965	$C_{14}H_{30}NO_2^+$	244.2271	244.2271	-0.09	1	
	12.583	$C_{14}H_{30}NO_2^+$	244.2271	244.2271	0.01	1	
	12.364	$C_{14}H_{30}NO_2^+$	244.2271	244.2271	-0.12	1	
	12.155	$C_{14}H_{30}NO_2^+$	244.2271	244.2272	-0.41	1	
	11.938	$C_{14}H_{30}NO_2^+$	244.2271	244.2271	0.15	1	
TP10	5.375	$C_{14}H_{28}NO_3^+$	258.2064	258.2053	4.34	2	
	4.615	$C_{14}H_{28}NO_3^+$	258.2064	258.2050	5.19	2	
	4.590	$C_{14}H_{28}NO_3^+$	258.2064	258.2053	4.21	2	
	4.205	$C_{14}H_{28}NO_3^+$	258.2064	258.2052	4.42	2	
	3.938	$C_{14}H_{28}NO_3^+$	258.2064	258.2051	4.78	2	
	3.663	$C_{14}H_{28}NO_3^+$	258.2064	258.2052	4.54	2	
TP11	13.533	$C_{14}H_{30}NO^+$	228.2322	228.2322	-0.07	1	
	13.032	$C_{14}H_{30}NO^+$	228.2322	228.2321	0.47	1	
	12.565	$C_{14}H_{30}NO^+$	228.2322	228.2321	0.45	1	
	12.130	$C_{14}H_{30}NO^+$	228.2322	228.2324	-1.05	1	
	11.805	$C_{14}H_{30}NO^+$	228.2322	228.2323	-0.27	1	
	11.587	$C_{14}H_{30}NO^+$	228.2322	228.2322	-0.25	1	
	11.354	$C_{14}H_{30}NO^+$	228.2322	228.2322	-0.07	1	
TP12	3.484	$C_{14}H_{28}NO_2^+$	242.2115	242.2113	0.60	2	

of ozone dosage is shown in Fig. 3.6. The amounts of TPs corresponded to intermediate products in series reactions, with their counts initially increasing to reach a maximum and then decreasing as a result of their further degradation. The generation pathway of these TPs is expected to include multiple routes due to the presence of different reactive sites. Despite this complexity, and in view of the information obtained from the literature, the results could be interpreted to propose the degradation pathway shown in Scheme 3.2 (Kroon *et al.*, 1994 and Patrauchan and Oriel, 2003). The degradation of BAC occurred on both its hydrophobic (*i.e.*, alkyl chain) and hydrophilic region (*i.e.*, benzyl and ammonium moiety), which explains the occurrence of transformation products TP_1–TP_6 (full symbols in Fig. 3.6) and TP_7–TP_{12} (empty symbols in Fig. 3.6), respectively.

On the hydrophobic region, the initiation step was hydrogen abstraction from alkyl chain by means of a hydroxyl radical leading to a carbon centered radical, its reaction with dissolved oxygen to yield a peroxyl radical and the subsequent

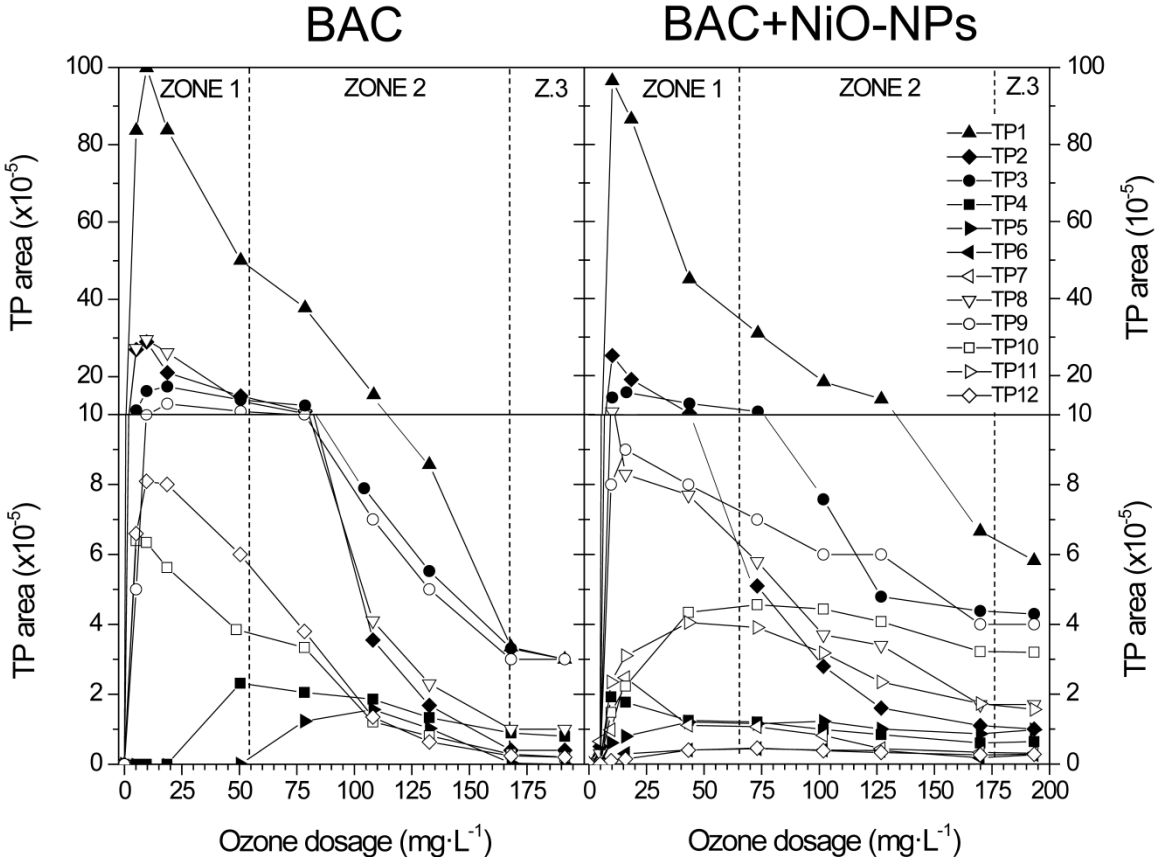
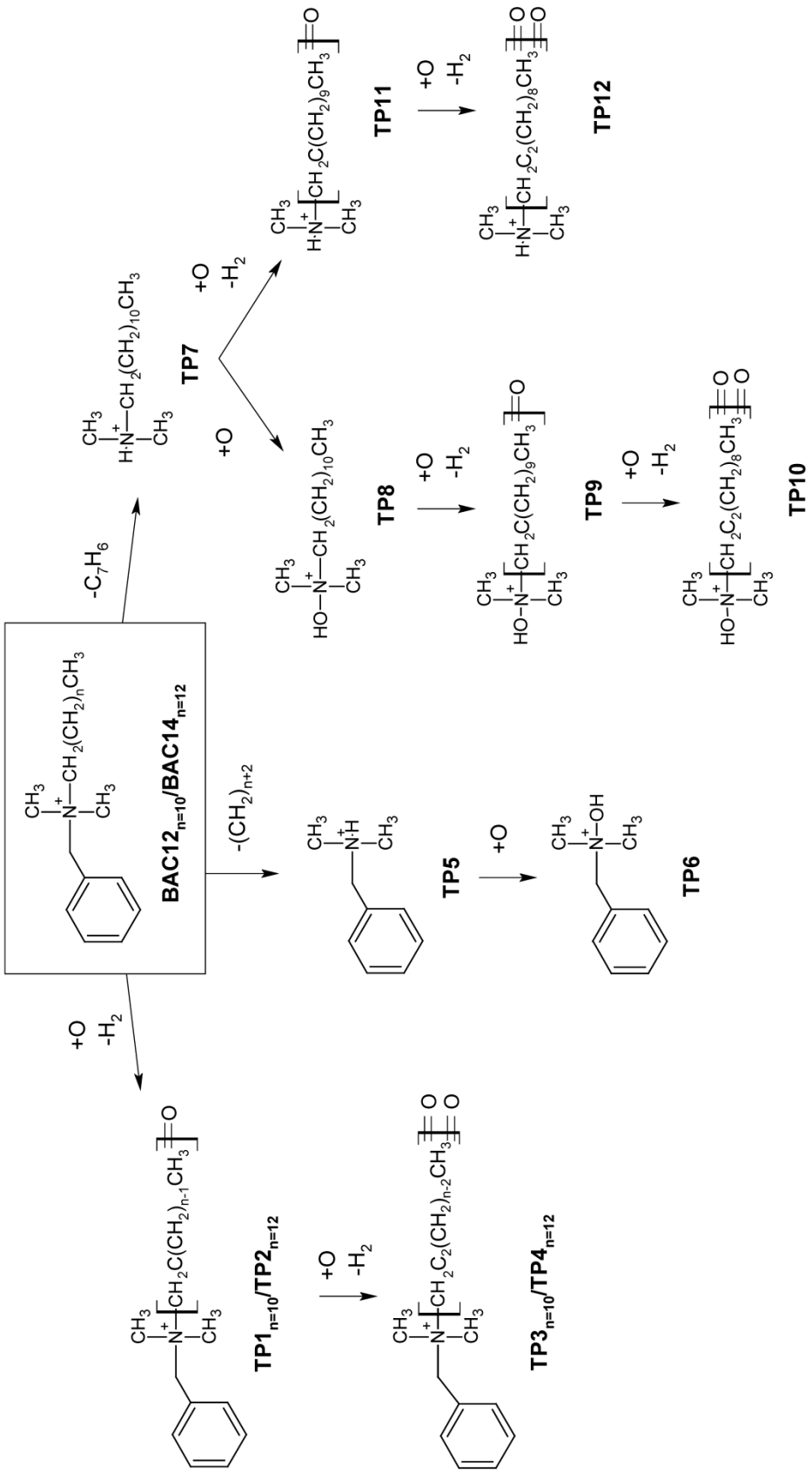


Fig. 3.6 Evolution of TPs of BAC for different ozone dosages in synthetic water matrix.

decomposition to carbonyl compounds TP_1 and TP_2 (van Geluwe *et al*., 2011). Carbonyl moiety may occur at different positions along the alkyl chain resulting in a series of isomers with similar counts: nine for TP_1 ($C_{21}H_{36}NO^+$, *m/z* 318.2791) and eight for TP_2 ($C_{23}H_{40}NO^+$, *m/z* 346.3104). In the same way, the alkyl chains of TP_1 and TP_2 may suffer hydrogen abstraction to yield TP_3 ($C_{21}H_{34}NO_2^+$, *m/z* 332.2584) and TP_4 ($C_{23}H_{38}NO_2^+$, *m/z* 360.2897), respectively. The occurrence of benzyldimethylamine (TP_5, $C_9H_{14}N^+$, *m/z* 136.1121) suggests a α-hydroxylation of the alkyl moiety followed by a central fission of the C_{alkyl}–N bond (dealkylation). Then, benzyldimethylamine can be hydroxylated to TP_6 ($C_9H_{14}NO^+$, *m/z* 152.1070). On the hydrophilic region, the initiation step would be the degradation of benzyl group to yield carboxylic acids (von Sonntag and von Gunten, 2012). These reactions lead to dodecyltrimethylamine (TP_7, $C_{14}H_{32}N^+$, *m/z* 214.2534). The degradation of TP_7 may give rise to TP_8 ($C_{14}H_{32}NO^+$, *m/z* 230.2478) through the addition of a hydroxyl radical. The aliphatic tertiary amine may undergo hydrogen abstraction along the aliphatic chain to yield a group of eight positional isomers (TP_9, $C_{14}H_{30}NO_2^+$, *m/z* 244.2271). Further hydrogen abstraction reactions on TP_9 could give rise to TP_{10} ($C_{14}H_{28}NO_3^+$, *m/z* 258.2064). TP_6 could also be oxidized to TP_{11}, a group of seven isomers ($C_{14}H_{30}NO^+$, *m/z* 228.2322), which would be further transformed to TP_{12} ($C_{14}H_{28}NO_2^+$, *m/z* 242.2115).

BAC degradation pathways on both the hydrophobic and hydrophilic region justified the large amount of carboxylic acids detected at the upper operational limit. Acetic acid (4.4 $mg \cdot L^{-1}$) was a clear outcome of the aliphatic chain oxidation, whereas oxalic acid (1.7 $mg \cdot L^{-1}$) seems to be the final product of ring-opening reactions (von Sonntag and von Gunten, 2012). Nitrate reached the maximum concentration of 0.1 $mg \cdot L^{-1}$ at zone 3 (5% of the total nitrogen content in BAC molecules), indicating negligible nitrogen mineralization. These facts suggest that the remaining organic carbon contains a high amount of nitrogen in compounds such as amines, whose protonated species react slowly with ozone (k <0.1 $M^{-1} \cdot s^{-1}$, von Sonntag and von Gunten (2012)). The accumulation of transformation products such as TP_1, TP_9 and TP_{10} could explain the incomplete depletion of cationic surfactants (Fig. 3.2B and 3.4B).



Scheme 3.2 Proposed degradation pathway during the ozonation of BAC in synthetic water matrix.

All TPs aforementioned above were also detected in the presence of NiO-NPs, which suggest that the same degradation pathway took place. However, significantly lower area counts of TPs from reactions on the hydrophilic part of BAC (TP_7–TP_{12}), especially in zone 1, were found. This is consistent with the already explained adsorption of BAC on NiO-NPs, which should proceed through the benzyl group and prevent it from oxidation.

3.4. Matrix effects

The influence of water matrix on the ozonation of BAC+NiO-NPs was also studied in a real STP influent. Non-spiked raw wastewater required an instantaneous ozone demand of 77 $mg \cdot L^{-1}$ (Fig. 3.7A). High values of consumed ozone were also observed at the upper operational limit, 87 $mg \cdot L^{-1}$. Both facts were mainly a result of the oxidation of wastewater organic matter, the concentration of which was elevated (DOC_0 = 61 $mg \cdot L^{-1}$). A disintegration of suspended solids also took place during ozonation process, leading to an increase of inorganic anions such as nitrate, phosphate and sulphate, as well as DOC (Fig. 3.7B). In spite of increasing DOC, partial oxidation reactions were revealed by a sharp reduction of the specific ultraviolet absorption at 254 nm ($SUVA_{254}$).

The evolution of BAC, DOC, dissolved and consumed ozone during the ozonation in spiked STP influent is represented in Fig. 3.8A. Important differences were observed with regard to the synthetic water matrix. The amount of consumed ozone increased with ozone input and no dissolved ozone was detected (<0.01 $mg \cdot L^{-1}$). The profile of BAC depletion was also different. BAC decay in STP influent could be split in two parts. First, up to ozone exposures of 18 $mg \cdot L^{-1}$, BAC concentration decreased sharply (up to 65% removal). For higher ozone dosages, BAC concentration declined slowly, probably influenced by the elevated amount of DOC in solution. DOC rose from 67 to 80 $mg \cdot L^{-1}$ during the first part of the reaction because of the solubilization of suspended solids, which increased ozone demand. Under these conditions, an ozone consumption of 157 $mg \cdot L^{-1}$ (dosage of 300 $mg \cdot L^{-1}$) was required to achieve 0.4 $mg \cdot L^{-1}$ of BAC, a concentration that required 54 $mg \cdot L^{-1}$ of ozone in synthetic water. On the whole,

consumed ozone in real wastewater was close to five-fold the corresponding value in synthetic water matrix.

The high ozone demand of wastewater was also related to the lower depletion of cationic surfactants (Fig. 3.8B), which followed the same profile as BAC. A significant removal of cationic surfactants was reached for ozone dosage of 18 $mg \cdot L^{-1}$ but their concentration stabilized at 4.6 $mg \cdot L^{-1}$ without further reduction. The evolution of carboxylic acids followed a similar trend to that observed in synthetic matrix, but

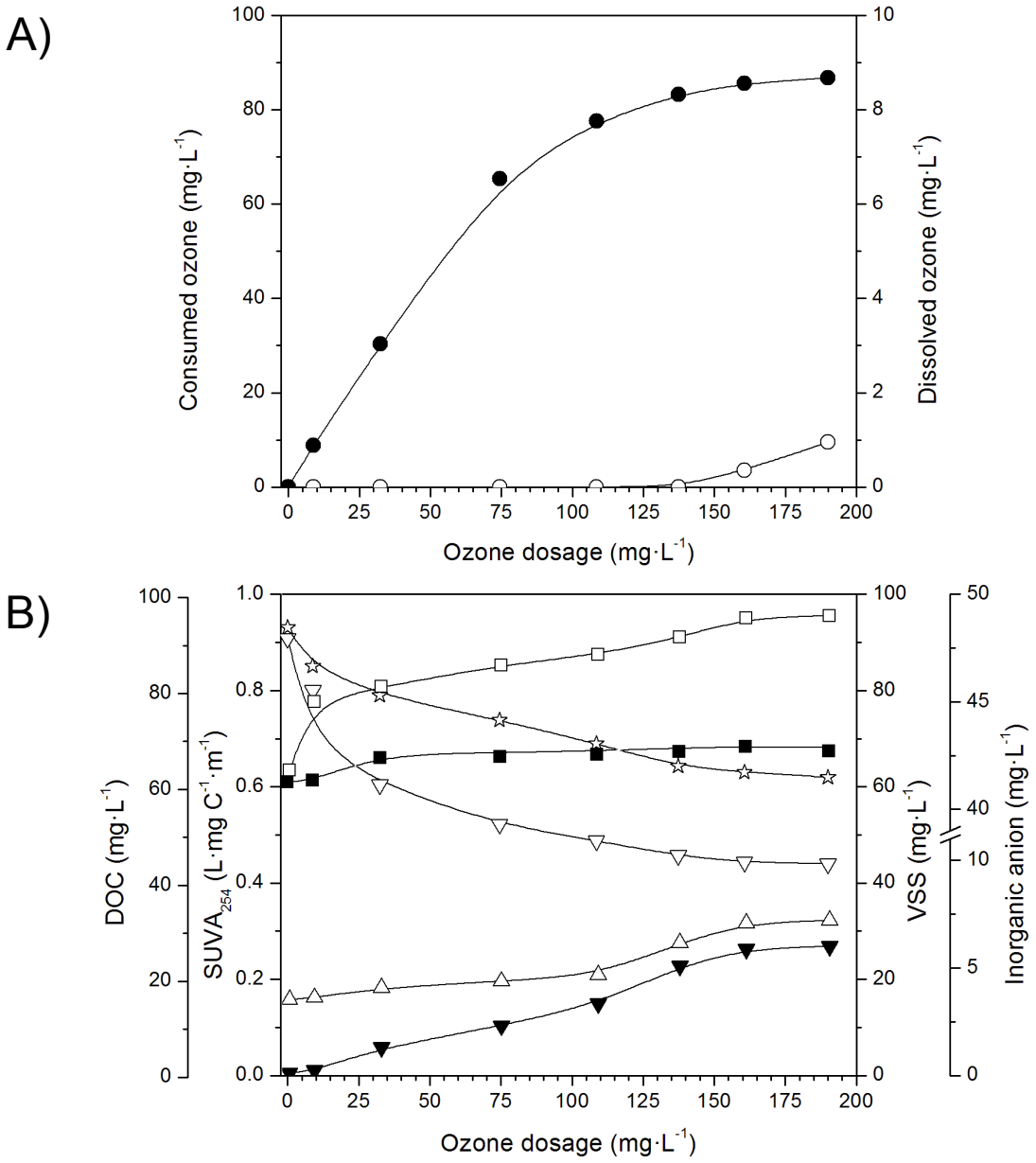


Fig. 3.7 Evolution of consumed (●) and dissolved ozone (○) (A) and DOC (■), $SUVA_{254}$ (▽), VSS (☆), nitrate (▼), sulfate (□) and phosphate (Δ) for different ozone dosages in STP influent.

displaying higher concentrations as a consequence of the oxidation reactions of dissolved organic matter (Zhang *et al.*, 2009 and van Geluwe *et al.*, 2011). Specifically, formic, acetic and oxalic acid achieved values of 14, 36 and 6.5 $mg \cdot L^{-1}$, representing altogether close to 40% of the remaining dissolved carbon. Meanwhile, ζ-potential displayed negative values during all the ozonation process, reaching -28 mV for an ozone dosage of 68 $mg \cdot L^{-1}$. A possible cause is that organic matter could adsorb on NP surfaces and confer them a negative charge (Zhang *et al.*, 2009). The concentration of dissolved

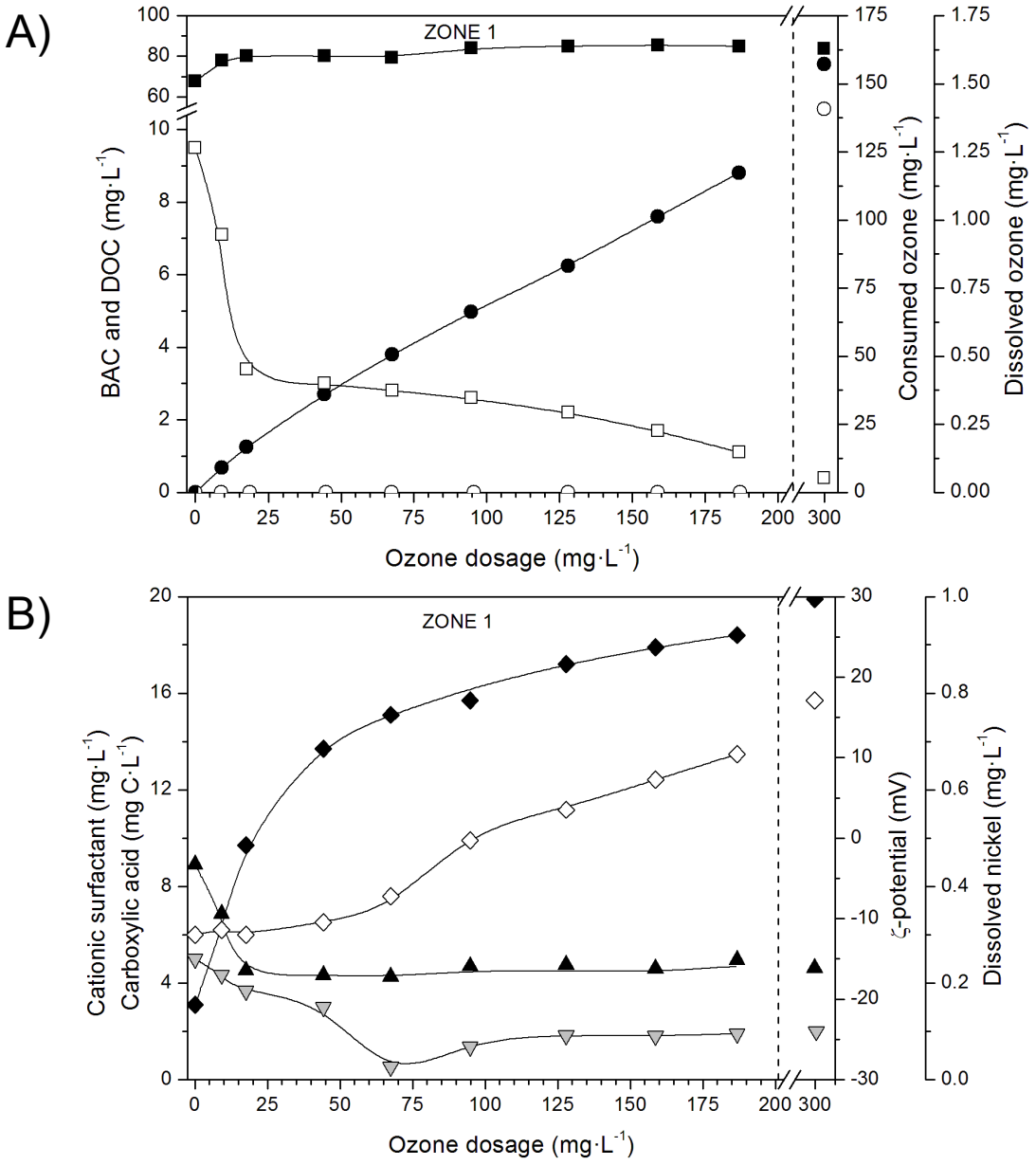


Fig. 3.8 Evolution of BAC (□), DOC (■), consumed (●) and dissolved ozone (○) (A) and cationic surfactants (▲), carboxylic acids (sum of oxalic, acetic and formic acids,♦), ζ-potential (▽) and dissolved nickel (◊) (B) for different ozone dosages in STP influent (BAC+NiO-NPs).

nickel reached a value significantly lower than that found in synthetic water (0.8 mg·L^{-1}). This fact is most likely due to the barrier caused by adsorbed organic matter on NiO-NPs, which stabilizes NP dispersion and reduces the rate of dissolution (Mirsa *et al.*, 2012 and Garner *et al.*, 2014).

3.5. Aquatic toxicity assessment

The toxicity of BAC and NiO-NPs to single species and activated sludge microorganisms was assessed by determining concentration-response curves as shown in Fig. 3.9. All the bioassays were sensitive to BAC with the following EC_{50} values: 0.26 mg·L^{-1} for *V. fischeri*, 8.40 mg·L^{-1} for *P. putida*, 4.28 mg·L^{-1} for *T. thermophila* and 3.43 mg·L^{-1} for activated sludge. The EC_{50} values are in good agreement with previously reported data and are consistent with the use of BAC as biocide (Nalecz-Jawecki *et al.*, 2003, Sütterlin *et al.*, 2008, Carbajo *et al.*, 2015a and ECHA, 2015). The growth inhibition tests using *P. putida* and *T. thermophila* were also sensitive to NiO-NPs with EC_{50} values of 4.25 and 0.58 mg·L^{-1} respectively, while *V. fischeri* and activated sludge assays displayed EC_{50} values >100 mg·L^{-1}. The same sensitivity pattern was observed for nickel ions (nickel as nickel sulfate), which displayed a concentration-response curve parallel to NiO-NPs for each bioassay (inset Fig. 3.9B). This fact suggests that the concentration of nickel ion released from the NP is the driver for the toxicity of NiO-NPs. The low sensitivity of *V. fischeri* and activated sludge to different NPs has also been reported elsewhere (Heinlaan *et al.*, 2008, García *et al.*, 2012 and Wang *et al.*, 2014).

Fig. 3.10 displays the evolution of the toxicity of untreated and treated water samples at different ozone exposures in synthetic matrix and STP influent for the organisms used in the present study. The aquatic toxicity of untreated synthetic water (BAC = 10 mg·L^{-1} and/or NiO-NPs = 20 mg·L^{-1}) displayed significant inter-bioassay differences, which essentially corresponded to the already described sensitivity to BAC and NiO-NPs. The growth inhibition of *P. putida* and *T. thermophila* was severely inhibited because BAC and NiO-NPs concentrations were considerably higher than their EC_{50} values. Moreover, untreated synthetic water could be classified as *toxic*, or even

highly toxic, to subsequent biological treatment according to the scoring system defined by Persoone *et al.* (2003). *V. fischeri* and activated sludge tests were also strongly affected by water spiked with BAC (*toxic* or *highly toxic*), but not with single NiO-NPs, which is consistent with their lower sensitivity to the nanomaterial.

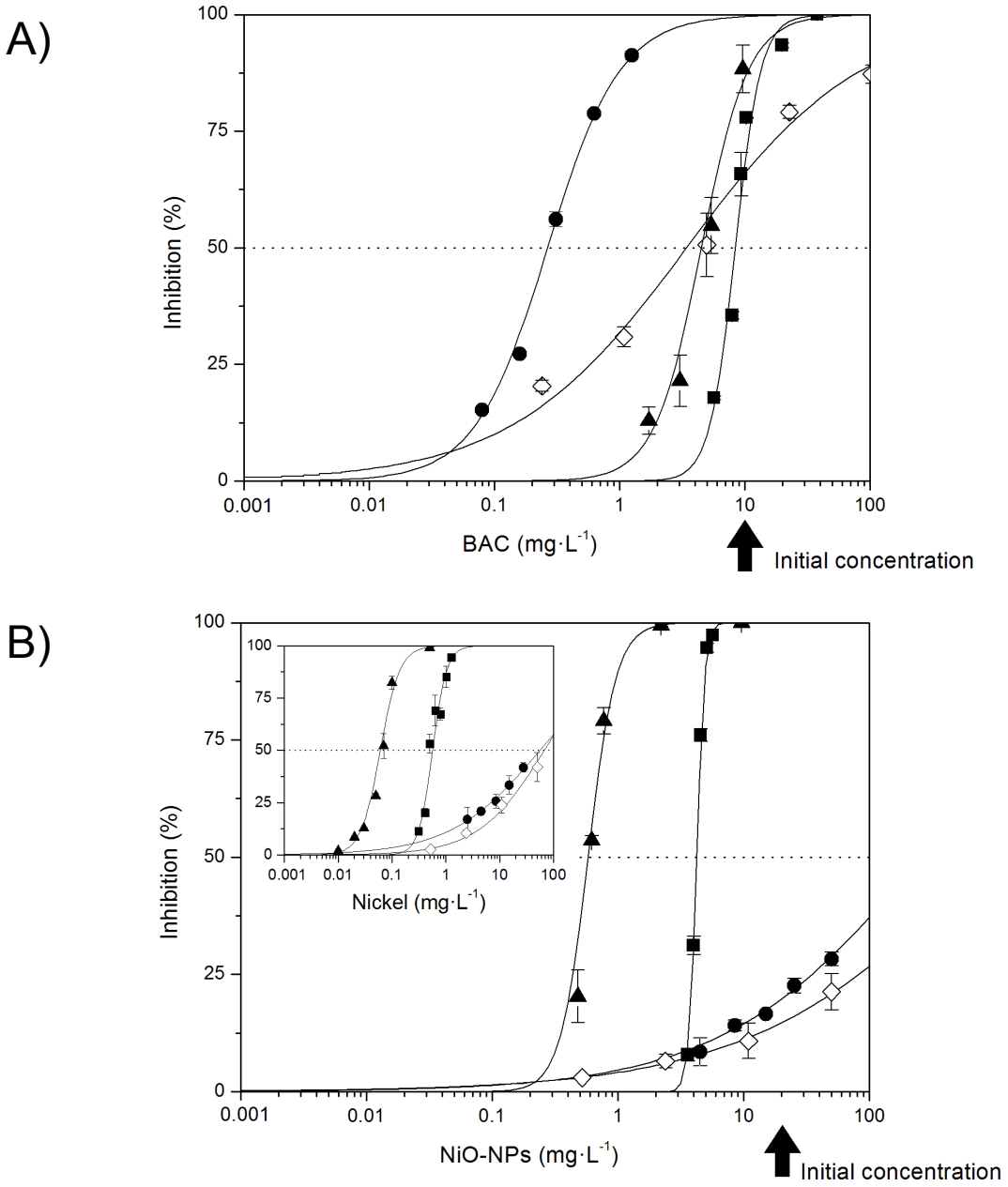


Fig. 3.9 Concentration-response curve of BAC, NiO-NPs and inset nickel as nickel sulfate for *V. fischeri* (●), *P. putida* (■) and *T. thermophila* (▲) and activated sludge assay (◊). Mean ± 95% confidence interval, lines gives nonlinear-regression sigmoidal dose-response curve model, arrows represent the initial BAC and NiO-NPs concentrations in spiked water (10 and 20 mg·L^{-1}, respectively).

The aquatic toxicity of BAC+NiO-NPs was lower in STP influent than in the synthetic matrix except to *V. fischeri*, which could be explained by the toxicity of the raw wastewater itself. For the rest of biotests, mixture toxicities of BAC+NiO-NPs were notably influenced by matrix (*i.e.*, high concentration of solids, organic and inorganic matter), which reduces the bioavailability of the cationic surfactants and dissolved metals (Nalecz-Jaecki *et al.*, 2003, Heinlaan *et al.*, 2008, Ismail *et al.*, 2010 and Mirsa *et al.*, 2012). Moreover, the high amount of nutrients contained in the STP influent (assimilable organic carbon, nitrogen, phosphate) could partially mask the toxic effects of the studied contaminants.

In the synthetic water matrix, the evolution of the aquatic toxicity throughout ozonation of BAC showed that the increase of ozone dosage up to 54 $mg \cdot L^{-1}$ (zone 1) caused a gradual toxicity reduction. By the end of zone 1, the toxicity reached a constant value similar to that of non-spiked synthetic water for all bioassays. TU values remained essentially constant thereafter in zone 2 and 3. Ozone treated water could then be classified as *non-toxic* for *P. putida* and activated sludge tests. Aquatic toxicity and BAC concentration follow a similar profile with increasing ozone dosage. Despite the fact that BAC was not completely depleted at the end of zone 1 (BAC = 0.4 $mg \cdot L^{-1}$), the reduction of its concentration brought about a considerable reduction of its toxic effects. The degradation reactions caused changes in the molecular structure of BAC, affecting moieties which were directly responsible for its biocide activity (Rusell, 2003). In fact, TOF/MS measurements of TPs showed that benzyl and alkyl groups were oxidized in parallel with the depletion of surface activity.

During the ozonation of NiO-NPs in synthetic water matrix, the toxicity was slightly enhanced at the lowest ozone dosage due to the increment of dissolved nickel that reached 3 $mg \cdot L^{-1}$. For higher ozone exposure, no toxicity changes were observed. The aquatic toxicity pattern of treated BAC+NiO-NPs in synthetic matrix was similar to that of BAC for the bioassays with low sensitivity to NiO-NPs. *V. fischeri* and activated sludge tests reached the same TU values of non-spiked synthetic water at the end of zone 1, with the toxicity remaining constant for further ozone dosages (zones 2 and 3). For *P. putida* and *T. thermophila*, the toxicity declined steadily with ozone exposure up

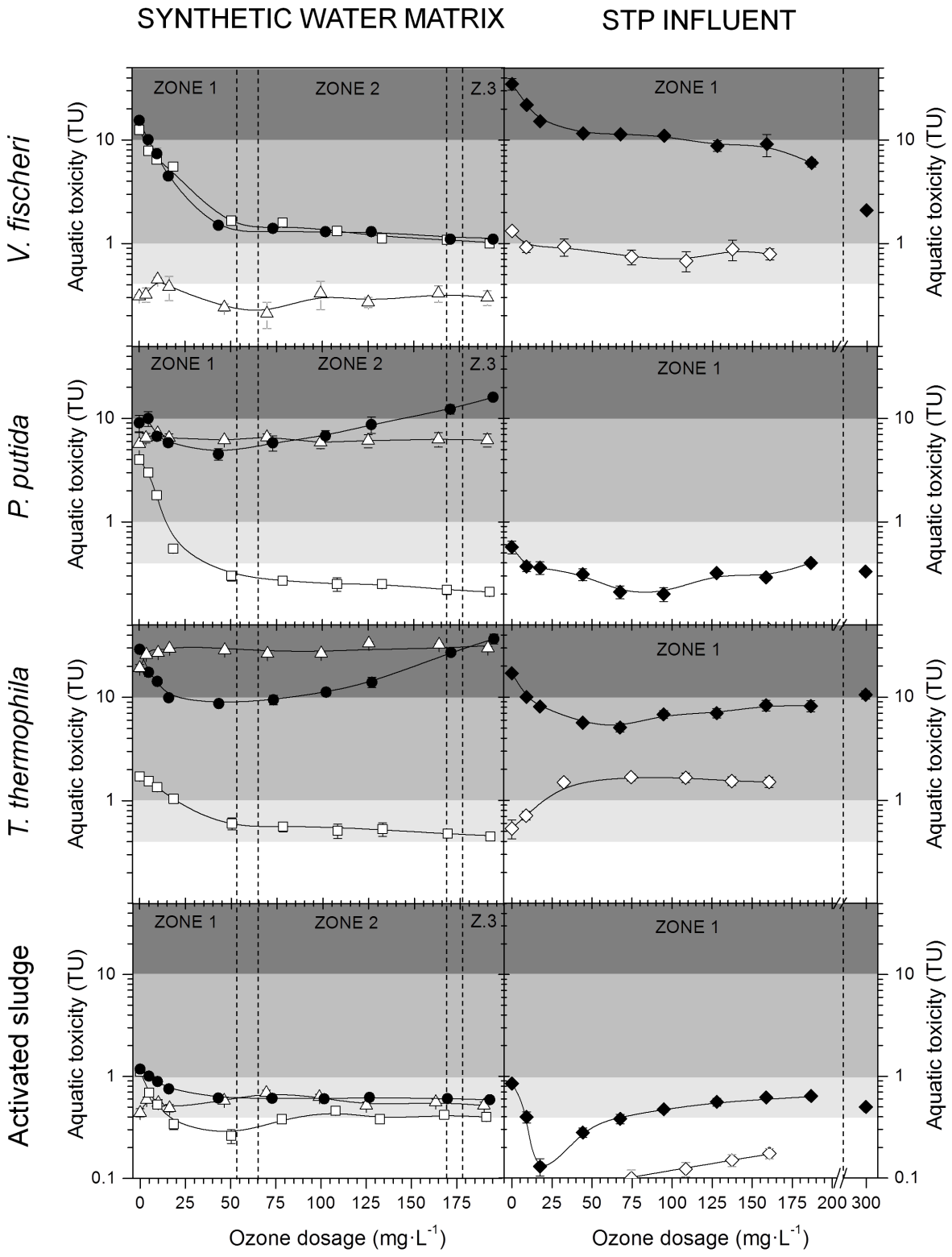


Fig. 3.10 Evolution of toxic units (TUs) of treated samples for different ozone dosages in synthetic water spiked with BAC (□), NiO-NPs (Δ), BAC+NiO-NPs (●), STP influent spiked with BAC+NiO-NPs (♦) and non-spiked STP influent (◊). Mean ± 95% confidence interval, *highly toxic, toxic, slightly toxic* and *non-toxic* influent to subsequent biological treatment according to the classification defined by Persoone *et al.* (2003).

to an ozone dosage of 58 mg·L^{-1} to sharply increase thereafter in zones 2 and 3. This increase was parallel with that of dissolved nickel, the concentration of which rose from 1 to 3 mg·L^{-1}. As a consequence, the ozonation yielded a *highly toxic* wastewater from a *toxic* influent to *P. putida*. As already described, *P. putida* and *T. thermophila* were highly sensitive to NiO-NPs and dissolved nickel as evidenced by the corresponding EC_{50} values: 0.57 and 0.061 mg·L^{-1}, respectively. It is worth mentioning that the toxicity towards *P. putida* at the highest ozone dosage (~190 mg·L^{-1}) was significantly higher in BAC+NiO-NPs than in NiO-NPs wastewater, even considering that both had the same amount of dissolved nickel (3 mg·L^{-1}). This suggests a synergistic effect between nickel and other mixture components.

The aquatic toxicity of non-spiked STP influent increased steadily with increasing ozone exposure for all biotests except *V. fischeri*, for which it slightly reduced. In spiked STP influent, aquatic toxicity steadily decreased in single-species tests up to 68 mg·L^{-1}, allowing treated wastewater to be classified as non-toxic to *P. putida*. For higher ozone exposures, the toxicity to *P. putida* and *T. thermophila* increased progressively in parallel with an increase in the amount of dissolved nickel. For the activated sludge test, ozonation resulted in a sharp toxicity reduction at low ozone dosage (18 mg·L^{-1}), allowing ozone treated wastewater to be considered *non-toxic*. For increasing ozone exposure, the toxicity of treated wastewater increased progressively equalling that of non-spiked STP influent. This fact, together with the low sensitivity of activated sludge to nickel (<5% inhibition at 1 mg·L^{-1}), suggests that ozonation by-products from STP influent matrix were the main source of toxicity to the activated sludge assay.

4. Conclusions

It was shown that the continuous ozonation with short reaction time and low ozone dosages is a suitable technology for sequential chemical-biological treatment regarding the reduction of toxicity caused by wastewater contaminated with BAC and NiO-NPs.

BAC was significantly removed (>95%) during ozonation independently of NiO-NP co-occurrence or water matrix characteristics. NiO-NPs and wastewater matrix notably increased the ozone dosage required for a given degree of BAC removal. BAC ozonation led to less hydrophobic molecules as a consequence of the reaction on both the hydrophobic (alkyl chain) and hydrophilic regions (benzyl and ammonium moieties) of the parent compound. The presence of NiO-NPs influenced the first steps of the degradation pathway of BAC preventing benzyl group from oxidation.

The aquatic toxicity of raw wastewater for the single-species tests (*V. fischeri*, *P. putida*, *T. thermophila*) and activated sludge assay was considerably reduced for an ozone dosage lower than that required for BAC abatement. Higher ozone dosage BAC+NiO-NPs caused an increase in nickel leaching from the nanomaterial and consequently, a toxicity enhancement of treated wastewater. Toxicity assessment was shown to be a critical parameter for the ozonation of wastewater.

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PART II

- Antibiotics
- Ozonation as polishing step
- Freshwater ecosystem

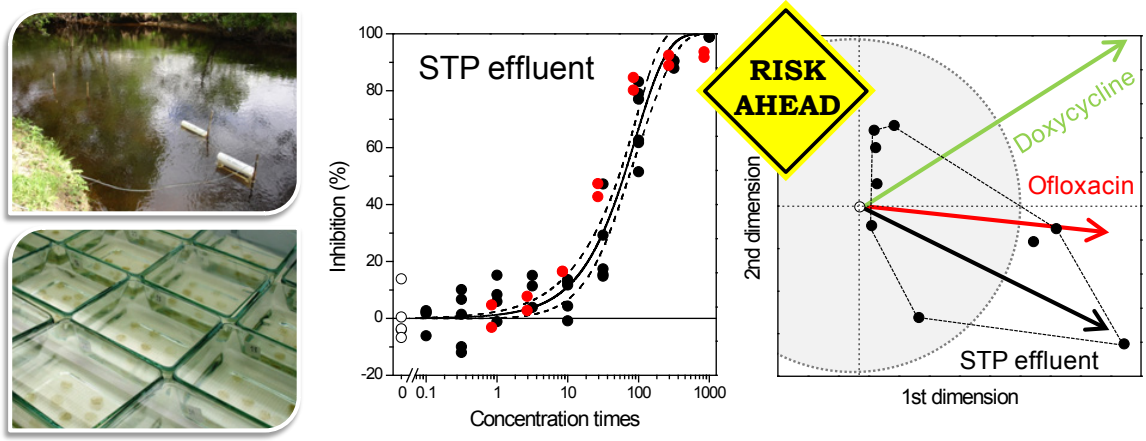
Chapter 4

Toxicity and environmental risk of antibiotics from STP effluents for limnic periphytic bacterial communities

Jose B. Carbajo, Åsa Arrhenius , Pedro Letón, Eloy García-Calvo and Thomas Backhaus

Environmental Pollution (submitted)

Graphical Abstract



TOXICITY AND ENVIRONMENTAL RISK OF ANTIBIOTICS FROM STP EFFLUENTS FOR LIMNIC PERIPHYTIC BACTERIAL COMMUNITIES

Abstract

The chronic toxicity of six antibiotics frequently detected in sewage treatment plant (STP) effluents and freshwaters (doxycycline, erythromycin, metronidazole, ofloxacin, sulfamethoxazole and trimethoprim) towards limnic bacterial periphytic communities was assessed. Only doxycycline and ofloxacin affected the carbon source metabolization in a concentration-dependent fashion at concentrations above 2.0 and 16 $\mu g \cdot L^{-1}$, respectively. Ofloxacin exposure has a more selective effect, resulting in clear changes in the bacterial carbon source utilization pattern, while doxycycline affects the bacterial utilization of a broader range of carbon sources with a similar concentration-response pattern. Indeed, both antibiotics cause different toxicant-induced succession (TIS) trajectories in the heterotrophic part of the communities, showing a dissimilar ecological mode of action. The combined effects of the six antibiotics were determined using a fixed ratio design according to their maximum detected concentrations in the effluent of two European STPs. Exposure to antibiotic mixtures reflecting effluents from the Swedish STP (Ryaverket, Gothenburg) and the Spanish STP (West-Alcalá, Madrid) affected the overall metabolic response of the heterotrophic communities to concentrations 8.9 and 250 times higher than the maximum detected concentrations, respectively. Furthermore, exposure to the mixture from the Spanish STP led to a re-arrangement of the carbon source utilization, indicating a change in community biodiversity and/or function with a pattern mainly influenced by ofloxacin. Results from screening level risk assessment indicate potential risk from ofloxacin and the antibiotic mixture under Spanish STP scenario. The results highlight that toxic effects of the antibiotic mixture, and especially ofloxacin (the risk driver of the mixture), should be assessed, in order to decide whether mitigation measures such as source control by targeted restrictions or STP upgrading for improving removal efficiencies are needed.

1. Introduction

Antibiotics are used extensively in human medicine for treating bacterial infections (Kümmerer, 2009a). More than 10 000 tonnes of antibiotics are consumed in the European Union each year (Valera *et al.*, 2013), with use patterns that vary greatly between countries (ECDC, 2014). Many antibiotics are metabolized only to a small extent, are poorly biodegraded and incompletely removed by conventional sewage treatment plants (STPs) (Verlicchi *et al.*, 2012). Thus, antibiotics are continuously released in wastewater effluents into the aquatic environment (Michael *et al.*, 2013), where they are detected in the $ng \cdot L^{-1}$ to lower $\mu g \cdot L^{-1}$ range (Kolpin *et al.*, 2002, Segura *et al.*, 2009, Santos *et al.*, 2010, Fatta-Kassinos *et al.*, 2011 and Rodríguez-Mozaz *et al.*, 2015).

Although the main concern of antibiotics is related to the development of resistance mechanisms by bacteria and its implications for human health (Ashbolt *et al.*, 2013, Rodríguez-Rojas, *et al.*, 2013 and Rodríguez-Mozaz *et al.*, 2015), their continuous release into environment and their bioactive properties also raise concerns about the chronic toxicity of antibiotics to aquatic organisms (Kümmerer, 2009a). Nevertheless, most research on the toxicity of antibiotics has been focused on investigations of their toxicity in single-species tests (Jjemba, 2008, Santos *et al.*, 2010 and Brausch, 2012). This approach is based on laboratory exposures that estimate the toxicity on single species by measuring the response as physiological or population-based parameters (*i.e.* mortality, growth, reproduction, mobility, and metabolism) (Proia *et al.*, 2013a). However, single-species tests do not take into account the interaction among species, often use genetically homogeneous populations of standard species that are not indigenous to the receiving water body and the tests are often conducted under experimental conditions very different from the receiving aquatic environment of concern (Geiszinger *et al.*, 2009). Therefore, ecotoxicological assessments should be completed by studies that use natural communities that are present in the receiving water body, in order to provide a more realistic indication of the toxicity of antibiotics in exposed ecosystems (Proia *et al.*, 2013b).

Evaluation of the toxicity of antimicrobials to complex communities is limited during the regulatory risk assessment to the activated sludge respiration inhibition test (OECD Guideline 209) (EMA, 2006). However, activated sludge communities have been established and live while exposed to concentrations of antibiotics that are significantly higher than those that occur in the environment (Verlicchi *et al.*, 2013). Therefore, sewage sludge bacterial communities are more tolerant towards antibiotics than bacteria in receiving waters. Moreover, the standard activated sludge respiration inhibition test is an acute assay that underestimates the toxic effects of antibiotics (Froehner *et al.*, 2000 and Kümmerer *et al.*, 2004).

Periphyton is an aquatic biofilm-forming community that develops on submerged surfaces. It comprises bacteria and other heterotrophs (*e.g.*, fungi and protozoa), and autotrophs (*e.g.*, diatoms, green algae and cyanobacteria) embedded in an extracellular polymeric matrix. Periphyton is a highly structured entity in which a diverse range of species compete for space and nutrients, each with its own strategy and sensitivity towards different stressors (Sabater *et al.*, 2007). Species-dependent changes in ecological fitness due to exposure to toxic compounds hence do not only change the overall physiological activity of the biofilm species, but also affect community biodiversity (Blanck, 2002). Furthermore, periphyton biofilms can be established in the natural environment and then transferred to the laboratory where they can be exposed to individual chemicals or complex mixtures under controlled conditions, combining high ecological realism with the precision and experimental capacity of laboratory-based studies (Porsbring *et al.*, 2007 and Johansson *et al.*, 2014).

Aquatic ecosystems are exposed to various multi-component mixtures (Backhaus, 2014) and also antibiotics do not occur as isolated, pure substances in the environment, although their toxicity has been mainly assessed substance by substance (Vasquez *et al.*, 2014). It is well established that chemical mixtures typically have higher toxicities than each its component alone (Kortenkamp *et al.*, 2009). Consequently, a mixture can have a considerable toxicity even if all components are present in low concentrations that do not induce toxic effects singly (Backhaus *et al.*, 2008), a pattern also observed for mixtures of antibiotics (Backhaus *et al.*, 2000). Therefore, mixture

effects must be taken into account when assessing the environmental risk of antibiotics, in order not to underestimate their hazard (Backhaus and Karlsson, 2014). Experimental findings suggest that the overall risk of a multi-component may often be driven by a few compounds (Price *et al.*, 2012 and Backhaus and Karlsson, 2014). The identification of such "drivers of mixture toxicity" has been put forward as a research priority in order to develop appropriate risk management and mitigation measures in order to safeguard the environment against adverse biological effects of anthropogenic chemical contamination (Altenburger *et al.*, 2015).

The present study assesses the chronic toxicities of six antibiotics that are frequently detected in STP effluents and the freshwater environment (doxycyline, erythromycin, metronidazole, ofloxacin, sulfamethoxazole and trimethoprim) to limnic periphytic bacterial communities. The joint toxicities of the six antibiotics, mixed in proportion to their occurrence in effluents from a Swedish STP (Ryaverket, Gothenburg) and a Spanish STP (West-Alcalá, Madrid), were studied in order to determine whether the wastewater effluents might impact the receiving freshwater ecosystem. On basis of toxicity data, it is then assessed whether the single antibiotics and their mixture might pose a risk to freshwater ecosystems, and identified the major risk driver of the mixture.

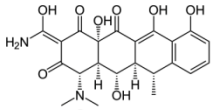
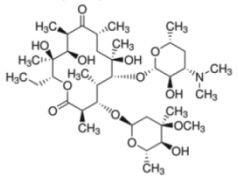
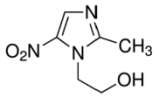
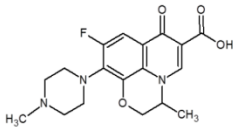
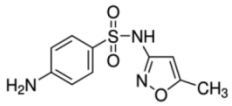
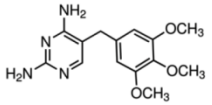
2. Materials and methods

2.1. Materials

The antibiotics used in this study belong to different chemical and mode-of-action classes and were selected based on their occurrence in STP effluents and freshwaters (see Table 4.1 and Supplementary data). The following six antibiotics were tested: doxycycline (DXY), erythromycin (ERY), ofloxacin (OFX), and trimethoprim (TMP) purchased from Sigma-Aldrich, and sulfamethoxazole (SMX) and metronidazole (MNZ) purchased from Fluka.

The principal mode of action of DXY (tetracycline) and ERY (macrolide) is inhibition of protein biosynthesis by binding to bacterial 30Sor 50S ribosomal subunits,

Table 4.1 Physical and chemical characteristics, STP removal efficiency and occurrence in STP effluents and freshwaters of the studied antibiotics.

Trade name (Abbreviation)	Group	CAS number	Structural formula	Molecular weight ($g \cdot mol^{-1}$)	$log\ K_{OW}$ [a]	pk_a [a]	STP removal efficiency (%) [b]	Occurrence [c]	
								STP effluent ($ng \cdot L^{-1}$)	Freshwater ($ng \cdot L^{-1}$)
Doxycycline (DXY)	Tetracycline	564-25-0		444.43	−0.02	3.0 8.0 9.2	57 (1) 14–100	71 (20) 915	33 (12) 400
Erythromycin (ERY)	Macrolide	114-07-8		733.93	3.06	8.9	19 (11) 0-75	150 (147) 6 316	18 (192) 3 847
Metronidazole (MNZ)	Nitroimidazole	44-48-1		171.15	-0.02	2.6	38 (2) 38–39	137 (40) 2 163	12 (62) 1 837
Ofloxacin (OFX)	Quinolone	82419-36-1		361.37	0.36	6.0 8.3	64 (11) 13–99	219 (128) 160 000	78 (104) 11 735
Sulfamethoxazole (SMX)	Sulfamide	723-46-4		253.28	0.89	1.9 5.6	44 (23) 0–100	183 (268) 9 460	40 (248) 11 920
Trimethoprim (TMP)	–	738-70-5		290.32	0.91	3.2 6.8	36 (20) 0–85	172 (220) 5 000	22 (189) 4 000

[a] According to Ying *et al.*, 2013.

[b] STP removal efficiency (aqueous phase removal) represent the median (number of values reported in brackets) and the interval (minimum-maximum) values reported according to Verlicchi *et al.* (2013).

[c] Ocurrence represent the median (number of values reported in brackets being > limit of quantification for each compound) and the maximum values reported in peer-reviewed literature (Supplementary data).

respectively (González-Pleiter *et al.*, 2013). OFX (quinolone) inhibits the enzyme DNA gyrase and topoisomerase IV, affecting replication and transcription. MNZ (nitroimidazole) is a pro-drug, whose reduced form is covalently bound to DNA inhibiting bacterial nucleic synthesis whose reduced form inhibits nucleic acid synthesis by disrupting the DNA of microbial cells (Kümmerer *et al.*, 2000). Finally, SMX (sulfonamide) and TMP inhibit the folate synthesis pathway in bacteria, but their inhibition sites are different. SMX inhibits dihydropteroate synthetase which catalyses the conversion of para-aminobenzic acid to dihydropteroic acid, a precursor of folate. On the other hand, TMP inhibits dihydrofolate reductase, which converts dihydrofolic acid to tetrahydrofolic acid, both active forms of folic acid suitable for utilization (Eguchi *et al.*, 2004).

Antibiotics were tested singly and in 6-component mixtures. The dilution series of single antibiotics were tested in concentrations ranging from the $ng \cdot L^{-1}$ to lower $mg \cdot L^{-1}$ range (up to 10 $\mu mol \cdot L^{-1}$). Two multi-component mixtures were studied, in which the six antibiotic were mixed relative to their occurrence in wastewater effluents from a Swedish STP (Ryaverket: 13 500 $m^3 \cdot h^{-1}$, 832 000 population equivalent, Gothenburg) and Spanish STP (West-Alcalá: 3 000 $m^3 \cdot h^{-1}$, 374 000 population equivalent, Madrid) (Table 4.2). Both STPs apply an activated sludge treatment followed by clarification. The main sources of the incoming waters are urban (included hospital wastewaters) and to a

Table 4.2 Concentration of studied antibiotics ($ng \cdot L^{-1}$) at the worst case scenario (maximum value detected in STP effluent) in the Swedish STP (Ryaverket, Gothenburg) and Spanish STP (West-Alcalá, Madrid).

Antibiotic	Concentration ($ng \cdot L^{-1}$)	
	Swedish STP	Spanish STP
Doxycycline	227[a]	61[c]
Erythromycin	160[a]	760[d]
Metronidazole	33[a]	127[d]
Ofloxacin	120[b]	3 594[d]
Sulfamethoxazole	20[b]	370[d]
Trimethoprim	231[a]	148[d]
Mixture	791	5 060

[a] Lindenberg *et al.*, 2005
[b] Andreozzi *et al.*, 2003
[c] Hijosa-Valsero *et al.*, 2011
[d] Rosal *et al.*, 2010

lesser extent industrial. Therefore, the antibiotics found in the effluents originate from their use in human medicine. It is important to note in this context that the two countries represent different antibiotic consumption patterns (Johanson *et al.*, 2015). Sweden used an average defined daily dose (DDD) of 13.0 per 1 000 inhabitants and per day in 2013, while Spain consumed 24.2 DDD, a 1.9-fold difference (ECDC, 2014). Both 6-compound mixtures were tested from a concentration of 0.1 to 1 000 times the maximum detected antibiotic concentrations in each STP effluent.

The tested antibiotics were dissolved in methanol in order to obtain a concentrated stock solution ($g \cdot L^{-1}$) for each compound and were stored at -20ºC in the dark. In order to prepare test solutions, the corresponding aliquot of the stock solution was pipetted into a 250 mL Pyrex bottle and the methanol was left to evaporate. Afterwards the test medium was added, which consisted of filtered river water (GF/F, Whatman, pore size 0.7 µm) amended with nutrients (Z8 medium, Scandinavian Culture Collection for Algae and Protozoa). The river water was collected from the periphyton sampling site one day prior to the start of the experiment, filtered and stored in the dark at 4ºC until use. Characteristics of the river water are included in Table 4.3. All toxicant dilutions were vigorously shaken at 4ºC in the dark for at least 12 h prior to use. New dilution series were prepared every 24 h and were used for changing the test medium in the periphyton incubation vessels in order to ensure constant toxicant and nutrient concentrations.

Table 4.3 Main physico-chemical parameters of Mölndalsån river at station no. 4 (Göta Älvs Vattenvårdsförbund, 2013) during the sampling period of the experiments.

Temperature (ºC)	16.8	Alkalinity (mg $CaCO_3 \cdot L^{-1}$)	39.0
Dissolved oxygen ($mg \cdot L^{-1}$)	8.9	N like NO_3^- ($\mu g \cdot L^{-1}$)	190
pH	7.0	Total N ($\mu g \cdot L^{-1}$)	500
Conductivity ($\mu S \cdot cm^{-1}$)	86.9	Total P ($\mu g \cdot L^{-1}$)	10.9
Coloration (mg $Pt \cdot L^{-1}$)	45.0	COD ($mg \cdot L^{-1}$)	8.5
Turbidity (FNU)	2.6	TOC ($mg \cdot L^{-1}$)	7.3

The stability of antibiotics under SWIFT periphyton test conditions was examined at the beginning and at the end of the exposure time according to the corresponding OECD Guidance (OECD, 2008). Analysis have been performed by means of LC-MS/MS using an Agilent 1260 LC equipped with an Agilent 6410 triple quadrupole detector. The

separation was performed on an Agilent Zorbax SB-C18 column (50 × 2.1 mm, 1.8 µm) equipped with a 5 mm guard column. A gradient of water (0.1% formic acid) [A] and acetonitrile (0.1% formic acid) [B] was applied as follows: 0 min, 5% [B]; 4 min, 10% [B]; 15 min, 100% [B]; 16 min 5% [B]. The electrospray source of the detector was operated in positive mode with an ionization voltage of 4 000 V at 250ºC with a nitrogen flow of 11 $L \cdot min^{-1}$ at 40 psi. The following main ions $[M+H]^+$ and one or more fragment ions for MS determination were chosen: for DXY *m/z* 445, 420 and 201; for ERY *m/z* 734, 158 and 116; for MNZ *m/z* 172 and 128; for OFX *m/z* 362, 318, and 261; for SMX *m/z* 254, 156 and 92, and for TMP *m/z* 291, 261 and 123. The concentrations of ERY, MNZ, SMX and TMP remained at 80–120% of their respective nominal concentration, therefore the effect concentrations are expressed as nominal concentrations in accordance with OECD Guidance (OECD, 2008). Concentrations of OFX and DXY after 24 hours exposure decreased to 76 and 52% of their nominal concentration, respectively. The limited stability of quinolones and tetracyclines in aqueous solution as a consequence of their photodegradability and complexing properties have been previously reported (Halling-Sørensen *et al.*, 2002 and Sukul and Spiteller, 2007). Thus, the exposure concentrations of OFX and DXY were calculated as the geometric mean of the measured concentrations during the experiment.

2.2. SWIFT periphyton test

Toxicant effects were studied in a slightly modified version of the semi-static SWIFT periphyton test, as described by Porsbring *et al.* (2007). Periphyton communities were sampled in Mölndalsån (N 57º 40´ 59´´ E 12º 13´ 7´´), a small river near Gothenburg (Sweden), which is neither recipient of STP effluent nor subjected to run offs from agricultural areas, and is hence considered free from antibiotic contamination. Biofilms were established on submerged glass discs (1.5 cm^2) that were mounted on polyethylene racks (Blanck and Wangberg, 1988) over seven days at an approximate depth of 0.5 m and then transferred to the lab. Eight colonized discs were placed in glass beakers (10 × 15 × 5 cm), into which 200 mL test solution were then added. The periphyton communities were then incubated for three days in a thermo constant room

at river temperature (15–17ºC) with a day-light cycle illumination (16 h light of ~125 µmol photon·m^{-2}·s^{-1}, 8 h darkness). During the exposure the periphyton samples were constantly shaken.

In order to assess effects on bacteria, three glass discs were sampled after 72 h from each test vessel. The discs were transferred to glass scintillation vials containing 20 mL of test solutions. Scintillation vials were sonicated three times for 15 seconds, followed by vigorous shaking over 15 seconds in order to detach the periphyton biolfims from the discs. Afterwards, the suspension was filtered through sterile paper (Kimcare, Kimberly-Clark Professional) into a sterile plastic Petri dish to remove large biofilm clumps. 150 µL of filtered suspension was pipetted into each well of a Biolog EcoPlates™ (referred as EcoPlates in the following), purchased from Dorte Egelung ApS, Roskilde (Denmark). These 96-well plates, pre-loaded with 31 different carbon-sources and a tetrazolium dye (Table 4.4), provide information on total metabolic activity and functional diversity of the bacteria growing in them. Optical densities were measured over 96 h (24, 42, 48, 66, 72, 86 and 96 h) at 595 and 700 nm using a microplate spectrophotometer (µ Quant™, Bio-Tek Instruments Inc.).

The recorded optical density (OD) was corrected first for turbidity by subtracting the absorbance at 700 nm from the absorbance at 595 nm (absorbance of the oxidized tetrazolium dye) for each well. The resulting OD was subsequently corrected for any unspecific colour formation by subtracting the median absorbance of the three wells without any pre-loaded carbon source (blanks) to yield the final correct OD for each carbon source (OD_{corr}) and exposure time. Negative values for OD_{corr} were set to zero for the following calculations. Average well colour (AWC) was then determined for each plate and exposure time by calculating the arithmetic mean of the OD_{corr} of all carbon source wells. The inhibition of AWC for each treatment was finally calculated in relation to the average AWC of the control plates as an indicator of general response of the whole bacterial communities. For this purpose, the data recorded after 66 h incubation were used. At this time a clear colour development was visible in most wells, but at the same time, the extensively metabolized carbon sources were not yet exhausted.

Table 4.4 Pre-loaded carbon source in Biolog EcoPlates™

Lable	Carbon Source	Guild
C1	Water	Water
C2	Pyruvic acid methyl ester	Carboxylic acid
C3	Tween 40	Polymer
C4	Tween 80	Polymer
C5	α-cyclodextrin	Polymer
C6	Glycogen	Polymer
C7	D-cellobiose	Carbohydrate
C8	α-D-lactose	Carbohydrate
C9	β-methyl-D-glucoside	Carbohydrate
C10	D-xylose	Carbohydrate
C11	i-erythritol	Carbohydrate
C12	D-mannitol	Carbohydrate
C13	N-acetyl-D-glucosamine	Carbohydrate
C14	D-glucosaminic acid	Carboxylic acid
C15	Glucose-1-phosphate	Carbohydrate
C16	D,L-α-glycerol phosphate	Carbohydrate
C17	D-galactonic acid γ-lactone	Carbohydrate
C18	D-galacturonic acid	Carboxylic acid
C19	2-hydroxy benzoic acid	Phenolic compound
C20	4-hydroxy benzoic acid	Phenolic compound
C21	γ-hydroxybutyric acid	Carboxylic acid
C22	Itaconic acid	Carboxylic acid
C23	α-ketobutyric acid	Carboxylic acid
C24	D-malic acid	Carboxylic acid
C25	L-arginine	Amino acid
C26	L-asparagine	Amino acid
C27	L-phenylalanine	Amino acid
C28	L-serine	Amino acid
C29	L-threonine	Amino acid
C30	Glycyl-L-glutamic acid	Amino acid
C31	Phenylethyl-amine	Amine
C32	Putrescine	Amine

Inhibition of AWC values were used for estimating concentrations-response curves using the Weibull model (Eq. 4.1), while significances between control and treatment were calculated using Dunnett's test, in order to determine No Observed Effect Concentrations ($NOEC$).

$$AWC\ inh = 1 - \exp\left(-\exp\ (\theta_1 + \theta_2 * log_{10}(conc))\right) \quad (4.1)$$

Curves describing the bacterial activity (colour development) of each carbon source over incubation time from 0 to 96 h were determined by fitting a Weibull model in the form of:

$$OD_{corr} = 1 - \exp\left(-\exp\left(\theta_1 + \theta_2 * log_{10}(time)\right)\right) \quad (4.2)$$

The area under each of the resulting curve (AUC) was calculated as an estimate of the total metabolization of each carbon source, using the classical simplex method. In order to gain insight into the overall metabolic diversity of the communities, data were ordained using nonmetric multidimensional scaling (nMDS), which reduces the multidimensional structure of the data into a 2-dimensional plot in which the distances between the individual samples reflect the multivariate dissimilarity between the original samples (Clarke and Warwick, 2001). Manhattan Distances (City-Block Metric) (Eq. 4.3) between all pairs of samples j, k were used as input data for the similarity matrix.

$$MD = \sum_{i=2}^{31} \left| OD_{corr\,j,i} - OD_{corr\,k,i} \right| \quad (4.3)$$

Calculations were implemented using PROXSCAL algorithm in SPSS software (v. 22, IBM SPSS, Chicago, USA).

2.3. Concept for predicting mixture toxicities

Predictive approaches based on the mathematical concepts of Concentration Addition (CA) and Independent Action (IA) have been performed. Both concepts predict the toxicity of a mixture based on the individual toxicities of the mixture components (Kortenkamp *et al.*, 2009).

CA can be mathematically formulated for an n-compound mixture as:

$$\sum_{i=1}^{n} \frac{c_i}{EC_{x_i}} = 1 \quad (4.4)$$

where c_i denotes the concentration of compound i in a mixture that is expected to cause x% effect, and EC_{x_i} gives the concentration at which the compound i alone causes the same x% effect. CA is based on the assumption that all components in the

mixture behave as if they are simple dilutions of one another, which is often taken to mean that CA describes the joint action of compounds with an identical mechanism of action (Kortenkamp *et al.*, 2009).

The competing concept of IA has been derived from probabilistic reasoning. Accordingly, the effect of a mixture comprised of n-compounds is calculated by applying the statistical concept of independent random events (Bliss, 1939):

$$E(c_{mix}) = 1 - \prod_{i=1}^{n}[1 - E(c_i)] \quad (4.5)$$

where $E(c_i)$ is the effect of compound i if applied alone at concentration c_i, the concentration at which it is present in the mixture. Due to its probabilistic basis, IA assumes that all substances in a mixture exert their effects completely independent of each other. This is usually interpreted as the compounds have dissimilar modes of action and affect different physiological process (Kortenkamp *et al.*, 2009).

2.4. Risk assessment

In order to estimate and assess the potential risk that antibiotics could cause on freshwater ecosystems, risk quotients (RQs) are calculated assuming the worst case scenario (*i.e.*, the maximum measured antibiotic concentrations) for Swedish and Spanish STP effluents (Table 4.2).

The toxic units (TUs, $TU_i = EnvConc_i/EC_{10_i}$) of single antibiotics were first calculated basis of EC_{10} due to the well-known shortcoming of classic $NOEC$ determinations (van Dam *et al.*, 2012). Multiplying TUs by the assessment factor (AF) of 10 (EMA, 2006) were calculated RQ for each single antibiotic. If no EC_{10} values were available for a particular antibiotic, the highest tested concentration was used.

On the basis of single antibiotic data, the expected joint risk of the antibiotic mixture is then estimated using the strategy for the component-based risk assessment

of chemical mixtures (Backhaus and Faust, 2012), which is primarily based on CA. In fact, the sum of toxic units (STUs) was calculated for each scenario as follows:

$$STUs = \sum_{i=1}^{n} \left(\frac{EnvConc_i}{EC_{10_i}} \right) \quad (4.6)$$

The final RQ for the mixture then equals the STU multiplied by the AF (EMA, 2006). The antibiotic mixtures studied by means of a whole-mixture approach were also assessed their risk as if they were a single chemical (Backhaus *et al.*, 2010) and then compared with the RQ from component-based strategy in order to determinate the risk predict capacity of the latter approach. RQ higher than 1 suggests that antibiotic risk would be inadequately controlled for the microorganisms present in a freshwater ecosystem.

3. Results and discussion

3.1. Toxicity of single antibiotics

The effects of antibiotics on the overall metabolic activity (inhibition of average well colour development, AWC) of the limnic periphytic bacterial communities are shown in Fig. 4.1. Table 4.5 provides $NOEC$, EC_{10}, EC_{50} and EC_{90} values together with the parameter estimates for the Weibull fits. The individual antibiotics clearly differed in their potencies: periphytic bacteria were highly sensitive to DXY and OFX, which caused effects on their metabolic activity at µg·L^{-1} concentrations, with EC_{10} and EC_{50} values of 2.0 and 94.3 µg·L^{-1} for DXY and 15.9 and 117 µg·L^{-1} for OFX. A comparison of the EC_{50} to the EC_{10} values reveals, however, that the concentration-reponse curves have different slopes with DXY (EC_{50}/EC_{10} = 47) having a clearly flatter curve than OFX (EC_{50}/EC_{10} = 7.4). A flat concentration-response curve might pose more of a challenge for risk mitigation measures, as huge reductions in the concentration would be necessary to decrease toxicity. The most significant factor affecting steepness of the concentration-response slope at community-level is, apart from the mechanism/mode of action of the antibiotic, the biodiversity of the organisms within DXY and OFX might also be driven by

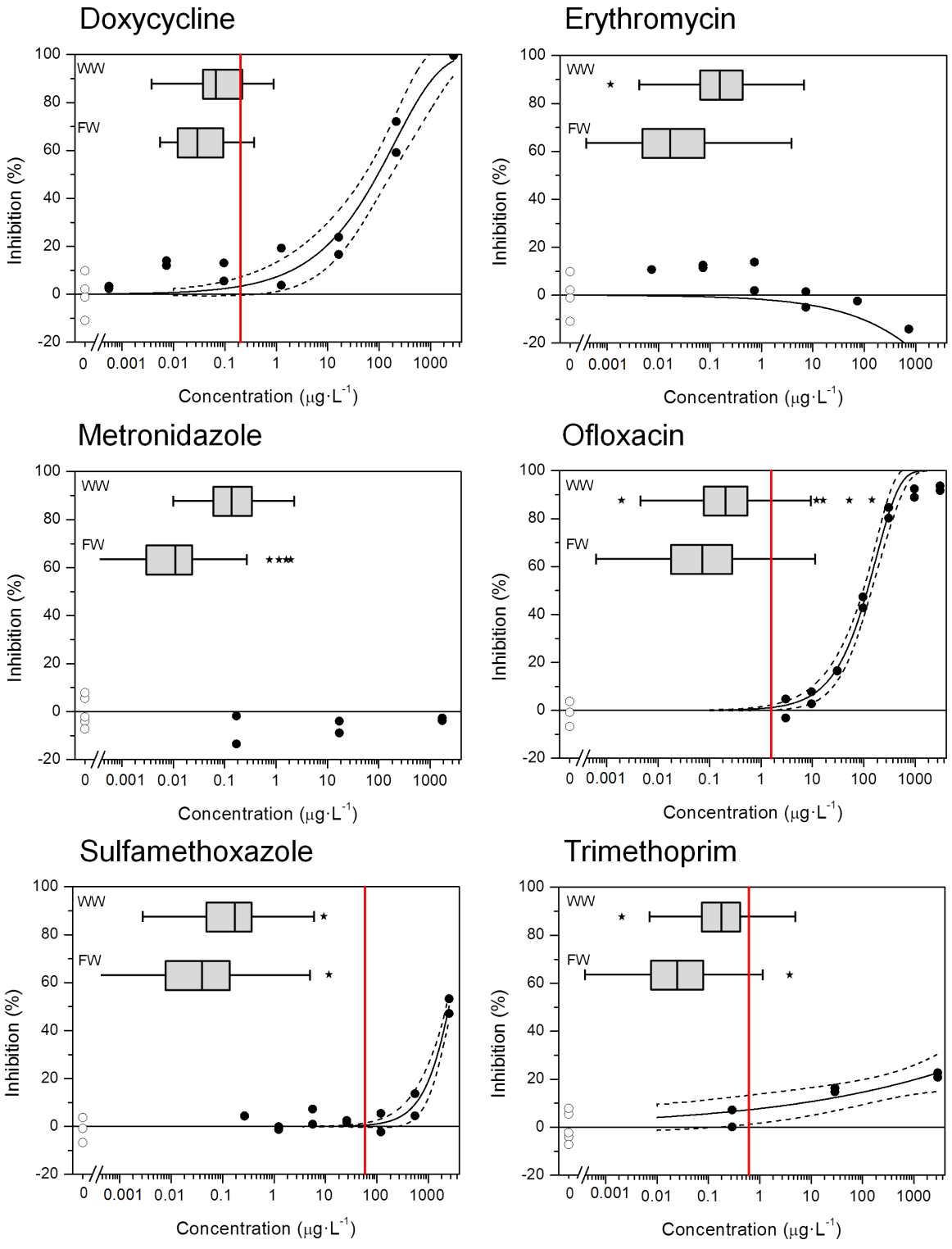


Fig 4.1 Concentration-response curves for the individual antibiotics for the endpoint "inhibition of average well colour development" (AWC) (●) with corresponding controls (○). Solid lines give the Weibull model fit and dashed lines their 95% confidence intervals. Box-plots represent occurrence of antibiotics in wastewater (WW) and freshwater (FW) reported in peer-reviewed literature (Supplementary data), and vertical red lines represent the $PNEC$ value determined for bacterial communities in the present study.

Table 4.5 Effect on periphytic bacteria exposed to single antibiotics. The effect is described as the inhibition of the average well color (AWC) in EcoPlates. Estimated parameters of the Weibull fits ($\hat{\theta}_1$,$\hat{\theta}_2$) that were used for estimating EC_{10}, EC_{50} and EC_{90} values are given together with approximate 95% confidence intervals and the No Observed Effect Concentrations ($NOECs$) determinate using Dunnett's test, α=0.05. Concentration values are expressed in µg·L^{-1}.

Antibiotic	$\hat{\theta}_1$	$\hat{\theta}_2$	EC_{10}	EC_{50}	EC_{90}	$NOEC$
Doxycycline	-2.58812	1.12460	2.00 [0.47–8.39]	94.3 [52.6–173]	1 100 [452–2 530]	1.25
Erythromycin	–	–	–	–	–	≥7 339
Metronidazole	–	–	–	–	–	≥1 712
Ofloxacin	-4.86374	2.17578	15.9 [9.96–25.5]	117 [96.9–140]	416 [311–549]	9.60
Sulfamethoxazole	-10.4506	2.96410	584 [358–909]	2 520 [2 220–2 980[a]]	6 390[a]	551
Trimethoprim	-2.51279	0.33351	6.13 [0.02–133]	2.73 10^{6} [b]	1.09 10^{10} [b]	0.29

[a] estimation of the upper limit of confidence band and EC_{90} value outside the concentration range. Maximum inhibition of 50% was recorded at 2 532 µg·L^{-1}

[b] estimation of EC_x value outside the concentration range. Maximum inhibition of 22% was recorded at 2 903 µg·L^{-1}

other factors such as ecological the exposed community and their different sensitivities towards the toxicant (Kümmerer *et al.*, 2009b). However, the different slope of the concentration-response curves of interactions between biofilm-inhabiting organisms (*i.e.*, algae, bacteria, fungi, protozoa) (Geiszinger *et al.*, 2009). Similar concentration-response relationships were found for tetracycline (chlortetracycline) and quinolone (ciprofloxacin) antibiotics on bacterial communities from natural environments (Brosche and Backhaus, 2010 and Johansson *et al*., 2014).

SMX and TMP affected limnic bacterial communities at $mg \cdot L^{-1}$ concentrations, while MNZ and ERY did not inhibit the carbon source metabolism in the tested concentration range (Fig. 4.1). Instead, a significant stimulation of the AWC was visible at ERY concentrations higher than 73 $\mu g \cdot L^{-1}$. These patterns are consistent with the spectrum of activity of the tested antibiotics. OFX, DXY, SMX and TMP have a broad spectrum of activity against gram-positive and gram-negative bacteria, whereas metronidazole is mainly effective against anaerobes (Kümmerer *et al.*, 2000). ERY is most effective against gram-positive bacteria (Alexy, 2003), while limnic bacterial communities are dominated by gram-negative bacteria (Manz *et al.*, 1999). Its stimulatory effects might therefore indicate indirect effects, *i.e.* the suppression of the few gram-positive species present which might lead to an increased metabolic activity of the unaffected gram-negative species.

The exposed periphytic bacterial communities had a similar sensitivity as various gram-negative bacteria that were tested in single-species tests (Table 4.6), which is consistent with the prevalence of these bacteria in freshwater ecosystems (Manz *et al.*, 1999). SMX was a notable exception, the periphytic bacterial biofilm was clearly more sensitive than the gram-negative bacterium *Pseudomonas putida* (EC_{50} values of 12 700 and 58 700 $\mu g \cdot L^{-1}$) and the gram-positive *Enterococcus faecalis* (EC_{50} >800 000 $\mu g \cdot L^{-1}$). The European Medicines Agency (EMA) suggests using blue-green algae for the toxicity testing of antimicrobials (EMA, 2006). These are already affected at lower concentrations of ERY, OFX and SMX, but show higher tolerance to TMP than the bacterial communities of the present study (Table 4.6). The limnic bacterial communities exposed in the current study seem to be more tolerant both to SMX and ERY than the

Table 4.6 Aquatic toxicity of the six selected antibiotics to multi-generational single species-test of freshwater bacteria and cyanobacteria. Values are expressed as $\mu g \cdot L^{-1}$ (continued on next page).

Antibitoic	Species	Taxonomic group	Exposure	Parameter	Value	Reference
Doxycycline	*Pseudomonas putida*	Bacteria (gram-negative)	16 h	EC_{10}	9.6	Carbajo *et al.*, 2015a
	Pseudomonas putida	Bacteria (gram-negative)	16 h	EC_{50}	40	Carbajo *et al.*, 2015a
	Escherichia coli	Bacteria (gram-negative)	24 h	EC_{50}	58	Suda *et al.*, 2012
	Bacillus subtilis	Bacteria (gram-positive)	24 h	EC_{50}	8.9	Suda *et al.*, 2012
Erythromycin	*Pseudomonas putida*	Bacteria (gram-negative)	16 h	EC_{10}	10 700	Alexy, 2003
	Pseudomonas putida	Bacteria (gram-negative)	16 h	EC_{50}	1 870	Alexy, 2003
	Enterococcus faecalis	Bacteria (gram-positive)	6 h	EC_{10}	562	Alexy, 2003
	Enterococcus faecalis	Bacteria (gram-positive)	6 h	EC_{50}	1 870	Alexy, 2003
	Anabaena sp. CPB4337	Algae (cyanobacteria)	72 h	EC_{10}	5	González-Pleiter *et al.*, 2013
	Anabaena sp. CPB4337	Algae (cyanobacteria)	72 h	EC_{50}	22	González-Pleiter *et al.*, 2013
	Anabaena cylindrica	Algae (cyanobacteria)	144 h	$NOEC$	3.1	Ando *et al.*, 2007
	Anabaena cylindrica	Algae (cyanobacteria)	144 h	EC_{50}	35	Ando *et al.*, 2007
	Anabaena flos-aquae	Algae (cyanobacteria)	144 h	$NOEC$	47	Ando *et al.*, 2007
	Anabaena flos-aquae	Algae (cyanobacteria)	144 h	EC_{50}	270	Ando *et al.*, 2007
	Anabaena variabilis	Algae (cyanobacteria)	144 h	$NOEC$	47	Ando *et al.*, 2007
	Anabaena variabilis	Algae (cyanobacteria)	144 h	EC_{50}	430	Ando *et al.*, 2007
	Microcystis aeruginosa	Algae (cyanobacteria)	144 h	$NOEC$	10	Ando *et al.*, 2007
	Microcystis aeruginosa	Algae (cyanobacteria)	144 h	EC_{50}	23	Ando *et al.*, 2007
	Microcystis wesenbergii	Algae (cyanobacteria)	144 h	$NOEC$	4.7	Ando *et al.*, 2007
	Microcystis wesenbergii	Algae (cyanobacteria)	144 h	EC_{50}	23	Ando *et al.*, 2007
	Nostoc sp. PCC7120	Algae (cyanobacteria)	144 h	$NOEC$	100	Ando *et al.*, 2007
	Nostoc sp. PCC7120	Algae (cyanobacteria)	144 h	EC_{50}	200	Ando *et al.*, 2007
	Synechococcus leopoldensis	Algae (cyanobacteria)	144 h	$NOEC$	2.0	Ando *et al.*, 2007
	Synechococcus leopoldensis	Algae (cyanobacteria)	144 h	EC_{50}	160	Ando *et al.*, 2007
	Synechococcus sp. PCC7002	Algae (cyanobacteria)	144 h	$NOEC$	7.8	Ando *et al.*, 2007
	Synechococcus sp. PCC7002	Algae (cyanobacteria)	144 h	EC_{50}	230	Ando *et al.*, 2007
Metronidazole	*Pseudomonas putida*	Bacteria (gram-negative)	16 h	EC_{10}	342 000	Alexy, 2003
	Pseudomonas putida	Bacteria (gram-negative)	16 h	EC_{50}	>800 000	Alexy, 2003
	Enterococcus faecalis	Bacteria (gram-positive)	6 h	EC_{10}	474 000	Alexy, 2003
	Enterococcus faecalis	Bacteria (gram-positive)	6 h	EC_{50}	>800 000	Alexy, 2003
Ofloxacin	*Pseudomonas putida*	Bacteria (gram-negative)	16 h	EC_{10}	90	Alexy, 2003
	Pseudomonas putida	Bacteria (gram-negative)	16 h	EC_{50}	265	Alexy, 2003
	Pseudomonas putida	Bacteria (gram-negative)	16 h	EC_{10}	67	Akhyany, 2013
	Pseudomonas putida	Bacteria (gram-negative)	16 h	EC_{50}	123	Akhyany, 2013

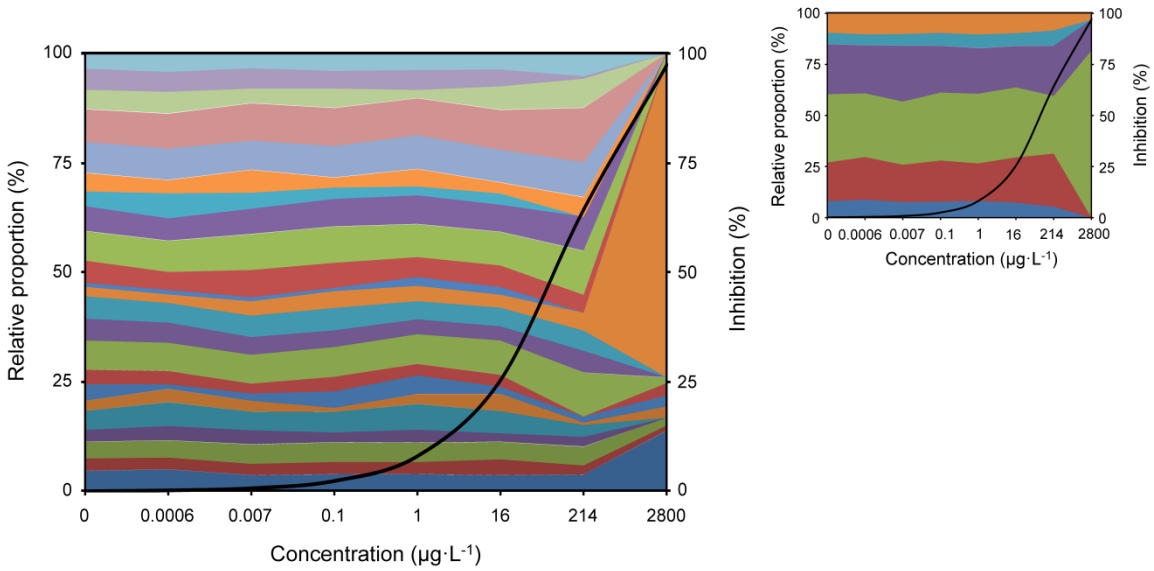
	Pseudomonas putida	Bacteria (gram-negative)	16 h	EC_{10}	31	Carbajo *et al.*, 2015b
	Pseudomonas putida	Bacteria (gram-negative)	16 h	EC_{50}	113	Carbajo *et al.*, 2015b
	Enterococcus faecalis	Bacteria (gram-positive)	6 h	EC_{10}	632	Alexy, 2003
	Enterococcus faecalis	Bacteria (gram-positive)	6 h	EC_{50}	2 420	Alexy, 2003
	Synechococcus leopolensis	Algae (cyanobacteria)	96 h	$NOEC$	5	Ferrari *et al.*, 2004
	Synechococcus leopolensis	Algae (cyanobacteria)	96 h	EC_{50}	16	Ferrari *et al.*, 2004
	Microcystis aeruginosa	Algae (cyanobacteria)	168 h	EC_{50}	21	Robinson *et al.*, 2005
Sulfamethoxazole	*Pseudomonas putida*	Bacteria (gram-negative)	16 h	EC_{10}	17 400	Alexy, 2003
	Pseudomonas putida	Bacteria (gram-negative)	16 h	EC_{50}	58 700	Alexy, 2003
	Pseudomonas putida	Bacteria (gram-negative)	16 h	EC_{10}	2 250	Akhyany, 2013
	Pseudomonas putida	Bacteria (gram-negative)	16 h	EC_{50}	12 700	Akhyany, 2013
	Enterococcus faecalis	Bacteria (gram-positive)	6 h	EC_{10}	154 000	Alexy, 2003
	Enterococcus faecalis	Bacteria (gram-positive)	6 h	EC_{50}	>800 000	Alexy, 2003
	Synechococcus leopolensis	Algae (cyanobacteria)	96 h	$NOEC$	5.9	Ferrari *et al.*, 2004
	Synechococcus leopolensis	Algae (cyanobacteria)	96 h	EC_{50}	26.8	Ferrari *et al.*, 2004
Trimethoprim	*Pseudomonas putida*	Bacteria (gram-negative)	16 h	EC_{10}	95 800	Alexy, 2003
	Pseudomonas putida	Bacteria (gram-negative)	16 h	EC_{50}	190 000	Alexy, 2003
	Pseudomonas putida	Bacteria (gram-negative)	16 h	EC_{10}	11 500	Akhyany, 2013
	Pseudomonas putida	Bacteria (gram-negative)	16 h	EC_{50}	75 500	Akhyany, 2013
	Enterococcus faecalis	Bacteria (gram-positive)	6 h	EC_{10}	91	Alexy, 2003
	Enterococcus faecalis	Bacteria (gram-positive)	6 h	EC_{50}	240	Alexy, 2003
	Anabaena cylindrica	Algae (cyanobacteria)	144 h	$NOEC$	≥200 000	Ando *et al.*, 2007
	Anabaena cylindrica	Algae (cyanobacteria)	144 h	EC_{50}	>200 000	Ando *et al.*, 2007
	Anabaena flos-aquae	Algae (cyanobacteria)	144 h	$NOEC$	≥200 000	Ando *et al.*, 2007
	Anabaena flos-aquae	Algae (cyanobacteria)	144 h	EC_{50}	>200 000	Ando *et al.*, 2007
	Anabaena variabilis	Algae (cyanobacteria)	144 h	$NOEC$	3 100	Ando *et al.*, 2007
	Anabaena variabilis	Algae (cyanobacteria)	144 h	EC_{50}	11 000	Ando *et al.*, 2007
	Microcystis aeruginosa	Algae (cyanobacteria)	144 h	$NOEC$	100 000	Ando *et al.*, 2007
	Microcystis aeruginosa	Algae (cyanobacteria)	144 h	EC_{50}	150 000	Ando *et al.*, 2007
	Microcystis aeruginosa	Algae (cyanobacteria)	168 h	EC_{50}	112 000	Holten-Luzhøft *et al.*, 1999
	Microcystis wesenbergii	Algae (cyanobacteria)	144 h	$NOEC$	3 100	Ando *et al.*, 2007
	Microcystis wesenbergii	Algae (cyanobacteria)	144 h	EC_{50}	>200 000	Ando *et al.*, 2007
	Nostoc sp. PCC7120	Algae (cyanobacteria)	144 h	$NOEC$	3 100	Ando *et al.*, 2007
	Nostoc sp. PCC7120	Algae (cyanobacteria)	144 h	EC_{50}	53 000	Ando *et al.*, 2007
	Synechococcus leopoldensis	Algae (cyanobacteria)	144 h	$NOEC$	13 000	Ando *et al.*, 2007
	Synechococcus leopoldensis	Algae (cyanobacteria)	144 h	EC_{50}	>200 000	Ando *et al.*, 2007
	Synechococcus sp. PCC7002	Algae (cyanobacteria)	144 h	$NOEC$	50 000	Ando *et al.*, 2007
	Synechococcus sp. PCC7002	Algae (cyanobacteria)	144 h	EC_{50}	>200 000	Ando *et al.*, 2007

freshwater biofilms studied by Yergeau *et al.* (2012) in which first effects were observed at 0.5 µg·L^{-1}, although these differences might be at least partly caused by the different endpoints employed.

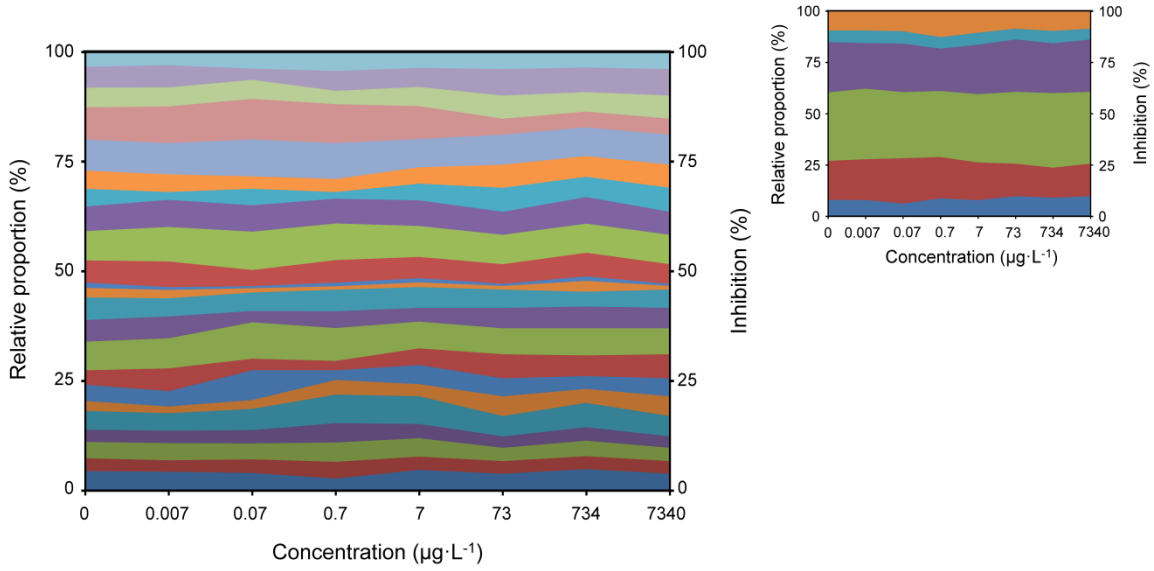
In order to further characterize the toxicant-induced changes of the bacterial communities, changes in the time-integrated metabolization of each individual carbon source during 96 hours were evaluated as changes in the area under the curve (AUC, see material and methods). The catabolic activity was in general unevenly distributed between the 31 carbon sources that are present on the EcoPlates, and not all carbon sources were utilized. Three carbon sources never reached an OD_{corr} of 0.05 or higher (C19 (2-hydroxy benzoic acid), C23 (α-ketobutyric acid) and C29 (L-threonine)) and were classified as inactive. Five additional carbon sources (C5 (α-cyclodextrin), C11 (i-erythritol), C21 (γ-hydroxybutyric acid), C27 (L-phenylalanine) and C30 (glycyl-L-glutamic acid)) were only slightly metabolized by unexposed communities (OD_{corr} <0.13 after 66 h incubation). Data from all these carbon sources were not used for the subsequent analyses.

In order to visualize the pattern of carbon source utilization, the relative proportion of each carbon source to the total carbon source utilization was plotted. DXY and OFX, the two most toxic antibiotics, induced the most substantial changes (Fig. 4.2). OFX changed the relative carbon source utilization, in relation to the chemical class ("guild") of the carbon sources (amines, amino acids, carbohydrates, carboxylic acids, phenolic compounds and polymers) already at low overall effect levels. First changes of the carbon source utilization pattern became visible at 96 µg·L^{-1}, corresponding to 44% effect on AWC. Utilization of phenolic compounds, amino acids and amines decreased notably at 304 µg·L^{-1} and higher, while the respiration of carbohydrates increased in parallel and they became the guild dominating the metabolic pattern at the higher concentrations. DXY exposure, in contrast, did not substantially affect the relative carbon source utilization at concentrations up to 214 µg·L^{-1}, which already caused 64% effect on AWC. Higher DXY concentrations then induced drastic changes. In fact, at the highest tested concentration of DXY, the carbohydrate C15 (glucose-1-phosphate) represents 73% of the carbon source utilization. Under the assumption that carbon

Doxycycline



Erythromycin



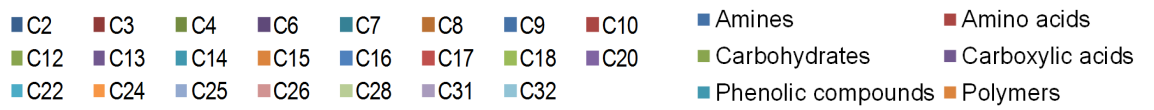
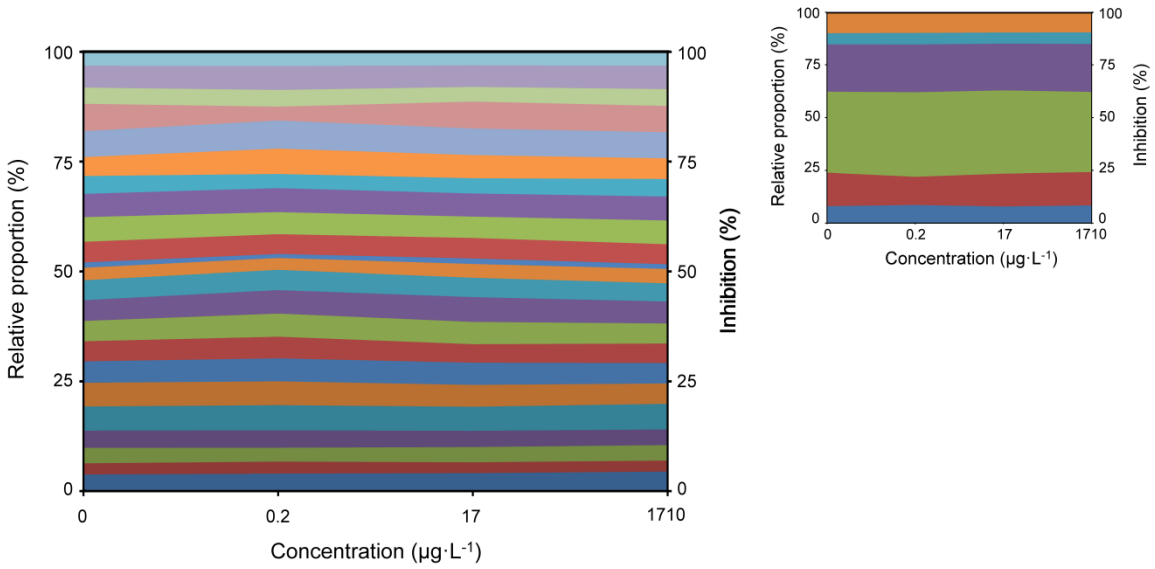


Fig.4.2 Relative area under the curve (AUC) of the individual carbon sources and the corresponding average well colour (AWC) Weibull function (continuous line) plotted against single antibiotic exposure concentrations. Inset plots represent relative AUC of individual guild and the corresponding AWC Weibull function (continued on next page).

Metronidazole



Ofloxacin

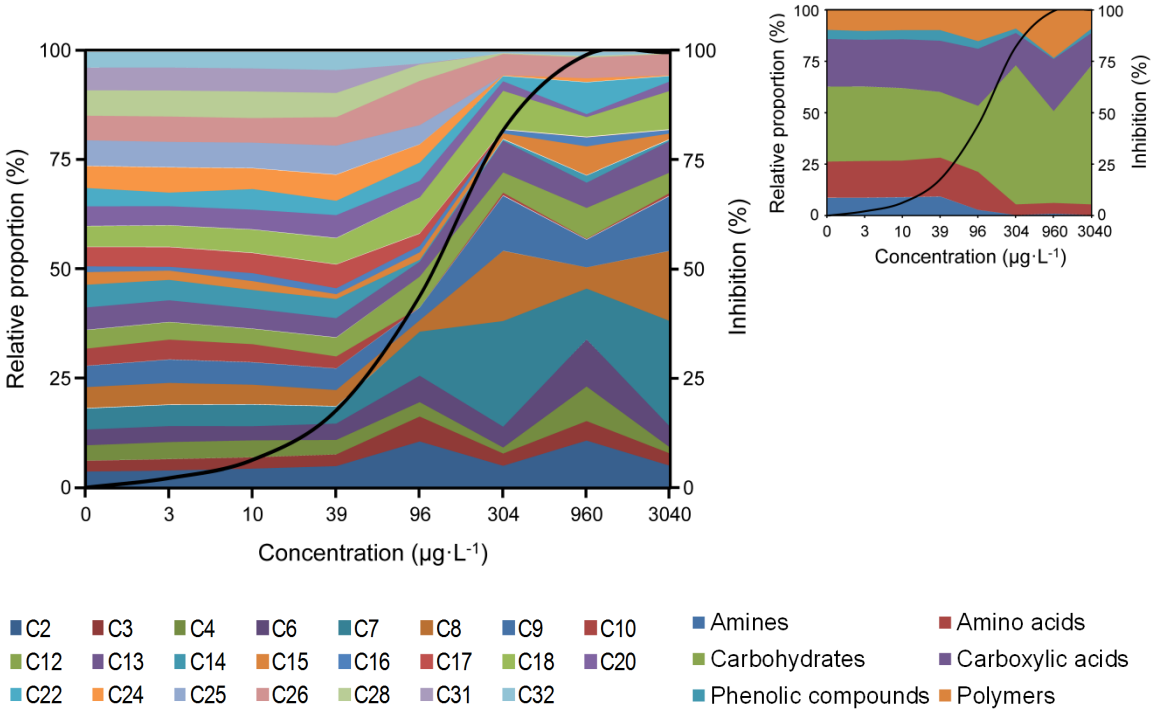


Fig. 4.2 Relative area under the curve (AUC) of the individual carbon sources and the corresponding average well colour (AWC) Weibull function (continuous line) plotted against single antibiotic exposure concentrations. Inset plots represent relative AUC of individual guild and the corresponding AWC Weibull function (continued on next page).

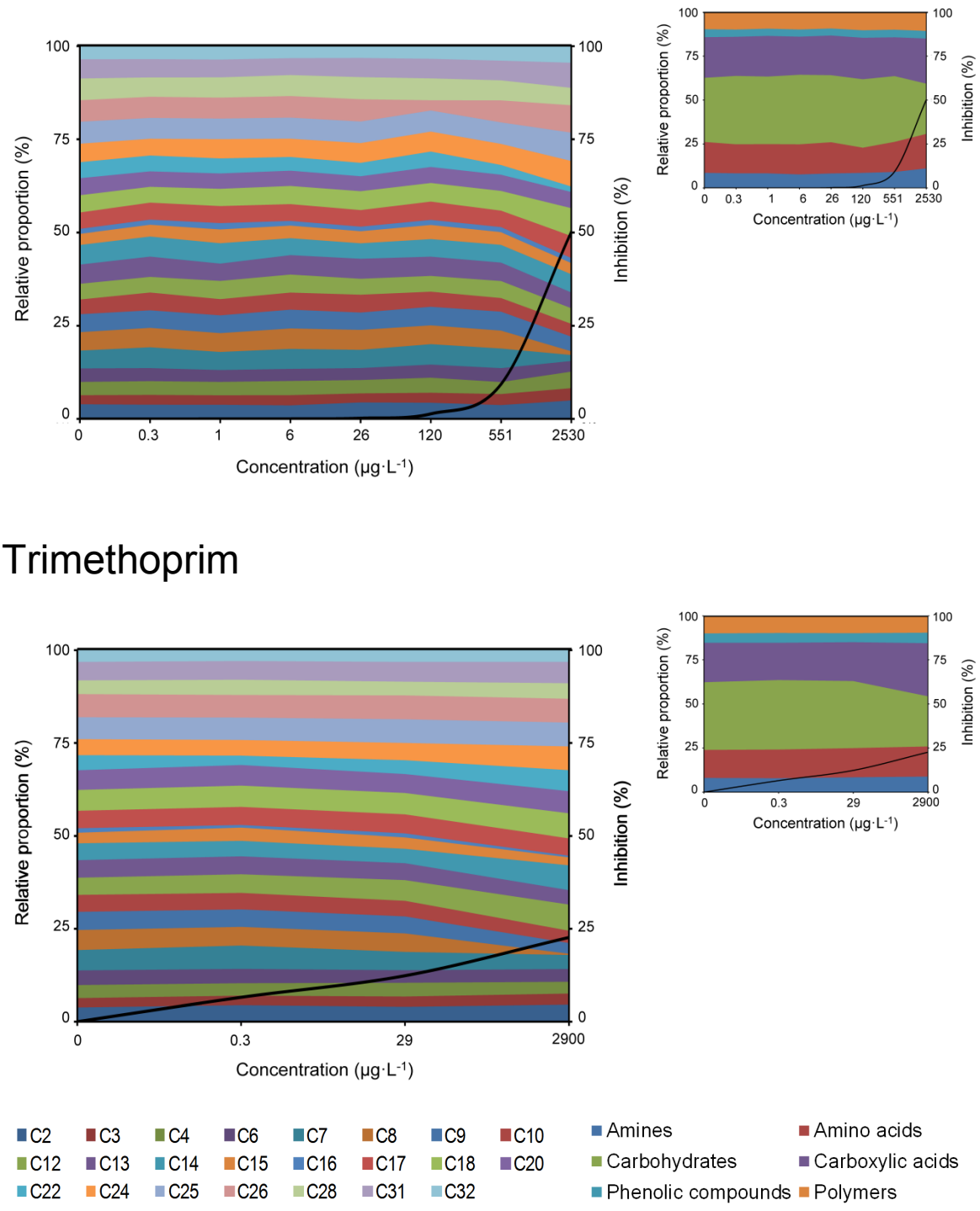
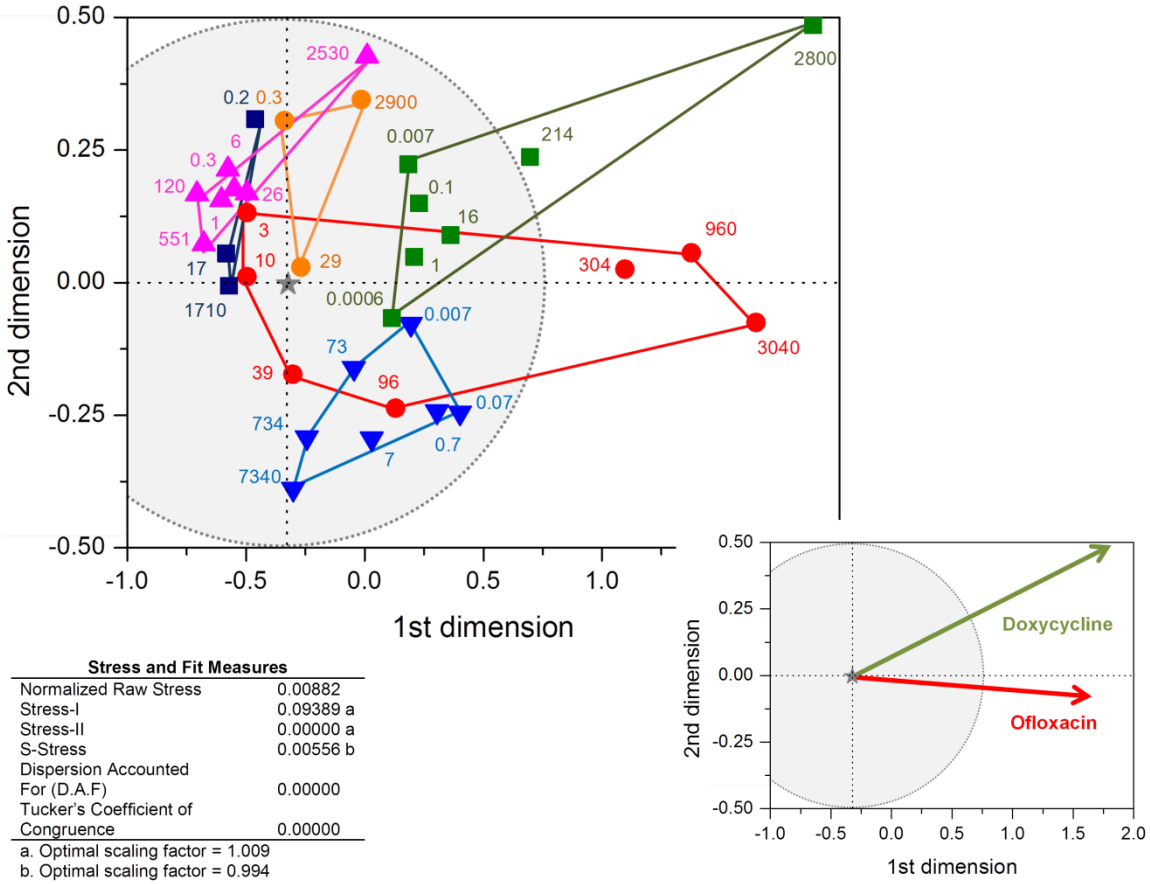


Fig. 4.2 Relative area under the curve (AUC) of the individual carbon sources and the corresponding average well colour (AWC) Weibull function (continuous line) plotted against single antibiotic exposure concentrations. Inset plots represent relative AUC of individual guild and the corresponding AWC Weibull function.

source utilization, reflects the structure of the bacterial community, this seems to indicate that DXY has a much broader spectrum of activity than OFX, as it affects carbon source utilization more evenly. That is, the sensitivities of the different bacterial species in the biofilm seem to be more equally sensitive to DXY than to OFX.

In addition, the AUC values of individual carbon sources allow for a multivariate data exploration. For this purpose, nonmetric multidimensional scaling (nMDS) was conducted, with the aim of visualizing the toxicant-induced succession (TIS) of the bacteria. The resulting graph is shown in Fig. 4.3, where the data are plotted in relation to the mean of all controls of all experiments (n = 29), in order to enable comparison across the studied antibiotics (single and mixtures, see below). The inhibitory effects of



Stress and Fit Measures	
Normalized Raw Stress	0.00882
Stress-I	0.09389 a
Stress-II	0.00000 a
S-Stress	0.00556 b
Dispersion Accounted For (D.A.F)	0.00000
Tucker's Coefficient of Congruence	0.00000

a. Optimal scaling factor = 1.009
b. Optimal scaling factor = 0.994

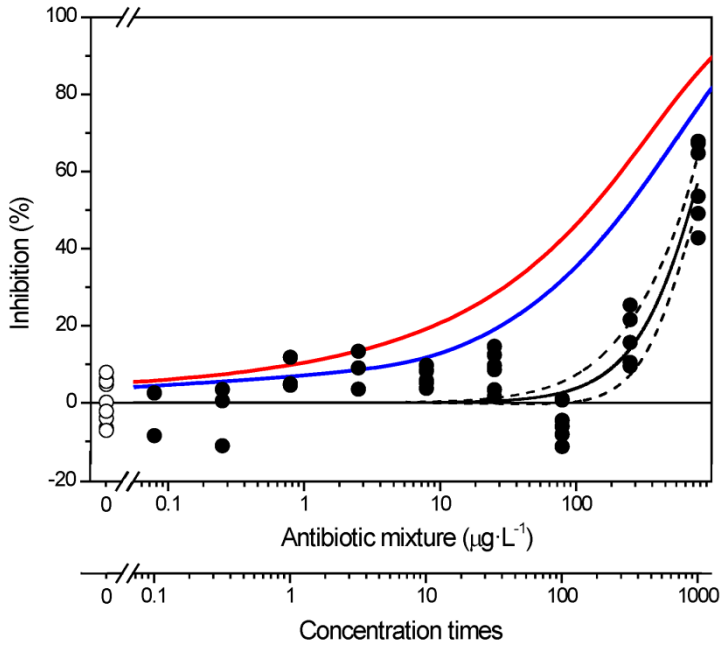
Fig. 4.3 Nonmetric multidimensional scaling (nMDS) of the time integrated carbon source utilization (AUC) using the PROXSCAL algorithm. The communities were exposed to doxycyline (■), erythromycin (▼), metronidazole (■), ofloxacin (●), sulfamethoxazole (▲) and trimethoprim (●). Numbers represent antibiotic exposure concentrations (µg·L^{-1}) and the grey circle indicates no significant differences of between exposed and non-exposed communities. The arrows indicate toxicant-induced succession (TIS) trajectories for doxycycline and ofloxacin, starting from the control (★) to the highest test concentration.

OFX and DXY to limnic bacteria led to a clear trajectory from left to right and left-down to right-up, respectively. The different TIS trajectories at the same effect range (*i.e.*, low to high influence on the community succession, inset Fig. 4.3) show that OFX and DXY do not only have different molecular modes of action (inhibition of DNA gyrase *versus* inhibition of protein synthesis), but that they also have different ecological modes of action, which leads to different species being affected and replaced by more tolerant ones. The other antibiotics that were included in the study (ERY, MNZ, SMX and TMP) did not affect the structure of the bacterial community at the tested concentrations (inside grey circle in Fig. 4.3) and consequently, their ecological mode of action cannot be assessed (Porsbring, 2009).

3.2. Toxicity of antibiotic mixture

The joint toxicity of all six antibiotics on limnic biofilm communities was studied in two different mixtures, corresponding to their occurrence in a Swedish and a Spanish STP. The outcome of the mixture experiments on the overall metabolic activity (AWC) is shown in Fig. 4.4. Details of fit parameters of Weibull model, EC_x and $NOEC$ values for the two studied mixtures are given in Table 4.7. The tested concentration range (0.1–1 000x the effluent concentration) covers the full concentration-response curve of the Spanish scenario, whereas a maximum inhibition of 68% was recorded at 1 000x the effluent concentration for the Swedish scenario. That is, the sensitivity of the bacterial communities to the mixtures was markedly different with EC_{10} and EC_{50} values of 8.87x and 62.1x the effluent concentration for the Spanish scenario and 248x and 867x the effluent concentration for the Swedish mixture. This fact is not only a consequence of the higher antibiotic concentrations detected (Table 4.2) in the Spanish wastewater (5 060 ng·L^{-1}) compared to the Swedish effluent (791 ng·L^{-1}), but is also be related to the different fraction of each compound in the mixtures. For instance, in the Spanish STP effluent, 71% of total concentration corresponds to OFX (3 594 ng·L^{-1}), a highly toxic substance towards the bacterial communities, while in Swedish scenario, the six antibiotics are more equally distributed and TMP (231 ng·L^{-1}), a far less toxic compound, is the most abundant (29%).

Swedish STP



Spanish STP

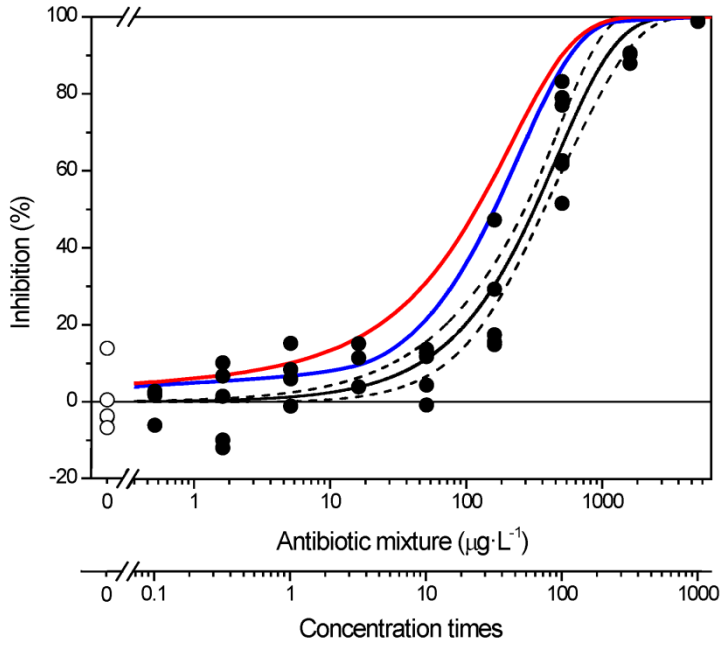


Fig. 4.4 Concentration-response curves for the Swedish and Spanish antibiotic mixtures, for the endpoint "inhibition of average well colour development" (AWC) (●) with corresponding controls (○). Black solid line give the Weibull fit, black dashed lines their 95% confidence intervals and blue and red lines prediction according to Concentration Addition and Independent Action, respectively.

Table 4.7 Effect on periphytic bacteria exposed to antibiotic mixture at maximum detected concentration in effluents from the Swedish STP (Ryaverket, Gothenburg) and Spanish STP (West-Alcalá, Madrid). The effect is described as the "inhibition of the average well color" (AWC) in EcoPlates for bacterial communities. Estimated parameters of the Weibull fits ($\hat{\theta}_1$,$\hat{\theta}_2$) that were used for estimating EC_{10}, EC_{50} and EC_{90} values are given together with approximate 95% confidence intervals and the No Observed Effect Concentrations ($NOECs$) determinate using Dunnett's test, $\alpha = 0.05$. Concentration values are expressed in concentration times of the antibiotic mixture in STP effluents.

Mixture	$\hat{\theta}_1$	$\hat{\theta}_2$	EC_{10}	EC_{50}	EC_{90}	$NOEC$
Swedish STP	-10.5358	3.46286	248 [164–364]	867 [764–980]	1 925[a]	100
Spanish STP	-4.36213	2.22791	8.87 [5.61–14.0]	62.1 [52.7–73.4]	215 [161–285]	10

[a] estimation of EC_{90} value outside the concentration range. Maximum inhibition of 68% was recorded at 1 000x.

As shown Fig. 4.4, mixture toxicity was also predicted using Concentration Addition (CA) and Independent Action (IA) (Eqs. 4.4 and 4.5, respectively). For Spanish STP mixture, full concentration-response curve could be approximated well by CA concept although, the differences between both predictive concepts are quite small: the factor of 3.7 and 1.9 between predicted EC_{10} and EC_{50} values. It is interesting to note the higher predictive power of CA compared to IA, despite the fact that the antibiotics comprising the mixture have distinctly different mechanisms of action. Differences between simple CA concept and complex biological realities can be observed (the factor of observed to CA-predicted EC_{10} and EC_{50} values 2.5 and 1.9), however its predictive power is sufficient for the mixture risk assessment purpose (Junghans *et al.*, 2006). On the other hand, significant higher deviation was observed in the Swedish STP scenario at low effects (EC_{10}), where both CA and IA clearly overestimated the observed mixture toxicity. The ratio of observed to predicted EC_{10} values is 39 for CA and 230 for IA, but differences between predictive concepts are small (the factor of 5.9 between predicted EC_{10} values). This experimental finding would be interpreted as an antagonism at low effect levels. It would also correspond to the results of Yeh and co-workers, who demonstrated Bliss-antagonistic mixture toxicities in binary combinations of chloramphenicol together with either tetracycline or streptomycin (Yeh *et al.*, 2006).

Results of the chronic effects on the periphytic bacteria of two studied mixture were also plotted as the relative proportion of single carbon source utilization (Fig. 4.5). Different responses were observed for the two mixtures. The antibiotic mixture that reflects the antibiotic content of the Spanish STP effluent changed the relative carbon source utilization of the community notably at concentration from 100x the effluent concentration and above. Especially the utilization of phenolic compounds and amines decreased markedly and came to a complete halt at a concentration equalling 316x the effluent concentration. Consumption of amino acids and polymers also decreased drastically at the highest tested concentration, and carbohydrates were the guild dominating the metabolic pattern at 1 000 times the effluent concentrations. No major re-arrangement of carbon source utilization was observed in the antibiotic mixture reflecting the Swedish STP effluent up to a total concentration equal to 1 000x the effluent concentration.

The AUC data of the individual carbon sources was further analyzed using nMDS (Fig. 4.6) as for single antibiotics. The bacterial communities exposed to individual antibiotics (Fig. 4.3) were removed in order to improve the clarity of the plot. For the studied mixtures, there are two clusters of exposures close to the control for concentrations up to 10 times for Spanish STP effluent and 316 times for Swedish STP effluent. From those points, the main trend in the data is once again a movement from the left to the right side of the graph when antibiotic mixture concentration is increased. It is important to stress that the multivariate Anosim-based $NOECs$ for the AUC of the different carbon sources were determined at higher concentration than the AWC-based $NOEC$: 32 and 1 000 times for Spanish and Swedish scenarios, respectively. Under the assumption that differences in relative carbon source utilization are indicative of changes in community biodiversity (species composition, physiological activity of each species), the Swedish mixture does not seems to significantly affect the bacterial biodiversity in the tested concentration range, while Spanish effluent causes a clear changes at concentrations 32 times higher than the maximum detected concentrations and above. In fact, heterotrophic communities' exposure at concentration between 100 and 316 times in Spanish STP effluent, resulting in communities similar to those

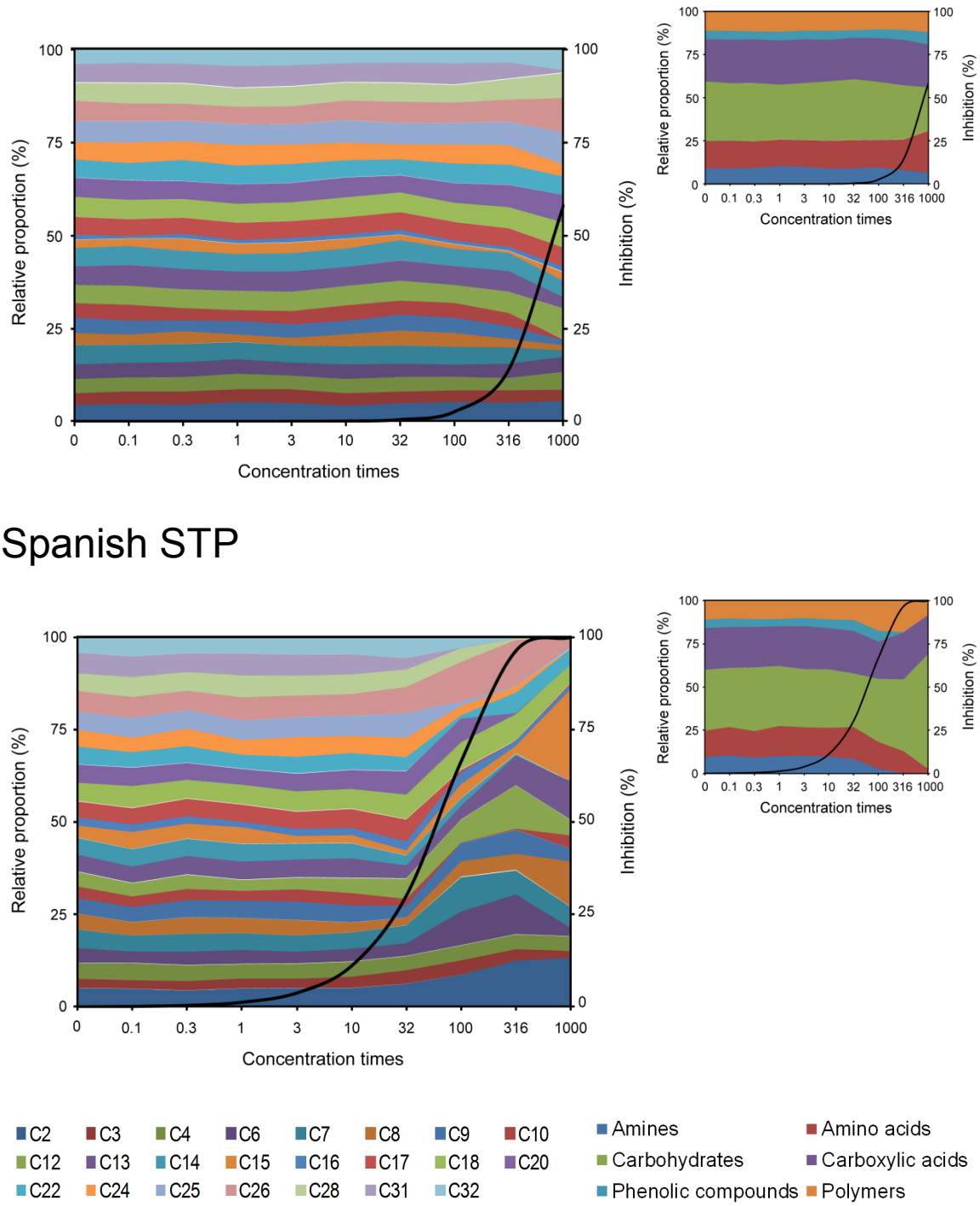
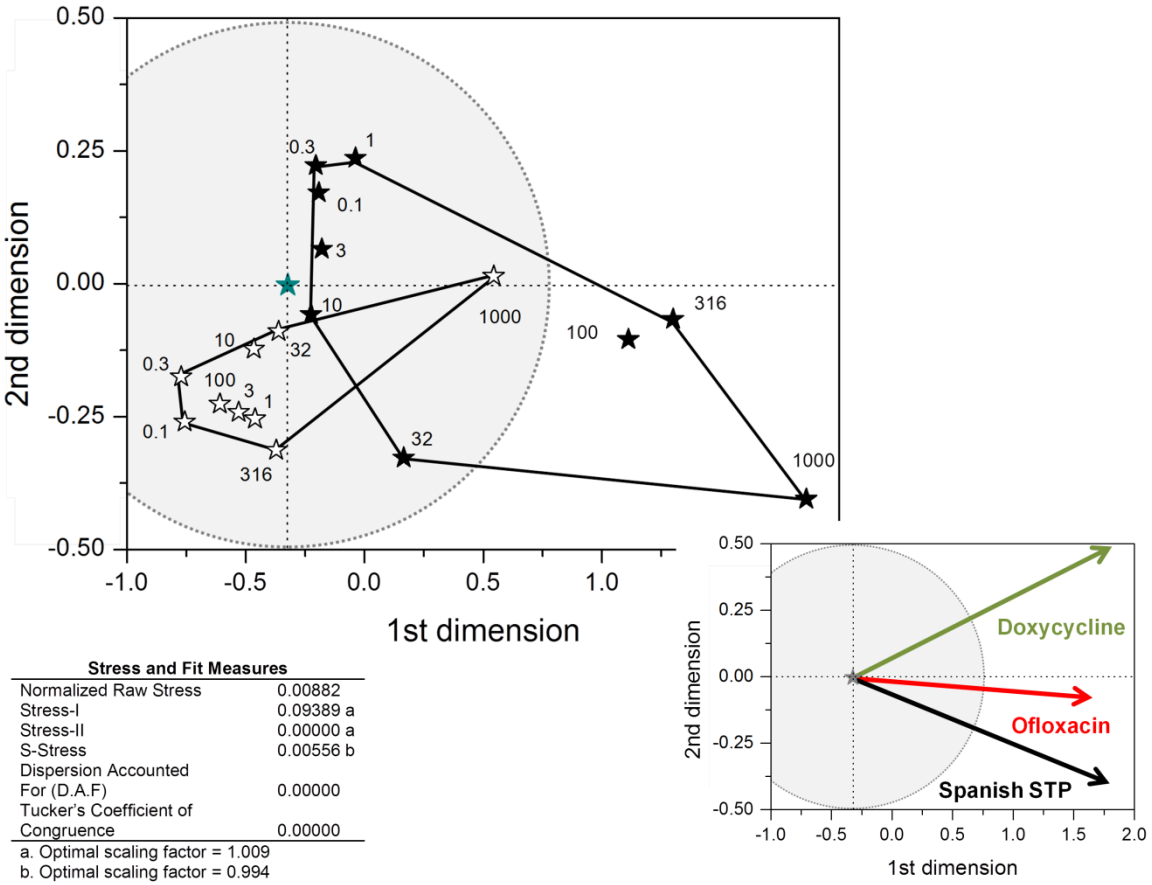


Fig. 4.5 Relative average under the curve (AUC) of individual carbon sources and the corresponding average well colour (AWC) Weibull function (continuous line) plotted against Swedish and Spanish STP mixtures exposure concentration times. Inset plots represent relative AUC of individual guild and the corresponding AWC Weibull function.



Stress and Fit Measures	
Normalized Raw Stress	0.00882
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S-Stress	0.00556 b
Dispersion Accounted For (D.A.F)	0.00000
Tucker's Coefficient of Congruence	0.00000

a. Optimal scaling factor = 1.009
b. Optimal scaling factor = 0.994

Fig. 4.6 Nonmetric multidimensional scaling (nMDS) of the time integrated carbon source utilization (AUC) using the PROXSCAL algorithm. The communities were exposed to antibiotic mixtures reflecting the mixtures in Swedish STP (☆) and Spanish STP (★) effluents. Numbers represent antibiotic mixture exposure concentrations (concentration times) and the grey circle indicates no significant differences of between exposed and non-exposed communities. The arrows indicate toxicant-induced succession (TIS) trajectories for doxycycline, ofloxacin and Spanish STP, starting from the control (★) to the highest test concentration.

OFX induced succession (inset Fig. 4.6). The nMDS plot indicates OFX as the compound largely determining the structure of the limnic bacterial communities exposed to the Spanish scenario, that is, the driver of the mixture toxicity with respect to the structural endpoint. Interestingly, the trajectory of the bacteria exposed to the Spanish antibiotic mixture does not fall between the trajectories of the two ecotoxicologically dominant mixture components, OFX and DXY. This might be considered a reflection of the input from the four less toxic mixture components, whose presence seems to drive the trajectory of the mixture downwards.

3.3. Hazard and risk of single and mixed antibiotic to limnic bacterial communities

First, the environmental risk of the individual antibiotics assuming the worst case scenario for Swedish and Spanish STP effluents (Table 4.2) is briefly assessed. Table 4.8 shows TUs calculated from EC_{10} values for the inhibition of AWC (as the more sensitive and better quantifiable endpoint) for the natural bacterial communities and the corresponding RQs. The results show that OFX and DXY are clearly hazardous for bacterial communities in the freshwater environment. In particular, OFX concentrations exceed the corresponding $PNEC$ in the Spanish scenario, if based on EC_{10} values for AWC and an assessment factor of 10 (EMA, 2006). Similarly, DXY concentrations in the Swedish scenario exceed their corresponding $PNEC$.

Table 4.8 Risk quotients (RQs) of the tested antibiotics to bacterial communities for effluents from the Swedish STP (Ryaverket, Gothenburg) and Spanish STP (West-Alcalá, Madrid). RQ higher than one are emphasized in bold.

Antibiotic	Swedish STP		Spanish STP	
	TUs	RQ	TUs	RQ
Doxycycline	0.1135	**1.1**	0.0305	0.31
Erythromycin	0.0000	0.00	0.0000	0.00
Metronidazole	0.0000	0.00	0.0000	0.00
Ofloxacin	0.0076	0.08	0.2260	**2.3**
Sulfamethoxazole	0.0000	0.00	0.0006	0.01
Trimethoprim	0.0377	0.38	0.0241	0.24
Mixture (component-based)	0.1588	**1.6**	0.2815	**2.8**
Mixture (whole-mixture)	–	0.04	–	**1.1**

The published literature on occurrence in STP effluents and freshwaters is fairly extensive for most of the studied antibiotics in peer-reviewed literature (Supplementary data). These data allow us performing a graphical comparison between toxic effects on the studied bacterial communities with the occurrence of the investigated antibiotics in different scenarios (Fig. 4.1). The $PNECs$ for OFX and DXY concentrations shows a clear overlap with the concentrations measured in wastewaters and freshwaters, *i.e.* the two antibiotics seem to be problematic not only for the two scenarios analysed in the present study. However, the large local and regional differences in exposure

concentrations do not allow a general conclusion on the environmental risk of OFX and DXY, but call for site-specific assessments. In fact, it is noteworthy that concentration detected of OFX in wastewater effluents located in India (160 µg·L^{-1}, Larsson *et al.*, 2007) would provoke a strong inhibition of 61% whereas, its median concentration in STP effluents (219 ng·L^{-1}) will not cause effects on the overall response of the periphytic bacterial communities. The high variability of the concentrations found in surface waters might also be a result of different factors like sampling location (*e.g.*, close to effluent discharge or upstream sampling), different river flows (*e.g.* dilution factors), and the time of the sampling (season); facts that make it difficult to reach a general conclusion on the presence or absence of risk.

Also TMP might exceed its $PNEC$ in several exposure scenarios (Fig. 4.1). These data are not in line with Straub (2013), who concluded that TMP does not pose a significant risk to freshwater systems. This is because the $PNEC$ estimated by Straub (2013) is markedly higher (240 µg·L^{-1}) than the value estimated in the present work (0.61 µg·L^{-1}). This indicates the need for further studies with natural microbial communities, especially as the available data from the present study only roughly describe the concentration-response pattern of TMP. For SMX, there is a low likelihood that exposure concentrations and effect concentration overlap. These data are consistent with recently reported studies about aquatic environmental risk assessment of SMX (Kosma *et al.*, 2014 and Straub, 2015), in which they were concluded that there is no significant risk in the majority of cases. No $PNECs$ were calculated for ERY and MNZ.

A comparison of the observed toxicity of both mixtures with environmental concentrations yields a ratio between the EC_{10} and the measured environmental concentrations of 0.004 (Swedish mixture) and 0.11 (Spanish mixture). This indicates a low direct environmental risk from the Swedish scenario. For the Spanish scenario, however, conclusions on the environmental risk depend on the applied assessment factor. An assessment factor of 10 is suggested by EMA (2006) for data from “the antimicrobial effect study”, which relates to the activated sludge respiration assay. The EMA document does not provide suggestions for assessment factors for studies with

natural microbial communities. As the application of an assessment factor of 10 would result in a risk quotient exceeding 1, an improvement of the regulatory guidance is warranted. From an ecological perspective, in view of the seasonality of microbial activities and biodiversity in natural environment, an assessment factor of 10 certainly does not seem excessively high. On the basis of TUs of the individual antibiotics, the CA-expected joint risk can be estimated for both mixtures, by summing up the toxic units (TUs) for each scenario, based on EC_{10} values for the inhibition of AWC. Using an assessment factor of 10 (EMA, 2006), the results yield a final risk quotient of 1.6 for the Swedish STP effluent and 2.8 for the Spanish STP scenario. The comparison between CA-based and empirical risk quotients, again, shows a very good prediction of the toxicity of the Spanish mixture (RQ_{STU} = 2.8 *vs.* $RQ_{empirical}$ = 1.1), while the toxicity of the Swedish mixture is overestimated (RQ_{STU} = 1.6 *vs.* $RQ_{empirical}$ = 0.04).

A component-based strategy using CA allows the ranking of the mixture components according to their TUs (Backhaus and Karlsson, 2014). Indeed, it can be clearly observed that ofloxacin (80%) contributes most to the overall STUs in the Spanish scenario, whereas the rest of the studied compounds has only a negligible contribution. This fact is illustrated in Fig. 4.7, in which the bacterial communities shows similar sensitivities when the concentration of OFX is scaled to its corresponding occurrence in the Spanish STP effluent. The differences between both fits to the data and CA-prediction become indiscriminate in lower effect levels (overlapping of the confidence belts). The figure confirms OFX as the compound largely determining the bacterial toxicity of the whole Spanish mixture.

Finally, it is worth pointing out that final assessment of the environmental risk due to the total antibiotic load in the studied STP scenarios depends on the dilution of the effluent in the recipient river. Between the two studied countries, the differences in national annual dilution factors (and hence chemical concentrations) are significant; there are nearly 2 orders of magnitude between the annual median dilution factors in Sweden (1 825) and Spain (26) (Keller *et al.*, 2014). However, the spatial variability of dilution factors within a country warrants consideration. The effluent of Ryaverket STP (the studied Swedish STP) is diluted by a factor close to 150 in the catchment of the Göta

river (mean annual flow, 550 $m^3 \cdot s^{-1}$, Göta Älvs Vattenvårdsförbund, 2013). That is, the Swedish STP effluent is diluted to concentrations significantly below the mixture $PNEC$ determined based on the data presented in this study. The Spanish STP (West-Alcalá STP) discharges its effluents into the Henares river (mean annual flow, 10.7 $m^3 \cdot s^{-1}$, CEDEX, 2015), which dilutes the total wastewater by an average factor of 13 (the monthly dilution factor generally varies between 3.4 and 25). In dry weather conditions, the Henares river can reach a wastewater content in the creek downstream from West-Alcalá STP close to 30% (minimum mean monthly flow, 2.86 $m^3 \cdot s^{-1}$, CEDEX, 2015). This would result in a total concentration of the antibiotic mixture of 1 518 $\mu g \cdot L^{-1}$, which is only a factor of 30 lower than the EC_{10}, *i.e.* the concentration at which effects on the carbon utilization became directly visible. Applying any assessment factor, in order to account for spatial and temporal changes in the sensitivity of the exposed bacterial community would then indicate a potential environmental risk, at least during the dry months of the year.

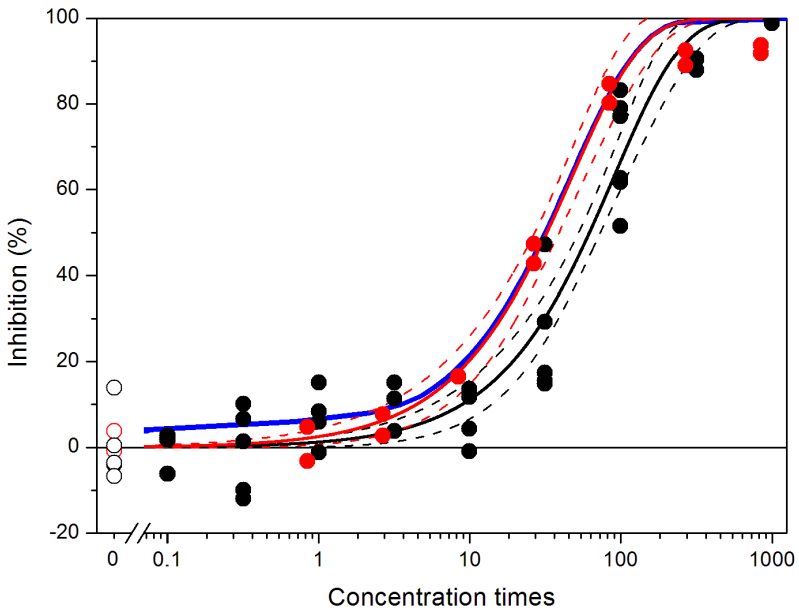

Fig. 4.7. Concentration-response curves for the Spanish STP mixture (●) compared to ofloxacin (⬬) exposure for the endpoint "inhibition of average well colour development" (AWC) with corresponding controls (○, ○). Solid black and red lines gives the Weibull fit, dashed lines their 95% confidence intervals and blue line prediction according to Concentration Addition.

4. Conclusions

The results clearly demonstrate that among the studied antibiotics (doxycycline, erythromycin, metronidazole, ofloxacin, sulfamethoxazole and trimethoprim), doxycycline and ofloxacin affect the carbon source metabolization of limnic periphytic bacteria in a concentration-dependent fashion after chronic exposure to concentrations above 2.0 and 16 µg·L^{-1} (EC_{10} values), respectively. Ofloxacin exposure has a more selective effect, resulting in clear changes in the relative bacterial carbon source utilization pattern, while doxycycline affects the bacterial utilization of a broader range of carbon sources with a similar concentration-response pattern. Indeed, both antibiotics cause different TIS trajectories on the bacterial communities, indicating that they also have dissimilar mode of action on ecological level.

The joint toxicity of the six studied antibiotics for the Swedish and Spanish STP scenarios shows that their chronic exposure affects the bacterial carbon source utilization at concentration above 8.9 and 250 times the maximum detected effluent concentrations. However, only the Spanish mixture exposure led to a re-arrangement of the carbon source utilization, indicating a change in community biodiversity and/or function with a pattern mainly influenced by ofloxacin.

Results from screening level risk assessment show potential risk for ofloxacin and the antibiotic mixture under the Spanish STP scenario. Despite final risk to freshwater organisms depends on the dilution, the removal and the degradation rates in surface waters, the results highlight that the toxic effects of antibiotic mixtures, and especially ofloxacin (*i.e.*, the risk driver of the mixture), should be assessed, in order to decide whether mitigation measures such as source control by target restrictions or STP upgrading for improving removal efficiencies are need.

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SUPPLEMENTARY DATA

Table S1 Occurrence of studied antibiotic in STP effluents (continued on next page).

Antibiotic	Country	Location	Concentration ($ng \cdot L^{-1}$)			Reference
			Range	Median	Mean	
DXY	Australia		ND-40			Watkinson *et al.*, 2007
	Australia	5 STPs	ND-15	10		Watkinson *et al.*, 2009
	Canada	8 STPs	ND-46	38		Miao *et al.*, 2004
	Spain	León STP			61	Hijosa-Valsero *et al.*, 2011
	Sweden		ND-220			Andersson *et al.*, 2005
	Sweden	Henriksdal STP	ND-915			Lindberg *et al.*, 2005
	Sweden	Ryaverket STP	ND-227			Lindberg *et al.*, 2005
	Sweden	Umea STP	ND-78			Lindberg *et al.*, 2005
	Sweden	Kalmar STP	ND-424			Lindberg *et al.*, 2005
	Sweden	Floda STP	72-880			Lindberg *et al.*, 2005
	USA	Northern Colorado STP			90	Yang *et al.*, 2003
ERY [a]	Canada	8 STPs	ND-838	80		Miao *et al.*, 2004
	China	Kaifuqu STP			430	Xu *et al.*, 2007a
	China	Guangzhou STP			2054	Xu *et al.*, 2007a
	China	New Territory STP			216	Xu *et al.*, 2007a
	China	Kowloon STP			259	Xu *et al.*, 2007a
	China	Wan Chai STP			850	Gulkowska *et al.*, 2008
	China	Tai Po STP			520	Gulkowska *et al.*, 2008
	China	Shatin STP			600	Gulkowska *et al.*, 2008
	China	Stonecutters Island STP			510	Gulkowska *et al.*, 2008
	China	Shatin STP			96.3	Li *et al.*, 2009
	China	Stanley STP			37.9	Li *et al.*, 2009
	Germany		ND-6000	2500		Hriscch *et al.*, 1999
	Germany				620	Ternes *et al.*, 2003
	Italy	8 STPs		47.4		Zuccato *et al.*, 2005
	Italy	Milan STP			34	Zuccato *et al.*, 2010
	Italy	Varese STP			27	Zuccato *et al.*, 2010
	Italy	Lugano STP			59	Zuccato *et al.*, 2010
	Italy	Como STP			6.5	Zuccato *et al.*, 2010
	Spain	Alcalá STP	<LOD-760		331	Rosal *et al.*, 2010
	Spain	León STP			61	Hijosa-Valsero *et al.*, 2011
	Spain	Girona STP1			17	Gros *et al.*, 2012
	Spain	Girona STP2			14	Gros *et al.*, 2012
	Spain	Almeria STP	236-1250		613	Martínez-Bueno *et al.*, 2012
	Spain	Cantabria STP	99-1112		371	Martínez-Bueno *et al.*, 2012
	Spain	Madrid STP 1	100-6316		997	Martínez-Bueno *et al.*, 2012
	Spain	Madrid STP 2	260-2695		694	Martínez-Bueno *et al.*, 2012
	Spain	Barcelona STP	99-3934		720	Martínez-Bueno *et al.*, 2012
	Spain	Alcalá STP			330	Rodríguez *et al.*, 2012
	Spain	Alcazar de San Juan STP			110	Rodríguez *et al.*, 2012
	Spain	Alicante STP	10-10		10	Ibañez *et al.*, 2013
	Spain	Murcia STP	10-200		20	Ibañez *et al.*, 2013
	Spain	Alcalá STP			670	Carbajo *et al.*, 2015
	Sweden	3 STPs	53-530			Fick *et al.*, 2011
	Switzcherland	Kloten-Opfikon STP	110-199			McArdell *et al.*, 2003
	Switzcherland	Kloten-Opfikon STP	3.4-9.5		6	Göbel *et al.*, 2004
	Switzcherland	2 STPs	60-110	70		Göbel *et al.*, 2005
	Taiwan	5 STPs			695	Lin *et al.*, 2008
	Taiwan	4 STPs	226-811			Lin *et al.*, 2009

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Table S1 Occurrence of studied antibiotic in STP effluents (continued on next page).

Antibiotic	Country	Location	Concentration ($ng \cdot L^{-1}$)			Reference
			Range	Median	Mean	
	UK	5 STPs	<LOQ-1842			Ashton *et al.*, 2004
	UK	Howdon STP		202		Roberts and Thomas, 2006
	UK	Howdon STP		202		Roberts and Thomas, 2006
	UK	Cilfynydd STP	292-2841		1385	Kasprzyk-Hordern *et al.*, 2009
	UK	Coslech STP	23-2772		696	Kasprzyk-Hordern *et al.*, 2009
	USA	Northern Colorado STP			80	Yang and Carlson., 2004
	USA	10 STPs	ND-610	35		Glassmeyer *et al.*, 2005
	USA	7 STPs		270		Karthikeyan and Meyer, 2006
MNZ	Spain	Alcalá STP			212	Rosal *et al.*, 2008
	Spain	3 STPs	<LOQ-295		164	Gros *et al.*, 2009
	Spain	Alcalá STP	<LOQ-127		55	Rosal *et al.*, 2010
	Spain	Barcelona STP	36-1801		327	Martínez-Bueno *et al.*, 2012
	Spain	Almería STP	25-337		81	Martínez-Bueno *et al.*, 2012
	Spain	Cantabria STP	17-1081		200	Martínez-Bueno *et al.*, 2012
	Spain	Madrid STP 1	27-2163		225	Martínez-Bueno *et al.*, 2012
	Spain	Madrid STP 2	21-331		199	Martínez-Bueno *et al.*, 2012
	Spain	Girona STP1			121	Gros *et al.*, 2012
	Spain	Girona STP 1			58	Gros *et al.*, 2013
	Spain	Girona STP 2			17	Gros *et al.*, 2013
	Spain	Girona STP 3			83	Gros *et al.*, 2013
	Spain	Alcalá STP			118	Herrera *et al.*, 2014
	Spain	Alcalá STP			330	Carbajo *et al.*, 2015
	Spain	Girona STP	ND-144			Rodríguez-Mozaz *et al.*, 2015
	Switzerland	Lausanne STP			567	Margot *et al.*, 2013
	Taiwan	5 STPs			100	Lin *et al.*, 2008
	Taiwan	4 STPs	10-126			Lin *et al.*, 2009
	UK	Cilfynydd STP	60-421		265	Kasprzyk-Hordern *et al.*, 2009
	UK	Coslech STP	129-561		353	Kasprzyk-Hordern *et al.*, 2009
OFX	Canada	8 STPs	ND-506	94		Miao *et al.*, 2004
	Canada	8 STPs	32-548	179		Lee *et al.*, 2007
	China	Kaifuqu STP			41	Xu *et al.*, 2007a
	China	Guangzhou STP			137	Xu *et al.*, 2007a
	China	New Territory STP			48	Xu *et al.*, 2007a
	China	Kowloon STP			165	Xu *et al.*, 2007a
	China	Gao Beidian STP			503	Xiao *et al.*, 2008
	China	Shatin STP			556.4	Li *et al.*, 2009
	China	Stanley STP			2.1	Li *et al.*, 2009
	France	Pierre Bénite STP			330	Andreozzi *et al.*, 2003
	France	Chatillon-sur-Chalaronne STP			510	Andreozzi *et al.*, 2003
	Greece	Iraklio STP			460	Andreozzi *et al.*, 2003
	India	Patancheru STP			55000	Fick *et al.*, 2009
	India	Patancheru STP			160000	Fick *et al.*, 2009
	Italy	Latina STP			580	Andreozzi *et al.*, 2003
	Italy	Roma STP			290	Andreozzi *et al.*, 2003
	Italy	Naples STP			310	Andreozzi *et al.*, 2003
	Italy	8 STPs		600		Zuccato *et al.*, 2005
	Italy	Varese Olona STP			183	Castiglioni *et al.*, 2008
	Italy	Milan STP			5.3	Zuccato *et al.*, 2010
	Italy	Varese STP			77	Zuccato *et al.*, 2010

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Table S1 Occurrence of studied antibiotic in STP effluents (continued on next page).

Antibiotic	Country	Location	Concentration ($ng \cdot L^{-1}$)			Reference
			Range	Median	Mean	
	Italy	Como STP			4.9	Zuccato *et al.*, 2010
	Spain	Alcalá STP			565	Rosal *et al.*, 2008
	Spain	3 STPs	13-367		95	Gros *et al.*, 2009
	Spain	Alcalá STP	<LOD-3594		816	Rosal *et al.*, 2010
	Spain	Girona STP1			191	Gros *et al.*, 2012
	Spain	Girona STP2			157	Gros *et al.*, 2012
	Spain	Almeria STP	321-13426		1569	Martínez-Bueno *et al.*, 2012
	Spain	Cantabria STP	43-81		55	Martínez-Bueno *et al.*, 2012
	Spain	Madrid STP 1	181-16574		2837	Martínez-Bueno *et al.*, 2012
	Spain	Madrid STP 2	217-10019		2572	Martínez-Bueno *et al.*, 2012
	Spain	Barcelona STP	228-1024		499	Martínez-Bueno *et al.*, 2012
	Spain	Girona STP 1			67	Gros *et al.*, 2013
	Spain	Girona STP 2			101	Gros *et al.*, 2013
	Spain	Girona STP 3			63	Gros *et al.*, 2013
	Spain	Alicante STP	100-490		220	Ibañez *et al.*, 2013
	Spain	Murcia STP	10-430		210	Ibañez *et al.*, 2013
	Spain	Alcalá STP			4700	Carbajo *et al.*, 2015
	Spain	Girona STP	ND-172			Rodríguez-Mozaz *et al.*, 2015
	Sweden	Ryaverket STP			120	Andreozzi *et al.*, 2003
	Sweden	Henriksdal STP	ND-7			Lindberg *et al.*, 2005
	Sweden	Kalmar STP	ND-52			Lindberg *et al.*, 2005
	Sweden	Floda STP	ND-45			Lindberg *et al.*, 2005
	Sweden	Kristianstad STP			10	Zorita *et al.*, 2009
	Switzerland	Lausanne STP			84	Margot *et al.*, 2013
	Taiwan	5 STPs			123	Lin *et al.*, 2008
	Taiwan	4 STPs	53-991			Lin *et al.*, 2009
	USA	STP 1	<LOQ-50	45		Renew and Huang, 2004
	USA	STP 2	100-210	180		Renew and Huang, 2004
	USA	East Lansing STP			100	Nakata *et al.*, 2005
	USA	Albulquerque STP			110	Brown *et al.*, 2006
	USA	50 STPs	ND-660		160	Kostich *et al.*, 2014
SMX	18 European countries	90 STP	ND-1691	164	280	Loss *et al.*, 2013
	18 European countries	90 STP	ND-1147	67.5	142	Loss *et al.*, 2013
	Australia	2 STPs	ND-270	320		Watkinson *et al.*, 2007
	Australia	5 STPs	ND-200	50		Watkinson *et al.*, 2009
	Canada	Eight STPs	ND-871	243		Miao *et al.*, 2004
	China	Kaifuqu STP			16	Xu *et al.*, 2007a
	China	Guangzhou STP			78	Xu *et al.*, 2007a
	China	New Territory STP			12	Xu *et al.*, 2007a
	China	Kowloon STP			9	Xu *et al.*, 2007a
	China	Shatin STP			46.6	Li *et al.*, 2009
	China	Stanley STP			15.3	Li *et al.*, 2009
	France	Pierre Bénite STP			90	Andreozzi *et al.*, 2003
	France	Chatillon-sur-Chalaronne STP			70	Andreozzi *et al.*, 2003
	Germany		ND-2000	400		Hirsch *et al.*, 1999
	Germany	Berlin STP 1			300	Hartig *et al.*, 1999
	Germany	Berlin STP 2			1500	Harig *et al.*, 1999
	Germany				620	Ternes *et al.*, 2003
	Greece	Iraklio STP			90	Andreozzi *et al.*, 2003
	Greece	8 STPs	<LOQ-481		47.2	Kosma *et al.*, 2014
	Italy	Latina STP			10	Andreozzi *et al.*, 2003
	Italy	Naples STP			30	Andreozzi *et al.*, 2003
	Italy	8 STPs		127		Zuccato *et al.*, 2005

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Table S1 Occurrence of studied antibiotic in STP effluents (continued on next page).

Antibiotic	Country	Location	Concentration ($ng \cdot L^{-1}$)			Reference
			Range	Median	Mean	
	Italy	Milan STP			16	Zuccato *et al.*, 2010
	Italy	Varese STP			11	Zuccato *et al.*, 2010
	Italy	Lugano STP			15	Zuccato *et al.*, 2010
	Italy	Como STP			30	Zuccato *et al.*, 2010
	Rep. of Korea	TanCheon STP	63-193	180		Choi *et al.*, 2008a
	Rep. of Korea	JungRang STP	25-275	185		Choi *et al.*, 2008a
	Rep. of Korea	NanJi STP	31-316	148		Choi *et al.*, 2008a
	Rep. of Korea	SeoNam STP	185-492	219		Choi *et al.*, 2008a
	Spain		0-580		250	Carballa *et al.*, 2004
	Spain	Alcalá STP			150	Rosal *et al.*, 2008
	Spain	3 STPs	13-448		208	Gros et al., 2009
	Spain	Alcalá STP	104-370		231	Rosal *et al.*, 2010
	Spain	León STP			60	Hijosa-Valsero *et al.* , 2011
	Spain	Alcalá STP			230	Rodríguez *et al.*, 2012
	Spain	Alcazar de San Juan STP			90	Rodríguez *et al.*, 2012
	Spain	Girona STP1			222	Gros *et al.*, 2012
	Spain	Almeria STP	191-1142		548	Martínez-Bueno *et al.*, 2012
	Spain	Cantabria STP	39-543		246	Martínez-Bueno *et al.*, 2012
	Spain	Madrid STP 1	56-498		208	Martínez-Bueno *et al.*, 2012
	Spain	Madrid STP 2	103-390		257	Martínez-Bueno *et al.*, 2012
	Spain	Barcelona	227-486		328	Martínez-Bueno *et al.*, 2012
	Spain	Girona STP 1			198	Gros *et al.*, 2013
	Spain	Girona STP 2			27	Gros *et al.*, 2013
	Spain	Girona STP 3			19	Gros *et al.*, 2013
	Spain	Alcalá STP			552	Herrera *et al.*, 2014
	Spain	Alcalá STP			670	Carbajo *et al.*, 2015
	Spain	Girona STP	ND-73			Rodríguez-Mozaz *et al.*, 2015
	Spain	Alicante STP	30-80		60	Ibañez *et al.*, 2013
	Spain	Murcia STP	60-120		80	Ibañez *et al.*, 2013
	Sweden	Ryaverket STP			20	Andreozzi *et al.*, 2003
	Sweden	Henriksdal STP	ND-193			Lindberg *et al.*, 2005
	Sweden	Umea STP	ND-135			Lindberg *et al.*, 2005
	Sweden	Kalmar STP	ND-304			Lindberg *et al.*, 2005
	Sweden	Floda STP	ND-302			Lindberg *et al.*, 2005
	Sweden	3 STPs	30-290			Fick *et al.*, 2011
	Switzcherland	Kloten-Opfikon STP	6-15		11	Göibel *et al.*, 2004
	Switzcherland	2 STPs	211-860	290		Göbel *et al.*, 2005
	Switzerland	Lausanne STP			171	Margot *et al.*, 2013
	Taiwan	5 STPs			226	Lin *et al.*, 2008
	Taiwan	4 STPs	47-964			Lin *et al.*, 2009
	UK	5 STPs	<LOQ-132			Ashton *et al.*, 2004
	UK	Cilfynydd	<LOQ-23		10	Kasprzyk-Hordern *et al.*, 2009
	UK	Coslech	4-44		19	Kasprzyk-Hordern *et al.*, 2009
	USA	Northern Colorado STP			320	Yang *et al.*, 2003
	USA	STP 1	<LOQ-70	60		Renew and Huang, 2004
	USA	STP 2	330-2140	660		Renew and Huang, 2004
	USA	10 STPs	ND-763	68		Glassmeyer *et al.*, 2005
	USA	7 STPs		195		Karthikeyan and Meyer, 2006
	USA	Lackawana STP		900		Batt *et al.*, 2006
	USA	East Aurora STP		410		Batt *et al.*, 2006

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Table S1 Occurrence of studied antibiotic in STP effluents (continued on next page).

Antibiotic	Country	Location	Concentration ($ng \cdot L^{-1}$)			Reference
			Range	Median	Mean	
	USA	Holland STP		1300		Batt *et al.*, 2006
	USA	Albulquerque STP			310	Brown *et al.*, 2006
	USA	Amherst STP			680	Batt *et al.*, 2007
	USA	East Aurora STP			220	Batt *et al.*, 2007
	USA	Holland STP			500	Batt *et al.*, 2007
	USA	Lackawana STP			380	Batt *et al.*, 2007
	USA	Northwest Ohio STP		274		Spongberg and Witter, 2008
	USA	Omaha STP			141	Bartelt-Hunt *et al.*, 2009
	USA	Nortern Colorado STP 1			1261	Ferrer and Thurman, 2013
	USA	Nortern Colorado STP 2			133	Ferrer and Thurman, 2013
	USA	50 STPs	ND-910		2900	Kostich *et al.*, 2014
TMP	18 European countries	90 STPs	ND-800	178	229	Loss *et al.*, 2013
	Australia		ND-70		50	Watkinson *et al.*, 2007
	Australia	5 STPs	ND-250	10		Watkinson *et al.*, 2009
	China	Wan Chai STP			170	Gulkowska *et al.*, 2008
	China	Tai Po STP			140	Gulkowska *et al.*, 2008
	China	Shatin STP			120	Gulkowska *et al.*, 2008
	China	Stonecutters Island STP			230	Gulkowska *et al.*, 2008
	China	Shatin STP			66.2	Li *et al.*, 2009
	China	Stanley STP			10.8	Li *et al.*, 2009
	France	Pierre Bénite STP			40	Andreozzi *et al.*, 2003
	France	Chatillon-sur-Chalaronne STP			20	Andreozzi *et al.*, 2003
	Germany		ND-660	320		Hrisch *et al.*, 1999
	Germany				340	Ternes *et al.*, 2003
	Greece	Iraklio STP			80	Andreozzi *et al.*, 2003
	Greece	8 STPs	<LOQ-533		47.4	Kosma *et al.*, 2014
	India	Patancheru STP			4400	Fick *et al.*, 2009
	Italy	Latina STP			40	Andreozzi *et al.*, 2003
	Italy	Roma STP			30	Andreozzi *et al.*, 2003
	Italy	Naples STP			130	Andreozzi *et al.*, 2003
	Rep. of Korea	TanCheon STP	<LOQ-87			Choi *et al.*, 2008a
	Rep. of Korea	JungRang STP	<LOQ-119	13		Choi *et al.*, 2008a
	Rep. of Korea	NanJi STP	<LOQ-108			Choi *et al.*, 2008a
	Rep. of Korea	SeoNam STP	31-174	110		Choi *et al.*, 2008a
	Rep. of Korea	Han River STP	ND-79.9		153	Choi *et al.*, 2008b
	Rep. of Korea	Kyung-Ahn Stream STP	ND-96.3		89.3	Choi *et al.*, 2008b
	Spain	Alcalá STP			69	Rosal *et al.*, 2008
	Spain	3 STPs	<LOQ-116		37	Gros *et al.*, 2008
	Spain	Alcalá STP	<LOD-148		99	Rosal *et al.*, 2010
	Spain	Girona STP1			100	Gros *et al.*, 2012
	Spain	Girona STP2			10	Gros *et al.*, 2012
	Spain	Almeria STP	29-1416		371	Martínez-Bueno *et al.*, 2012
	Spain	Cantabria STP	56-257		129	Martínez-Bueno *et al.*, 2012
	Spain	Madrid STP 1	29-403		118	Martínez-Bueno *et al.*, 2012
	Spain	Madrid STP 2	29-387		196	Martínez-Bueno *et al.*, 2012
	Spain	Barcelona STP	64-624		193	Martínez-Bueno *et al.*, 2012
	Spain	Girona STP 1			108	Gros *et al.*, 2013
	Spain	Girona STP 3			69	Gros *et al.*, 2013
	Spain	Alcalá STP			850	Herrera *et al.*, 2014

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Table S1 Occurrence of studied antibiotic in STP effluents.

Antibiotic	Country	Location	Concentration ($ng \cdot L^{-1}$)			Reference
			Range	Median	Mean	
	Spain	Girona STP	ND-125			Rodríguez-Mozaz *et al.*, 2015
	Spain	Alcalá STP			430	Carbajo *et al.*, 2015
	Sweden	Ryaverket STP			50	Andreozzi *et al.*, 2003
	Sweden	Henriksdal STP	214-225			Lindberg *et al.*, 2005
	Sweden	Ryaverket STP	66-231			Lindberg *et al.*, 2005
	Sweden	Umea STP	644-1340			Lindberg *et al.*, 2005
	Sweden	Kalmar STP	561-700			Lindberg *et al.*, 2005
	Sweden	Floda STP	230-777			Lindberg *et al.*, 2005
	Sweden	3 STPs	60-510			Fick *et al.*, 2011
	Switzcherland	Kloten-Opfikon STP	3-7		4	Göbel *et al.*, 2004
	Switzcherland	2 STPs	20-310	70		Göbel *et al.*, 2005
	Switzerland	Lausanne STP			158	Margot *et al.*, 2013
	Taiwan	5 STPs			321	Lin *et al.*, 2008
	Taiwan	4 STPs	200-415			Lin *et al.*, 2009
	UK	5 STPs	<LOQ-1288	70		Ashton *et al.*, 2004
	UK	Howdon STP		271		Roberts and Thomas, 2006
	UK	Cilfynydd STP	625-6052		1152	Kasprzyk-Hordern *et al.*, 2009
	UK	Coslech STP	385-1218		876	Kasprzyk-Hordern *et al.*, 2009
	USA	STP 2	<LOQ-1760	1070		Renew and Huang, 2004
	USA	10 STPs	ND-414	11		Glassmeyer *et al.*, 2005
	USA	7 STPs		170		Karthikeyan and Meyer, 2006
	USA	Albulquerque STP			180	Brown *et al.*, 2006
	USA	Lackawana STP		315		Batt *et al.*, 2006
	USA	East Aurora STP		90		Batt *et al.*, 2006
	USA	Holland STP		160		Batt *et al.*, 2006
	USA	Amherst STP			2400	Batt *et al.*, 2007
	USA	East Aurora STP			210	Batt *et al.*, 2007
	USA	Holland STP			540	Batt *et al.*, 2007
	USA	Lackawana STP			360	Batt *et al.*, 2007
	USA	Nortern Colorado STP 1			1531	Ferrer and Thurman, 2013
	USA	Nortern Colorado STP 2			15.3	Ferrer and Thurman, 2013
	USA	50 STPs	ND-370		170	Kostich *et al.*, 2014

[a] Erythromycin (and $-H_2O$)

UK: United Kingdom

USA: States Unitates of America

ND: Not detect

LOD: Limit of detection

LOQ: Limit of quantification

Table S2 Occurrence of studied antibiotic in freshwater (continued on next page).

Antibiotic	Country	Location	Concentration ($ng \cdot L^{-1}$)			Reference
			Range	Median	Mean	
DXY	Australia	6 river systems	ND-400	ND		Watkinson *et al.*, 2007
	Australia	81 surface waters	ND-40			Watkinson *et al.*, 2009
	China	Huangpu river	5.61-46.9	13.6		Jiang *et al.*, 2011
	China	Streams with livestock	ND-12.6			Zhou *et al.*, 2013
	USA	Poundre river			100	Yang *et al.*, 2003
	USA	Cache la Poudre river	10-50		30	Kim and Carlson, 2007
	USA	Choptank river	<LOQ-2			Arikan *et al.*, 2008
	USA	Subwatershed	<LOQ-146			Arikan *et al.*, 2008
ERY[a]	China	Victoria Harbour	ND-5.2			Xu *et al.*, 2007b
	China	Pearl river	ND-636			Xu *et al.*, 2007b
	China	Yellow river	<LOQ-102			Xu *et al.*, 2009
	China	Pearl river	ND-2070			Yang *et al.*, 2011
	China	Haihe river System	3-400	110	130	Heeb *et al.*, 2012
	China	Yangtze river	21-217	48	81	Qi *et al.*, 2014
	China	Wangyang river	ND-253	68.7	98.1	Jiang *et al.*, 2014
	France	Siene river	<LOQ-4			Dihn *et al.*, 2011
	France	Predecelle river	<LOQ-4.2			Dihn *et al.*, 2011
	France	Charmoise river	<LOQ-131			Dihn *et al.*, 2011
	Germany	River waters and drainages	ND-1700	150		Hirsch *et al.*, 1999
	Germany	Water slides-Westphalia	<190			Christian *et al.*, 2003
	Italy	Po River	1.40-15.9			Calmari *et al.*, 2003
	Italy	Lambro river			4.5	Zuccato *et al.*, 2005
	Italy	Po river	ND-15.9	3.2		Zuccato *et al.*, 2005
	Italy	Po river	0.78-4.62		2.9	Zuccato *et al.*, 2010
	Italy	Arno river	2.88-8.12		5.4	Zuccato *et al.*, 2010
	Italy				30.5	Meffe and de Bustamante, 2014
	Japan	Tamagawa river	21-120	78	32.9	Managaki *et al.*, 2007
	Japan	Nationwide survey	ND-27.8	0.01	2.55	Murata *et al.*, 2011
	Japan	Nationwide survey	ND-128	1.1	8.13	Murata *et al.*, 2011
	Spain	Llobregat river	ND-363			Osorio *et al.*, 2012
	Spain	Llobregat river	10-70		30	Ginebreda *et al.*, 2010
	Spain	Jarama river	ND-603			Valcárcel *et al.*, 2011
	Spain	Guadarrama river	ND-721			Valcárcel *et al.*, 2011
	Spain	Henares river	ND-284			Valcárcel *et al.*, 2011
	Spain	Tagus river	ND-3847			Valcárcel *et al.*, 2011
	Spain	Tagus river basin	<LOQ-326			Martínez-Bueno *et al.*, 2010
	Spain	Llobregat river (STP upstream)			4	Proia *et al.*, 2013
	Spain	Llobregat river (STP downstream)			32.3	Proia *et al.*, 2013
	Spain	Llobregat river basin	58.1-363		12.3	Osorio *et al.*, 2014
	UK	River (STP upstream)	<LOQ-57			Ashton *et al.*, 2004
	UK	River (STP downstream)	<LOQ-1022			Ashton *et al.*, 2004
	UK	Tyne river	<LOQ-70			Roberts and Thomas, 2006
	UK	Taff river	11-351		91	Kasprzyk-Hordern *et al.*, 2008

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Table S2 Occurrence of studied antibiotic in freshwater (continued on next page).

Antibiotic	Country	Location	Concentration ($ng \cdot L^{-1}$)			Reference
			Range	Median	Mean	
	UK	Ely river	<LOQ-141		50	Kasprzyk-Hordern *et al.*, 2008
	UK	Taff river (STP upstream)	<LOQ-20		4	Kasprzyk-Hordern *et al.*, 2009
	UK	Taff river (STP downstream)	11-121		52	Kasprzyk-Hordern *et al.*, 2009
	UK	Ely river (STP upstream)	<LOQ-2		0	Kasprzyk-Hordern *et al.*, 2009
	UK	Ely river (STP downstream)	<LOQ-72		15	Kasprzyk-Hordern *et al.*, 2009
	USA	Cache La Poudre river			170	Yang and Carlson, 2004
	USA	Cache la Pundre river	20-450		120	Kim and Carlson, 2007
	Vietnam	Urban drainage	29-41	35.6	36.5	Managaki *et al.*, 2007
	Vietnam	Mekong river	9-12	10.5	10.5	Managaki *et al.*, 2007
MNZ	China	Haihe river System	12-250	100	100	Heeb *et al.*, 2012
	China	Yangtze river	7-224	35	74	Qi *et al.*, 2014
	Italy				68	Meffe and Bustamante, 2014
	Spain	Ebro river basin	6-45		21	Gros *et al.*, 2009
	Spain	Jarama river	ND-1757			Valcárcel *et al.*, 2011
	Spain	Manzanares river	ND-1251			Valcárcel *et al.*, 2011
	Spain	Guadarrama river	ND-1834			Valcárcel *et al.*, 2011
	Spain	Tagus river	ND-182			Valcárcel *et al.*, 2011
	Spain	Tagus basin	<LOQ-32			Martínez-Bueno *et al.*, 2010
	Spain	Llobregat river basin	1.19-3.98		0.22	Osorio *et al.*, 2014
	Spain	Ter river (STP downstream)	ND-28.4			Rodríguez-Mozaz *et al.*, 2015
	UK	Taff river	<LOQ-14		5	Kasprzyk-Hordern *et al.*, 2008
	UK	Ely river	<LOQ-24		11	Kasprzyk-Hordern *et al.*, 2008
	UK	Taff river (STP upstream)	<LOQ-10		1	Kasprzyk-Hordern *et al.*, 2009
	UK	Taff river (STP downstream)	2-11		5	Kasprzyk-Hordern *et al.*, 2009
	UK	Ely river (STP downstream)	<LOQ-24		12	Kasprzyk-Hordern *et al.*, 2009
OFX	China	Pearl river	ND-108			Xu *et al.*, 2007b
	China	Major Pearl river	<LOQ-439			Peng *et al.*, 2008
	China	Tonghui river	149-535	176		Xiao *et al.*, 2008
	China	Yellow river	<LOQ-264			Xu *et al.*, 2009
	China	Streams	ND-14.5			Zhou *et al.*, 2013
	China	Wangyang river	ND-11735	668	15835	Jiang *et al.*, 2014
	Findland	Vantaa river			5	Vieno *et al.*, 2007
	France	Seine river	ND-55			Tamtam *et al.*, 2008
	France	Siene river	2.3-18			Dihn *et al.*, 2011
	France	Predecelle river	3.5-65			Dihn *et al.*, 2011
	France	Charmoise river	4.3-231			Dihn *et al.*, 2011
	India	Isakavagu-Nakkavagu rivers			10000	Fick *et al.*, 2009
	India	Lake			11000	Fick *et al.*, 2009
	Italy	Olona river	<LOQ-177			Castiglioni *et al.*, 2008
	Italy	Lambro river	19-306			Castiglioni *et al.*, 2008

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Table S2 Occurrence of studied antibiotic in freshwater (continued on next page).

Antibiotic	Country	Location	Concentration ($ng \cdot L^{-1}$)			Reference
			Range	Median	Mean	
	Italy	Po river	<LOQ-37			Castiglioni *et al.*, 2008
	Italy	Lambro river			4.5	Zuccato *et al.*, 2005
	Italy	Po river	ND-15.9	3.2		Zuccato *et al.*, 2005
	Italy	Po river	0.65-18.1		10.9	Zuccato *et al.*, 2010
	Italy	Arno river	<1.4-10.9		5	Zuccato *et al.*, 2010
	Italy				306	Meffe and Bustamante, 2014
	South Korea	Mankyung river	ND-87.4			Kim and Carlson, 2009
	Spain	Ebro river basin	<LOQ-50		11	Gros *et al.*, 2009
	Spain	Llobregat river	190-8770		2110	Ginebreda *et al.*, 2010
	Spain	Tagus river system	<LOQ-402			Martínez-Bueno *et al.*, 2010
	Spain	Jarama river	ND-336			Valcárcel *et al.*, 2011
	Spain	Manzanares river	ND-269			Valcárcel *et al.*, 2011
	Spain	Guadarrama river	ND-552			Valcárcel *et al.*, 2011
	Spain	Tagus river	ND-49			Valcárcel *et al.*, 2011
	Spain	Ter river			33	Gros *et al.*, 2012
	Spain	Onyar river			20	Gros *et al.*, 2012
	Spain	Ebro river basin	<LOQ-79.9		10.2	López-Serna *et al.*, 2012
	Spain	Llobregat river	<LOD-448			Osorio *et al.*, 2012
	Spain	Llobregat river (STP upstream)			30.0	Proia *et al.*, 2013
	Spain	Llobregat river (STP downstream)			208	Proia *et al.*, 2013
	Spain	Ter river (STP downstream)	<138			Rodríguez-Mozaz *et al.*, 2015
SMX	27 European countries		ND-4072	15	76	Loss *et al.*, 2009
	Australia	6 river systems	ND-2000	8		Watkinson *et al.*, 2007
	Australia	81 surface waters	ND-2000	8		Watkinson *et al.*, 2009
	China	Pearl River	ND-193			Xu *et al.*, 2007b
	China	Major Pearl river	<LOQ-510			Peng *et al.*, 2008
	China	Yellow river	<LOQ-56			Xu *et al.*, 2009
	China	Pearl river	ND-616			Yang *et al.*, 2011
	China	Huangpu river	16.9-55.2	28.3		Jiang *et al.*, 2011
	China	Haihe river System	17-600	140	180	Heeb *et al.*, 2012
	China	Wangyang river	ND-4870	78.9	529	Jiang *et al.*, 2014
	China	Yangtze river	5-36	12	16	Qi *et al.*, 2014
	China	Streams	3.58-11.9			Zhou *et al.*, 2013
	France	Seine river	<121			Tamtam *et al.*, 2008
	France	Siene river	3.6-18			Dihn *et al.*, 2011
	France	Predecelle river	<LOQ-25			Dihn *et al.*, 2011
	France	Charmoise river	5.6-1435			Dihn *et al.*, 2011
	France	Orne river	ND-6			Minguez *et al.*, 2014
	Germany	5 rivers		45		Hartig *et al.*, 1999
	Germany	River and drainages	ND-480	30		Hirsch *et al.*, 1999
	Germany	Water slides-Westphalia	40-200			Christian *et al.*, 2003
	Italy	Po river	1.83-2.39		2.1	Zuccato *et al.*, 2010
	Italy	Arno river	1.79-11.4		5.3	Zuccato *et al.*, 2010
	Italy				89	Meffe and Bustamante, 2014
	Japan	Tamagawa river	4-23	18.5	7	Managaki *et al.*, 2007
	Japan	Nationwide survey	ND-33.9	1.1	4.85	Murata *et al.*, 2011

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Table S2 Occurrence of studied antibiotic in freshwater (continued on next page).

Antibiotic	Country	Location	Concentration ($ng \cdot L^{-1}$)			Reference
			Range	Median	Mean	
	Rep. of Korea	Han river	<LOQ-82	21		Choi *et al.*, 2008b
	Spain	Ebro river basin	<LOQ-50		11	Gros *et al.*, 2009
	Spain	Henares-Jarama-Tagus river system	0.1-23.7	6.9		Fernández *et al.*, 2010
	Spain	Tagus river system	<LOQ-140			Martínez-Bueno *et al.*, 2010
	Spain	Llobregat river	30-11920		1110	Ginebreda *et al.*, 2010
	Spain	Jarama river	ND-952			Valcárcel *et al.*, 2011
	Spain	Manzanares river	ND-638			Valcárcel *et al.*, 2011
	Spain	Guadarrama river	ND-879			Valcárcel *et al.*, 2011
	Spain	Henares river	ND-32			Valcárcel *et al.*, 2011
	Spain	Tagus river	ND-82			Valcárcel *et al.*, 2011
	Spain	Llobregat river	0.2-1500			Osorio *et al.*, 2012
	Spain	Ter river			16	Gros *et al.*, 2012
	Spain	Onyar river			79	Gros *et al.*, 2012
	Spain	Llobregat river (STP upstream)			234	Proia *et al.*, 2013
	Spain	Llobregat river (STP downstream)			908	Proia *et al.*, 2013
	Spain	Henares river (STP upstream)			28	Herrera *et al.*, 2014
	Spain	Henares river (STP downstream)			171	Herrera *et al.*, 2014
	Spain	Llobregat river basin	33.9-151		5.83	Osorio *et al.*, 2014
	Spain	Ter river (STP upstream)	ND-7			Rodríguez-Mozaz *et al.*, 2015
	Spain	Ter river (STP downstream)	ND-71.8			Rodríguez-Mozaz *et al.*, 2015
	Sweden	Hoje river	ND-10			Bendz *et al.*, 2005
	Sweden		<LOD-44			Fick *et al.*, 2011
	UK	Downstream of STPs	<50			Ashton *et al.*, 2004
	UK	Taff river	<LOQ-2		1	Kasprzyk-Hordern *et al.*, 2008
	UK	Ely river	<LOQ-4		1	Kasprzyk-Hordern *et al.*, 2008
	UK	Taff river (STP upstream)	<LOQ-1		0	Kasprzyk-Hordern *et al.*, 2009
	UK	Taff river (STP downstream)	<LOQ-8		2	Kasprzyk-Hordern *et al.*, 2009
	UK	Ely river (STP upstream)	<LOQ-1		0	Kasprzyk-Hordern *et al.*, 2009
	UK	Ely river (STP downstream)	<LOQ-4		1	Kasprzyk-Hordern *et al.*, 2009
	USA	139 stream sites	ND-1900	150		Kolpin *et al.*, 2002
	USA	Buffalo river (East Aurora STP downstream)		20		Batt *et al.*, 2006
	USA	Buffalo river (Holland STP downstream)		56		Batt *et al.*, 2006
	USA	Rio Grande			300	Brown *et al.*, 2006
	USA	Cache la Poundre River	40-320		110	Kim and Carlson, 2007
	USA	Choptank River	<LOQ-7			Arikan *et al.*, 2008

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Table S2 Occurrence of studied antibiotic in freshwater (continued on next page).

Antibiotic	Country	Location	Concentration ($ng \cdot L^{-1}$)			Reference
			Range	Median	Mean	
	USA	Subwatershed	<LOQ-7			Arikan *et al.*, 2008
	USA	139 stream sites	<1900			Kolpin *et al.*, 2002
	USA	Grand Island river	<LOQ-29.4			Bartelt-Hunt *et al.*, 2009
	USA	Lincoln river	1.4-343			Bartelt-Hunt *et al.*, 2009
	USA	Hstings			173	Bartelt-Hunt *et al.*, 2009
	Vietnam	Urban drainage	37-360	153	179	Managaki *et al.*, 2007
	Vietnam	Mekong river	20-33	22	26.3	Managaki *et al.*, 2007
TMP	Australia	Six river systems	ND-150	3		Watkinson *et al.*, 2007
	Australia	81 surface waters	ND-150	3		Watkinson *et al.*, 2009
	China	Huangpu river	6.75-62.4	14.2		Jiang *et al.*, 2011
	China	Pearl river	ND-605			Yang *et al.*, 2011
	China	Haihe river system	8-340	60	82	Heeb *et al.*, 2012
	China	Streams	6.22-19.2			Zhou *et al.*, 2013
	China	Wangyang River	ND-1126	61.0	242	Jiang *et al.*, 2014
	France	Seine River	ND-45			Tamtam *et al.*, 2008
	France	Predecelle river	<LOQ-8			Dihn *et al.*, 2011
	France	Charmoise river	<LOQ-254			Dihn *et al.*, 2011
	France	Orne river	ND-2			Minguez *et al.*, 2014
	Germany	River and drainages	ND-200	ND		Hirsch *et al.*, 1999
	Germany	Water slides-Westphalia	6-70			Christian *et al.*, 2003
	Japan	Tamagawa river	19-54	29.5	13.7	Managaki *et al.*, 2007
	Japan	Nationwide survey	ND-3.6	0.02	2.50	Murat *et al.*, 2011
	Korea	Han river	ND-312		108	Choi *et al.*, 2008b
	Korea	Kyung-Ahn stream	ND-30.6		117	Choi *et al.*, 2008b
	Italy				25	Meffe and Bustamante, 2014
	India	Isakavagu-Nakkavagu rivers			4000	Fick *et al.*, 2009
	Rep. of Korea	Han river	<LOQ-26			Choi *et al.*, 2008b
	Spain	Ebro river basin	<LOQ-16		4	Gros *et al.*, 2009
	Spain	Llobregat river	20-470		140	Ginebrada *et al.*, 2010
	Spain	Henares-Jarama-Tagus river system	0.4-23.3	12.0		Fernández *et al.*, 2010
	Spain	Tagus river system	<LOQ-112			Martínez-Bueno *et al.*, 2010
	Spain	Jarama river	ND-690			Valcárcel *et al.*, 2011
	Spain	Manzanares river	ND-478			Valcárcel *et al.*, 2011
	Spain	Guadarrama river	ND-519			Valcárcel *et al.*, 2011
	Spain	Henares river	ND-38			Valcárcel *et al.*, 2011
	Spain	Tagus river	ND-61			Valcárcel *et al.*, 2011
	Spain	Ter river			5	Gros *et al.*, 2012
	Spain	Onyar river			9	Gros *et al.*, 2012
	Spain	Llobregat river	ND-35.6			Osorio *et al.*, 2012
	Spain	Ebro river basin	<LOQ-59.9		9.46	López-Serna *et al.*, 2012
	Spain	Llobregat river (STP upstream)			7.6	Proia *et al.*, 2013
	Spain	Llobregat river (STP downstream)			27.4	Proia *et al.*, 2013
	Spain	Llobregat river basin	7.88-20.5		0.70	Osorio *et al.*, 2014
	Spain	Henares river (STP upstream)			18	Herrera *et al.*, 2014
	Spain	Henares river (STP downstream)			249	Herrera *et al.*, 2014

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Table S2 Occurrence of studied antibiotic in freshwater.

Antibiotic	Country	Location	Concentration ($ng \cdot L^{-1}$)			Reference
			Range	Median	Mean	
	Spain	Ter river (STP downstream)	ND-92.7			Rodríguez-Mozaz *et al.*, 2015
	Sweden	Hoja river	<1-20			Bendz *et al.*, 2005
	Sweden		<LOD-8			Fick *et al.*, 2011
	UK	River (STP upstream)	<LOQ-36			Ashton *et al.*, 2004
	UK	River (STP downstream)	<LOQ-42			Ashton *et al.*, 2004
	UK	Downstream of STPs	<10-42		12	Ashton *et al.*, 2004
	UK	Tyne river	7-19			Roberts and Thomas, 2006
	UK	Taff river	2-120		71	Kasprzyk-Hordern *et al.*, 2008
	UK	Ely river	10-183		73	Kasprzyk-Hordern *et al.*, 2008
	UK	Taff river (STP upstream)	<LOQ-7		1	Kasprzyk-Hordern *et al.*, 2009
	UK	Taff river (STP downstream)	30-120		89	Kasprzyk-Hordern *et al.*, 2009
	UK	Ely river (STP upstream)	<LOQ-90		19	Kasprzyk-Hordern *et al.*, 2009
	UK	Ely river (STP downstream)	10-183		62	Kasprzyk-Hordern *et al.*, 2009
	USA	139 stream sites	ND-710	150		Kolpin *et al.*, 2002
	USA	Buffalo river (STP downstream)			80	Batt *et al.*, 2006
	Vietnam	Urban drainage	15-46	28	29.9	Managaki *et al.*, 2007
	Vietnam	Mekong river	7-19	17.5	15.3	Managaki *et al.*, 2007

[a] Erythromycin (and $-H_2O$)

UK: United Kingdom

USA: States Unitates of America

ND: Not detect

LOD: Limit of detection

LOQ: Limit of quantification

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Chapter 5

Continuous ozonation treatment of ofloxacin: Transformation products, water matrix effect and aquatic toxicity

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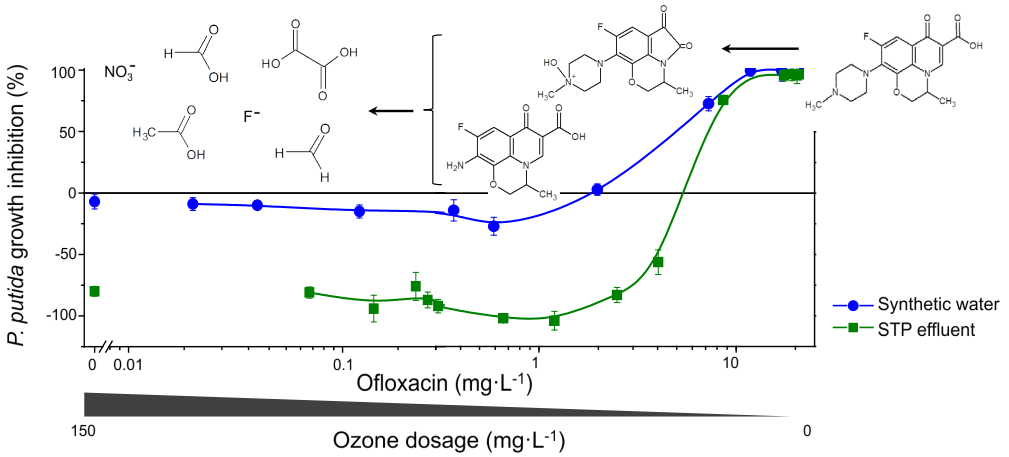
Graphical Abstract



CONTINUOUS OZONATION TREATMENT OF OFLOXACIN: TRANSFORMATION PRODUCTS, WATER MATRIX EFFECT AND AQUATIC TOXICITY

Abstract

The continuous ozonation of the antibiotic ofloxacin (OFX) has been performed using a synthetic water matrix and in a sewage treatment plant (STP) effluent. The aim was to study the effect of the water matrix on the ozonation with particular emphasis on the aquatic toxicity of treated water. OFX was completely removed in both water matrices, although the amount of ozone consumed for its depletion was strongly matrix-dependent. The extent of mineralization was limited and a number of intermediate transformation products (TPs) appeared, twelve of which could be identified. OFX reaction pathway includes the degradation of piperazinyl and quinolone moieties. The further oxidation of TPs gave rise to the formation and accumulation of carboxylic acids, aldehydes, nitrogen-containing organic compounds and inorganic ions. Aquatic toxicity of treated mixtures was assessed using four standard species: the bacteria *Vibrio fischeri* and *Pseudomonas putida* as target organisms and the protozoan *Tetrahymena thermophila* and the algae *Pseudokirchneriella subcapitata* as non-target organisms. OFX was toxic for the bacteria and the microalgae at the spiked concentration in untreated water. However, the continuous ozonation at the upper operational limit removed its toxic effects. *T. thermophila* was not affected by OFX, but was sensitive to STP effluent.

1. Introduction

Antibiotics are commonly used to treat infections in humans and are intensively applied for veterinary uses (van der Grinten *et al.*, 2010). As a consequence of their poor metabolization and their incomplete removal in sewage treatment plants (STPs), antibiotics are continuously released into the aquatic environment (Kümmerer, 2009a and Fatta-Kassinos *et al.*, 2011). Their occurrence in surface waters has generated human health and environmental concerns. Although found at sub-therapeutic levels, relatively low concentrations of these drugs can promote bacterial resistance (Kümmerer, 2009b and Rizzo *et al.*, 2013). Indeed, antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) have been found in STP effluents, surface and drinking waters (Schwartz *et al.*, 2003 and Yang *et al.*, 2014). Despite the fact that antibiotics are specifically applied to fight pathogenic bacteria, non-target environmental organisms which provide important ecosystem services are inevitably exposed, resulting in a potential risk of ecosystem disruption (van der Grinten *et al.*, 2010 and Brain *et al.*, 2009).

Ofloxacin (OFX), a quinolone, is a broad-spectrum antibacterial agent widely used for treating bacterial infections. Its major mode of action is the inhibition of DNA replication in bacteria via interference of the normal function of the A-subunit of the DNA gyrase protein (Sukul and Spiteller, 2007). It displays high activity not only against bacteria, but the detection of a gyrase-like protein in plants explains the high quinolone toxicity also found for algae (Brain *et al.*, 2009). In conventional STP, OFX is partially removed (apparent removal efficiency of 60%), mainly by adsorption onto activated sludge (Li and Zhang, 2010 and Verlicchi *et al.*, 2012), being the balance discharged with treated wastewater. In fact, OFX has frequently been detected in STP effluents and river basins in up to $\mu g \cdot L^{-1}$ and $ng \cdot L^{-1}$ levels, respectively (Segura *et al.*, 2009, Fatta-Kassinos *et al.*, 2011, Verlicchi *et al.*, 2012 and Michael *et al.*, 2013). As a consequence of its occurrence and toxicity, recent publications have concluded that OFX might pose a potential risk to aquatic organisms (Kümmerer *et al.*, 2000, Isidori *et al.*, 2005, Segura *et al.*, 2009 and Backhaus and Karlsson, 2014).

As conventional processes used in an STP are unable to act as a reliable barrier toward some pharmaceutical compounds, a great effort is currently directed to develop technologies capable of efficiently removing them (Ternes *et al.*, 2004). Among them, ozonation is known as an attractive alternative due to its effectiveness in the removal of a wide range of micropollutants with potential environmental risks (Andreozzi *et al.*, 2004, Huber *et al.*, 2005, Rosal *et al.*, 2010 and Rodríguez *et al.*, 2012). A further advantage of the ozonation is its disinfecting potential, which is able to deactivate ARG biological activities in addition to achieving ARB inactivation, preventing the dissemination of antibiotic resistance (Tyrrell *et al.*, 1995 and Dodd, 2012).

Using continuous processes working with real STP effluents have proven more useful than batch/semi-batch works performed in wastewater or simulated effluents for full-scale studies. Continuous treatment displays a closer approximation to a full-scale system and a better understanding of the fate of pollutants under oxidizing conditions (Huber *et al.*, 2005). In addition to the reaction time and ozone dose, the extent of oxidation depends mainly on the chemical nature of the micropollutant itself and water matrix composition (Katsoyiannis *et al.*, 2011). Moreover, it is important to take into account that the abatement of the target compound rarely leads to its total mineralization, but rather the formation of transformation products (TPs). The concern is whether or not these TPs keep the biological effects of the parent compounds or whether new and undesired biological effects are developed (Dantas *et al.*, 2008, Li *et al.*, 2008, Dodd *et al.*, 2009 and Gómez-Ramos *et al.*, 2011). This issue cannot be addressed merely elucidating the structures of the TPs by chemical analysis. Instead, the assessment of treated water toxicity and the influence of the water matrix are necessary for the optimization of continuous ozonation treatments.

In this work, the continuous ozonation of OFX in two different water matrices (synthetic water and STP effluent) was studied, elucidating its TPs in order to propose a reaction pathway. Aquatic toxicity of treated water was assessed using a biotest battery composed of two target (*Vibrio fischeri* and *Pseudomonas putida*) and two non-target (*Tetrahymena thermophila* and *Pseudokirchneriella subcapitata*) organisms.

2. Materials and methods

2.1. Materials

Ofloxacin (OFX) was purchased from Sigma-Aldrich (≥98%). Two water matrices spiked with OFX (22 $mg \cdot L^{-1}$) were used for ozonation process experiments: synthetic water and an STP effluent. The synthetic matrix was prepared in ultrapure water (resistivity ≥18 MΩ·cm at 25ºC) with the required amount of sodium bicarbonate to equal the alkalinity and pH values of the STP effluent. Wastewater was collected from the outlet of the secondary clarifier of an STP located in Alcalá de Henares (Spain). The plant treats domestic wastewater with a minor contribution of industrial effluents from facilities located near the city (374 000 population equivalent) and has a nominal capacity of 3 000 $m^3 \cdot h^{-1}$. Details on wastewater characterization are showed in Table 5.1.

Table 5.1 Main physico-chemical parameters of STP effluent.

pH	7.33	Na^+ ($mg \cdot L^{-1}$)	65.0	Cr ($\mu g \cdot L^{-1}$)	0.36
Conductivity ($\mu S \cdot cm^{-1}$)	750	NH_4^+ ($mg \cdot L^{-1}$)	4.14	Ni ($\mu g \cdot L^{-1}$)	11.5
TSS ($mg \cdot L^{-1}$)	11.4	K^+ ($mg \cdot L^{-1}$)	14.7	Cu ($\mu g \cdot L^{-1}$)	12.7
Turbidity (NTU)	7.00	Mg^{2+} ($mg \cdot L^{-1}$)	18.3	Zn ($\mu g \cdot L^{-1}$)	34.4
COD ($mg \cdot L^{-1}$)	27.8	Ca^{2+} ($mg \cdot L^{-1}$)	51.9	As ($\mu g \cdot L^{-1}$)	9.88
DOC ($mg \cdot L^{-1}$)	8.42	Cl^- ($mg \cdot L^{-1}$)	85.7	Se ($\mu g \cdot L^{-1}$)	0.05
BOD_5 ($mg \cdot L^{-1}$)	6.00	NO_2^- ($mg \cdot L^{-1}$)	5.61	Cd ($\mu g \cdot L^{-1}$)	ND
BOD_5/COD	0.22	NO_3^- ($mg \cdot L^{-1}$)	58.8	Sn ($\mu g \cdot L^{-1}$)	4.27
$SUVA_{254}$* ($L \cdot mg\ C^{-1}\ m^{-1}$)	2.61	PO_4^{3-} ($mg \cdot L^{-1}$)	3.34	Hg ($\mu g \cdot L^{-1}$)	ND
Alkalinity (mg $CaCO_3 \cdot L^{-1}$)	138	SO_4^{2-} ($mg \cdot L^{-1}$)	81.3	Pb ($\mu g \cdot L^{-1}$)	ND

*Specific ultraviolet absorption at 254 nm

ND: not detected

2.2. Experimental procedure

The experiments were carried out in a cylindrical reactor (internal diameter of 6.0 cm and working height of 51 cm) with a total working volume of 1.44 L, which operated in continuous co-current mode. The retention time distribution curve yielded an average retention time of 10.3 min. The reactor modelling using the continuous stirred tank reactor (CSTR) in series model (Burrows *et al.*, 1999) determined an equivalent value of 1.13 tanks, indicating that the bubble column can reasonably approach a perfect CSTR (Asenjo and Merchuck, 1995).

Water flow rate was 142 mL·min^{-1} and gas flow was 390 mL·min^{-1} with different inlet ozone concentrations. During the runs, the inlet ozone dosage was stepwise increased from 4.2 to 145 milligrams of ozone per liter of wastewater (mg·L^{-1}). For the different ozone dosages samples were withdrawn for analysis at the column outlet once the stationary state was reached. This was ensured by circulating the hydraulic retention time four times after a constant ozone concentration was obtained both in liquid and gas phases at the column outlet. Assuming CSTR behaviour and stationary state $dC_{O_3}^{liq}/dt = 0$), the amount of ozone consumed can be obtained by means of the following mass balance (Eq. (5.1)):

$$Consumed\ O_3 = F_{O_3}^{gas,in} - F_{O_3}^{gas,out} - F_{O_3}^{liq,out} \quad (5.1)$$

in which F_{O_3} is the rate of ozone entering the system in the gas phase (gas, in) or leaving either in the exhaust gases (gas, out) or dissolved in water (liq, out). Details about experimental set-up are given in Chapter 3 (section 2.2).

2.3. Analytical methods

OFX concentration was performed by HPLC, Agilent 1200, with reversed-phase C18 analytical column (Phenomenex Luna SCX, 250 × 4.6 mm, 5 µm) and operated at a flow rate of 0.5 mL·min^{-1}. An isocratic method, with 30% acetonitrile and 70% ultrapure water with 0.1 M phosphoric acid and 10 mM ammonium acetate mobile phase, was employed with detection of OFX at λ 294 nm. The structural elucidation of TPs was carried out using a hybrid quadrupole time-of-flight mass spectrometer TripleTOF 5600 system (AB SCIEX) with an ESI (electrospray ionization) source coupled to an Agilent 1200 Series HPLC system (LC/ESI-QTOF-MS). The ion source parameters were: Ion Spray Voltage Floating (ISVF), 5 500 V; Temperature (TEM), 550ºC; Curtain Gas (CUR), 25 (arbitrary units) and Ion Source Gas (GS1 and GS2) at 35 and 40 psi, respectively. The MS was operated in full scan TOF-MS and MS/MS mode through information dependent acquisition (IDA) in a single run analysis. In addition to the discriminative information based on mass accuracy of the molecular ions acquired in TOF-MS, MS/MS mode was

used for the characterization of the TPs. The declustering potential (DP) and collision energy (CE) were 70 V and 10 V in the full scan TOF-MS experiment. The LC analysis was performed with a reversed-phase C18 analytical column (Agilent Zorbax Eclipse XDB, 50 × 4.6 mm, 1.8 µm). Mobile phases [A] and [B] were, respectively, acetonitrile and HPLC-grade water with 0.1% formic acid. A linear gradient was set from 10% to 100% of [A] in 11 min, and then maintained at 100% for 5 min. Data acquisition and processing were carried out using Analyst® TF 1.5 and PeakView™ (AB SCIEX) software.

Dissolved Organic Carbon (DOC) was determined using a TOC-V_{CSH} Shimadzu TOC analyzer. Carboxylic acids were measured by a Dionex DX120 Ion Chromatograph with a conductivity detector. Oxalic and mesoxalic acid concentrations were analyzed by IonPac AS9-HC analytical column (4 × 250 mm) with ASRS-Ultra suppressor whereas, acetic and formic acid concentrations were measured using an IonPac ICE analytical column (9 × 250 mm) with AMMS-ICE II suppressor. Inorganic ions were determined by means of a Metrohm 861 Advance Compact IC with conductivity detector; a Metrosep A Supp 7-250 analytical column was used in anion analysis while, a Metrosep C3 column was used in cation analysis. Formaldehyde was measured spectrophotometrically using the acetylacetone method (Hach-Lange LCK 325).

2.4. Procedures for aquatic toxicity tests

Aquatic toxicity assessment was performed with a bioassay battery composed of single-species tests of the bacteria *V. fischeri* and *P. putida*, the algae *P. subcapitata* and the protozoan *T. thermophila*. This set of bioassays allowed both acute and chronic assays to be performed and the combined usage of target (prokaryotes) and non-target (eukaryotes) OFX organisms at different trophic levels.

V. fischeri acute test measure the decrease in bioluminescence induced in the cell metabolism. The bioassay was performed according to ISO 11348-3 standard protocol (ISO, 2007) using the commercial BioFix®Lumi test (*V. fischeri*, NRRL-B 11177 from Macherey-Nagel, Germany). Bioluminescence was measured at 15 ± 1ºC after 30 min in 96-well white polypropylene microplate by a Fluoroskan Ascent FL microplate

luminometer (Thermo Scientific). *P. putida* test determine the inhibitory effect of a substance on the bacteria (*P. putida*, NCIB 9494 from CECT, Spain) by means of cell growth inhibition. The bioassay was performed according to ISO guideline 10712 (ISO, 1995). Bacterial culture was exposed to test solutions at 23 ± 1ºC for 16 h in 10 mL glass incubation vials which were constantly shaken in the dark. The cell growth was determined by optical density (λ 600 nm) in 96-well clear polypropylene microplate using a Rayto RT-2100C microplate reader.

Growth inhibition assay with the ciliate protozoan *T. thermophila* was carried out according to the Standard Operational Procedure Guidelines of Protoxkit F^{TM} (1998). The test is based on the turnover of substrate into ciliate biomass. Substrate and reconstitution medium were purchased from MicroBioTest Inc. (Belgium) whereas *T. thermophila* (SB 210) was kindly supplied by D. Cassidy-Hanley (Tetrahymena Stock Center, USA). Ciliates were incubated with water samples and food suspension in test vessels at 30 ± 1ºC for 24 h in the dark. Growth inhibition was determined on the basis of turbidity changes (OD at λ 440 nm), at the beginning and at the end of the test.

Finally, algal growth inhibition test was carried out following the procedure described in the European Guideline OECD TG (Guideline) 201, using *P. subcapitata* open system (OECD, 2011). The algal stock culture for inoculation was taken from commercial test system Algaltoxkit F^{TM} (MicroBioTest Inc., Belgium). The cells of *P. subcapitata* were exposed to tested water samples at 23 ± 1ºC for 72 h in 10 mL glass incubation vials which were constantly shaken and illuminated in a chamber (∼100 µmol foton·m^{-2}·s^{-1}). Algal biomass was measured daily by chlorophyll-*a* content, whose extraction was carried out as following: 50 µL culture samples were transferred to a 96-well black polypropylene microplate, 200 µL of ethanol was added to each well and the plate was shaken for 3 h in the dark. Thereafter the fluorescence was measured using a Fluoroskan Ascent FL microplate fluorometer (Excitation 450 nm, Emission 672 nm) from Thermo Scientific.

$ZnSO_4 \cdot 7H_2O$ for *V. fischeri* test, 3,5-dichlorophenol for *P. putida* and $K_2Cr_2O_7$ for the rest of the bioassays were used as reference substances in order to check each test

procedures. Three independent experiments with duplicate samples were carried out to ensure reproducibility. All aquatic toxicity data are expressed as mean ± 95% confidence interval and data analysis were performed using a nonlinear-regression sigmoidal dose-response curve model provided in the GraphPad Prism 6.0 software (GraphPad software Inc., San Diego, USA).

3. Results and discussion

3.1. Synthetic water matrix

The continuous ozonation process was studied from different ozone dosages in order to achieve maximum OFX oxidation and mineralization degrees. Fig. 5.1 shows the evolution of OFX concentration and DOC in the synthetic matrix as a function of the amount of ozone supplied. OFX declined with ozone up to an exposure of 60 $mg \cdot L^{-1}$, where it was completely removed. Otherwise, despite DOC also decaying with ozone, ozonation did not lead to OFX mineralization, with maximum values slightly over 40%.

The evolution of the consumed and dissolved ozone is also represented in Fig. 5.1. Based on the evolution of both parameters, three different zones can be observed as a function of ozone dosage. In zone 1, up to 58 $mg \cdot L^{-1}$, ozone consumption linearly increased and no dissolved ozone was detected (<0.01 $mg \cdot L^{-1}$), which indicated that ozone was acting as limiting reactant. This behaviour occurred during the oxidation of more easily oxidizable compounds because ozone mass transfer rate was slower than ozone consumption. In this initial zone, total OFX degradation was reached, suggesting that the target pollutant is easily abated by ozonation. This result was in line with previous studies using semi-batch processes. Márquez *et al.* (2013) reported high second-order rate constants (>10^6 $M^{-1} \cdot s^{-1}$) at pH >7 and De Witte *et al.* (2009) found a half-life time of 12.8 min at pH 7 for ozone inlet of 0.58 $mg \cdot min^{-1}$.Runs carried out using *t*-butanol (30 mM) as a radical scavenger (Fig. 5.2), suggest that OFX is mainly degraded by molecular ozone attack. The direct ozonation reaction would occur with the fast reacting moieties present in the OFX molecule such as the deprotonated amine and the aromatic ring (10^3–10^{11} M^{-1} s^{-1}) (De Witte *et al.*, 2009, Márquez *et al.*, 2013 and El Najjar

et al., 2013). Dodd *et al.* (2006) also observed that the kinetics of other quinolone reactions was predominantly driven by molecular ozone oxidation.

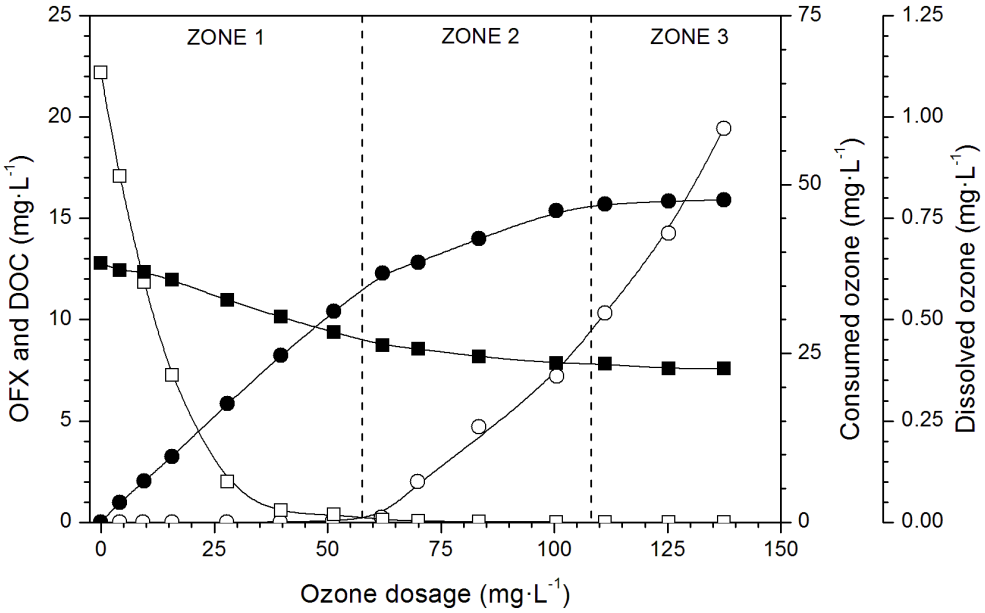


Fig. 5.1 Evolution of OFX (□), DOC (■), consumed (●) and dissolved ozone (○) for different ozone dosages in synthetic water matrix.

The DOC depletion achieved in zone 1 was 30%, which represented roughly three quarters of the maximum mineralization degree achieved along the runs. In zone 2, consumed ozone still increased and dissolved ozone began to be detected at the outlet stream. This fact is consistent with the oxidation of less easily oxidizable compounds, whose ozonation proceeded at a slower rate than ozone mass transfer. In this zone, the mineralization degree slightly rose from 30 to 41%, suggesting that the increase of consumed ozone was mainly due to the partial oxidation of organic matter. For dosages above 108 $mg \cdot L^{-1}$ (zone 3), ozone consumption remained constant, without further mineralization and a concentration of ozone at the reactor outlet (gas and liquid) which increased proportionally to ozone input. Under these conditions, the upper operational limit of the system, the consumed ozone value was 48 $mg \cdot L^{-1}$. Taking into account both consumed ozone and the abatement degree of OFX at the upper ozone dosage, the ozone consumed per milligram of OFX found in the synthetic water matrix was 2.15 $mg \cdot O_3 \cdot (mg\ OFX)^{-1}$. Considering the ozone consumed by the matrix (2.88 $mg \cdot L^{-1}$), the mass factor was 2.02 $mg\ O_3 \cdot (mg\ OFX)^{-1}$.

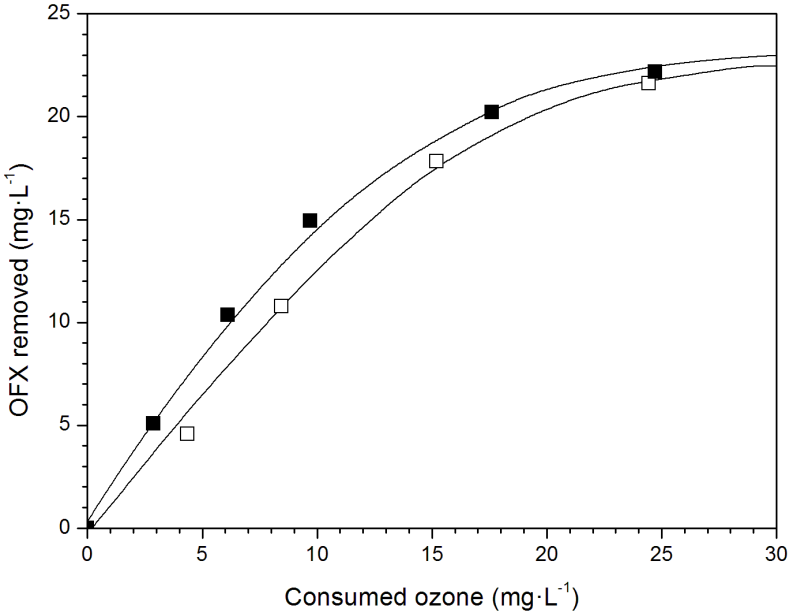
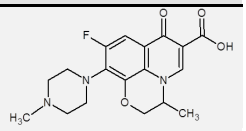
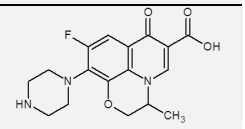
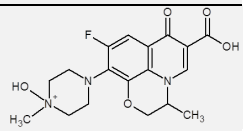
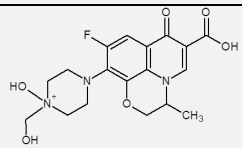
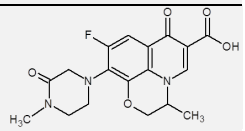
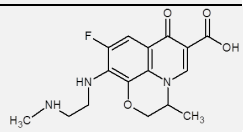
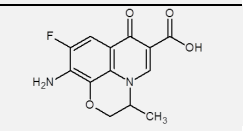
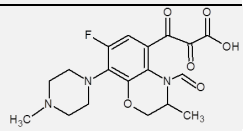
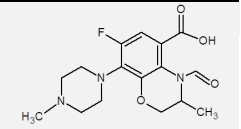
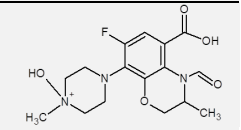
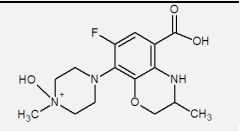


Fig. 5.2 Evolution of OFX in the synthetic water matrix without (■) and with *t*-butanol (30 mM) (□) for different levels of consumed ozone.

3.2. Elucidation of transformation products and degradation pathway

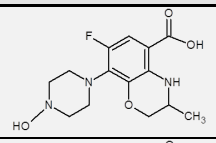
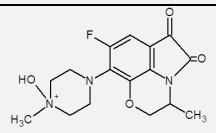
Twelve compounds were elucidated as TPs formed during the ozonation of OFX. Table 5.2 shows LC/ESI-QTOF-MS mass measurements of OFX and its TPs and structures proposed for them. The evolution of the corresponding TPs and OFX depletion as a function of the amount of ozone supplied are shown in Fig. 5.3. Relative amounts were calculated from the ion counts associated with each individual compound normalized by the ion count corresponding to the initial concentration of OFX. This approach allowed the yields and the evolution of TPs to be estimated, while their actual concentrations could not be determined due to the lack of standards (Liu *et al.*, 2012a). As can be seen in the figure, the TP amounts behaved as an intermediate product in series reactions, with their counts initially increasing to reach a maximum and then decreasing due to the further oxidation of these products by ozone. In fact, the maximum concentration of most TPs occurred for ozone dosages between 4.2 and 28 $mg \cdot L^{-1}$, and all of them disappeared at ozone exposure of 64 $mg \cdot L^{-1}$ (at the end of zone 1), demonstrating thus their high reactivity with ozone (Fig. 5.3A). It is worth mentioning that the yields of TPs were variable, with the highest ion counts corresponding to TP_2.

Table 5.2 LC/ESI-QTOF-MS mass measurements of ofloxacin (OFX) and its transformation products (TPs) and structures proposed for them (continued on next page).

Compound	R_t (min)	Elemental formula	Mass (m/z)		Error		Proposed structure
			Theoretical	Experimental	ppm	DBE	
OFX	4.170	$C_{18}H_{21}FN_3O_4^+$	362.1511	362.1518	2.0	9.5	
		$C_{17}H_{21}FN_3O_2^+$	318.1612	318.1608	-1.2	8.5	
		$C_{14}H_{14}FN_2O_2^+$	261.1034	261.1025	-3.4	8.5	
		$C_{11}H_{10}FN_2O_2^+$	221.0721	221.0713	-3.5	7.5	
TP1	4.150	$C_{17}H_{19}FN_3O_4^+$	348.1354	348.1367	3.7	9.5	
		$C_{17}H_{17}FN_3O_3^+$	330.1249	330.1256	2.3	9.5	
		$C_{16}H_{19}FN_3O_2^+$	304.1456	304.1468	3.9	8.5	
		$C_{16}H_{18}N_3O_2^+$	284.1394	284.1404	3.7	8.5	
		$C_{14}H_{14}FN_2O_2^+$	261.1034	261.1052	6.9	8.5	
TP2	4.550	$C_{18}H_{21}FN_3O_5^+$	378.1460	378.1471	2.9	9.5	
		$C_{18}H_{20}FN_3O_4^+$	361.1432	361.1421	-3.2	9.5	
		$C_{18}H_{19}FN_3O_4^+$	360.1354	360.1372	5.0	9.5	
		$C_{17}H_{21}FN_3O_3^+$	334.1562	334.1579	5.2	8.5	
		$C_{17}H_{21}N_2O_4^+$	317.1496	317.1530	11	8.5	
TP3	3.940	$C_{18}H_{21}FN_3O_6^+$	394.1409	394.1419	2.6	9.5	
		$C_{18}H_{19}N_2O_6^+$	359.1238	359.1285	13	7.5	
		$C_{17}H_{19}N_2O_4^+$	315.1339	315.1378	12	8.5	
		$C_{15}H_{12}FN_2O_3^+$	287.0827	287.0835	3.0	10.5	
		$C_{13}H_{12}FN_2O_2^+$	247.0877	247.0907	12	7.5	
TP4	3.435	$C_{18}H_{19}FN_3O_5^+$	376.1303	376.1323	5.3	10.5	
		$C_{18}H_{17}FN_3O_4^+$	358.1198	358.1205	2.1	11.5	
		$C_{15}H_{12}FN_2O_3^+$	287.0826	287.0825	-0.3	10.5	
		$C_{14}H_{14}FN_2O_2^+$	261.1034	261.1036	0.8	8.5	
TP5	4.117	$C_{16}H_{19}FN_3O_4^+$	336.1354	336.1346	-0.4	8.5	
		$C_{16}H_{17}FN_3O_3^+$	318.1249	318.1248	-0.2	9.5	
		$C_{13}H_{10}FN_2O_3^+$	298.1186	298.1188	0.7	10.5	
		$C_{13}H_{10}FN_2O_3^+$	261.0670	261.0692	8.4	9.5	
TP6	5.953	$C_{13}H_{12}FN_2O_4^+$	279.0775	279.0780	1.8	8.5	
		$C_{13}H_{10}FN_2O_3^+$	261.0670	261.0674	1.6	8.5	
		$C_{10}H_5FN_2O_3^+$	220.0279	220.0284	2.4	7.5	
		$C_{10}H_4FN_2O_3^+$	219.0200	219.0209	4.1	7.5	
TP7	4.533	$C_{18}H_{21}FN_3O_6^+$	394.1409	394.1426	4.3	9.5	
		$C_{15}H_{14}FN_2O_4^+$	305.0932	305.0944	3.9	9.5	
		$C_{14}H_{14}FN_2O_2^+$	261.1034	261.1059	9.6	8.5	
		$C_{13}H_{12}FN_2O_2^+$	247.0877	247.0884	2.8	6.5	
TP8	0.953	$C_{16}H_{21}FN_3O_4^+$	338.1511	338.1537	7.7	7.5	
		$C_{15}H_{21}FN_3O_3^+$	310.1562	310.1577	5.0	6.5	
		$C_{15}H_{19}FN_3O_2^+$	292.1456	292.1462	2.1	7.5	
		$C_{12}H_{12}FN_2O_2^+$	235.0877	235.0888	4.7	7.5	
TP9	2.322	$C_{16}H_{21}FN_3O_5^+$	354.1460	354.1469	2.6	7.5	
		$C_{16}H_{20}FN_3O_4^+$	337.1432	337.1453	6.1	7.5	
		$C_{15}H_{20}FN_3O_3^+$	309.1483	309.1466	-5.6	6.5	
		$C_{15}H_{19}N_3O_3^+$	289.1421	289.1443	7.6	5.5	
TP10	4.585	$C_{15}H_{21}FN_3O_4^+$	326.1511	326.1523	3.8	6.5	
		$C_{15}H_{20}FN_3O_3^+$	309.1483	309.1480	-1.0	6.5	
		$C_{15}H_{19}N_3O_3^+$	289.1421	289.1417	-1.3	5.5	
		$C_{14}H_{14}FN_2O_2^+$	261.1034	261.1078	17	8.5	
		$C_{11}H_{10}FN_2O_2^+$	221.0721	221.0713	-3.5	7.5	

(continued on next page)

Table 5.2 LC/ESI-QTOF-MS mass measurements of ofloxacin (OFX) and its transformation products (TPs) and structures proposed for them.

Compound	R_t (min)	Elemental formula	Mass (m/z)		Error		Proposed structure
			Theoretical	Experimental	ppm	DBE	
TP11	1.878	$C_{14}H_{19}FN_3O_4^+$	312.1354	312.1364	3.2	6.5	
		$C_{13}H_{17}FN_3O_2^+$	266.1299	266.1285	-5.4	5.5	
		$C_{12}H_{12}FN_2O^+$	219.0928	219.0917	-5.1	4.5	
			209.1085	209.1099	6.8	5.5	
TP12	4.441	$C_{16}H_{19}FN_3O_4^+$	336.1354	336.1350	-1.2	8.5	
		$C_{16}H_{18}FN_3O_3^+$	319.1327	319.1329	0.7	8.5	
		$C_{15}H_{18}FN_3O_2^+$	291.1378	291.1395	6.0	6.5	
		$C_{14}H_{15}FN_3O_2^+$	276.1143	276.1142	-0.3	8.5	
		$C_{13}H_{10}FN_2O_3^+$	261.0670	261.0692	8.4	9.5	

The generation pathway of these TPs is expected to include multiple routes due to the presence of several reactive sites in the parent compound and the occurrence of two oxidation mechanisms by both molecular ozone and hydroxyl radicals. Despite this complexity, the results presented above and the information available from reported data (De Witte *et al.*, 2009 and Liu *et al.*, 2012a) can be interpreted to propose the degradation pathway shown in Scheme 5.1. The degradation of OFX occurs on both piperazinyl (TP_1–TP_6, open symbols in Fig. 5.3) and quinolone ring (TP_7–TP_{12}, solid symbols in Fig. 5.3). No TPs were found corresponding to the degradation of the oxazinyl group, indicating that it remained unmodified by ozonation reactions.

On the one hand, the reactions of the piperazinyl ring were due to attacks to both the methyl group and the piperazine core. TP_1 is attributed to the demethylation of the piperazinyl ring at position 4′. TP_1 could be regarded as one of the intermediates for the formation of TP_6, which can be yield owing to the total oxidation of the piperazine ring to an amino group. The main transformation product TP_2 was a consequence of the initial ozone attack on N_4′ atom (Dodd *et al.*, 2006). The oxidation of TP_2 may yield TP_3 through the addition of a hydroxyl radical at 7′. OFX can also be oxidized to the keto-derivative TP_4, which would be transformed into TP_5 through the opening of the piperazine ring. Further oxidation of TP_3 and TP_5 would generate TP_6. These cited TPs, from the reaction of the piperazine group, seem to be formed primarily via molecular ozone attack (Liu *et al.*, 2012a). According to the proposed reaction pathway, the carbonyl and carboxyl groups at the quinolone moiety, which are essential for binding at

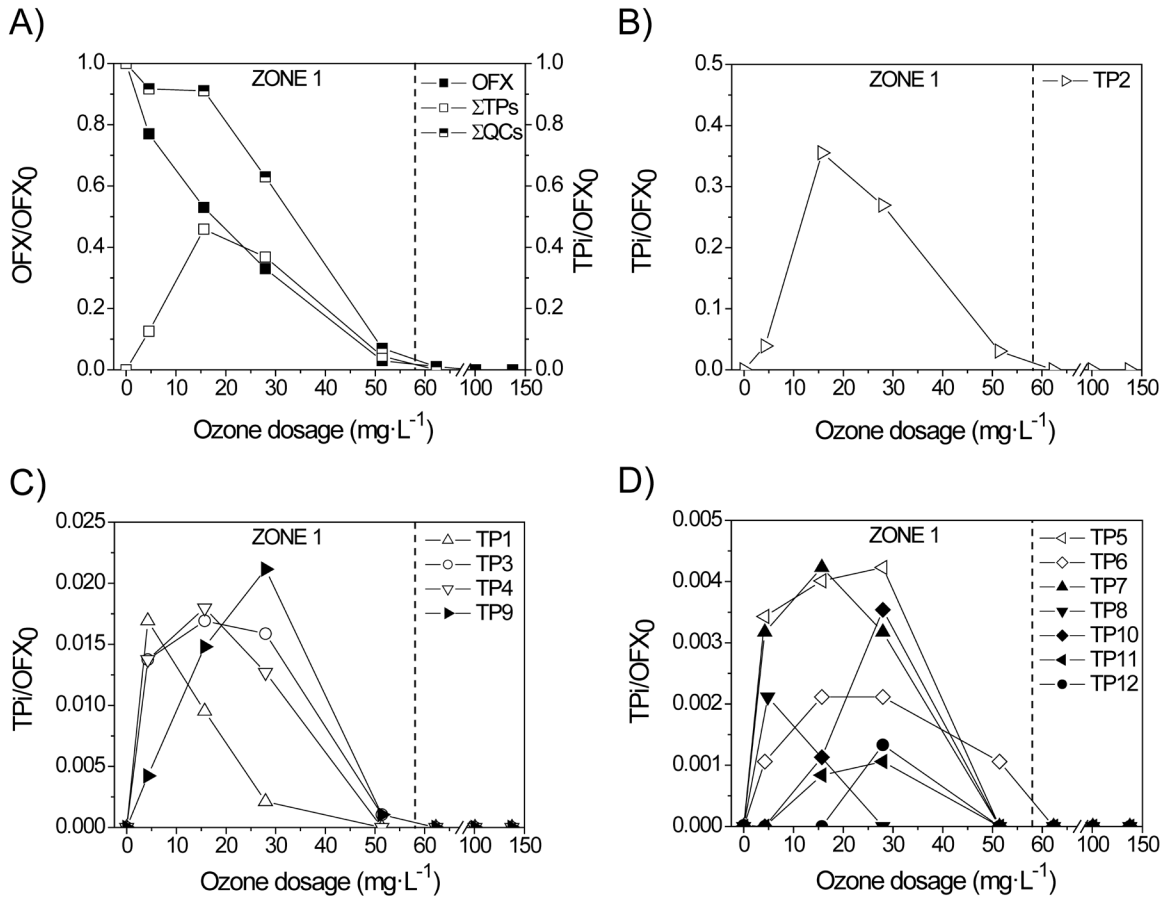
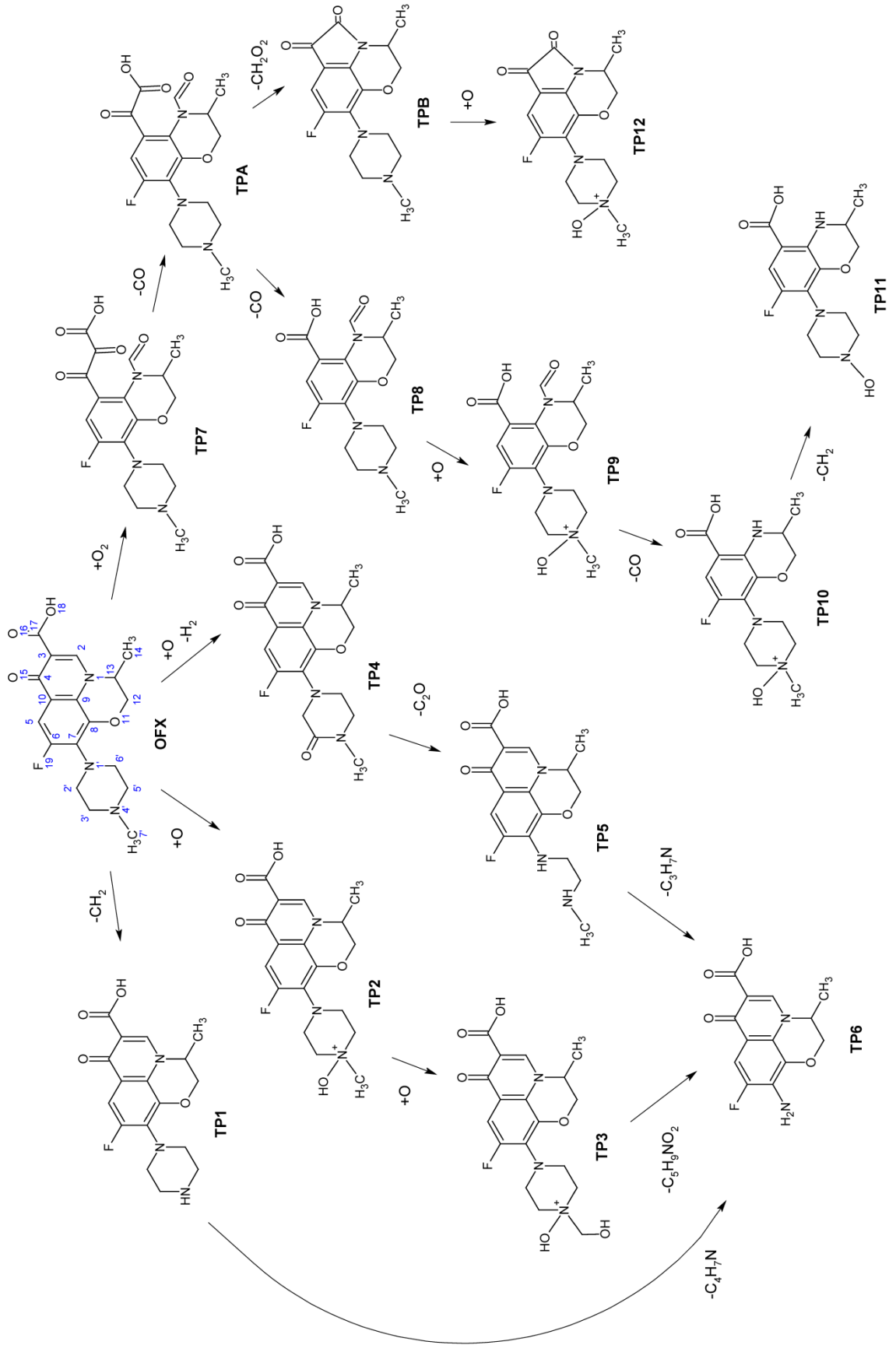


Fig. 5.3 Evolution of relative ion amount of ofloxacin, TPs and sum of TPs (ΣTPs) and quinolone core compounds (ΣQCs) for different ozone dosages in synthetic water matrix.

the DNA gyrase (Sukul and Spiteller, 2007), were not modified in TP_1–TP_6 so the direct ozonation mechanism is not likely inactivating the drug. Under this assumption, the sum of ion counts from TP_1–TP_6 and OFX, would correspond to biologically active compounds, non-monotonically decreased up to ozone dosages higher than 16 $mg \cdot L^{-1}$ as observed in Fig. 5.3A. On the other hand, the oxidation of quinolone moiety through the breaking of C_2=C_3 double bond led to TP_7. In agreement with Liu *et al.* (2012a), TP_7 should produce TP_A (non-observed in the present study), whose decarboxylation at C_3 yields anthranilic acid analogues (TP_8–TP_{11}), whereas deformylation at C_2 leads to isatin analogue formation (TP_B and TP_{12}). TPs from reactions at the quinolone moiety were a consequence of hydroxyl radical reactions according to reported data on fluoroquinolone degradation (De Witte *et al.*, 2009 and Liu *et al.*, 2012a). The proposed degradation pathway for the early oxidation stages of OFX is not only consistent with the



Scheme 5.1 Proposed degradation pathway for ofloxacin in ozonation process.

evolution and yield of TPs, but also supports that OFX oxidation was most likely due to direct ozonation reactions. In fact, TPs from reactions at piperizine group, mainly generated by molecular ozone (open symbols in Fig. 5.3), were more abundant than those at quinolone moiety, primarily consequence of radical reactions (full symbols in Fig. 5.3).

The further oxidation of detected TPs gave rise to the formation of species with lower molecular weight such as carboxylic acids. Fig. 5.4 represents the evolution of the main detected carboxylic acids (mesoxalic, oxalic, acetic and formic acid) found in ozonation runs. Their concentration increased in zone 2 due to the partial oxidation of organic matter. This explains the noticeable increase of consumed ozone in spite of OFX has been completely depleted. In zone 3, the concentration of carboxylic acids remained essentially constant together with mineralization degree. This fact is in good agreement with the well-known refractory character of these final ozonation products, which is the reason why their concentrations increased in the reaction mixture (von Sonntag and von Gunten, 2012 and Petre *et al.*, 2015). The organic acids only account for a third of DOC. As a consequence, other refractory organic compounds were not detectable by ionic chromatography, such as aldehydes or nitrogen-containing organic compounds, which should be present (Liu *et al.*, 2012b). Among aldehydes, formaldehyde was detected at a concentration close to 1.0 $mg \cdot L^{-1}$ at ozone exposures of 39 $mg \cdot L^{-1}$, probably as a result of the reaction yielding TPs such as TP_1.

Nitrogen was not completely mineralized as shown by the amount of nitrate detected, which achieved a maximum value corresponding to 30% of the initial nitrogen content of OFX (11.3 $mg \cdot L^{-1}$). This fact suggests that the remaining organic carbon contained a high amount of nitrogen in compounds such as quaternary amines which are species that are particularly refractory to ozonation (Muñoz and von Sonntag, 2000 and Nawrocki and Andrzejewski, 2011). OFX decay also led to the occurrence of other inorganic ion, fluoride, whose concentration reached a value corresponding to 100% initial fluorine in OFX.

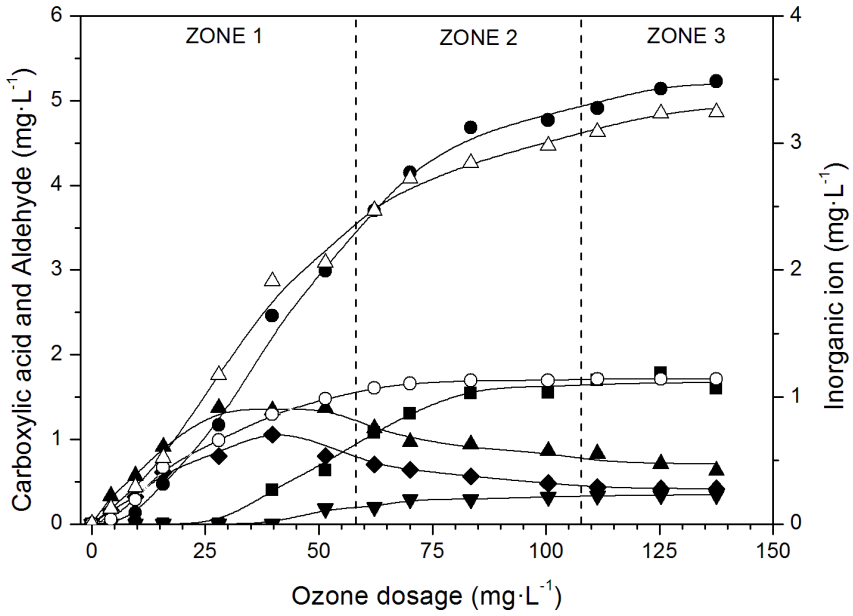


Fig. 5.4 Evolution of formaldehyde (♦), mesoxalic (■), oxalic (●), acetic (▼), formic acid (▲), nitrate (Δ) and fluoride (○) for different ozone dosages in synthetic water matrix.

3.3. Matrix effect

STP effluent showed an instantaneous ozone demand of 8.7 $mg \cdot L^{-1}$, in line with reported values for other wastewaters (Xu *et al.*, 2002 and Sharif *et al.*, 2012). The organic compounds (DOC = 8.4 $mg \cdot L^{-1}$) were mineralized at an extent of 20% at the upper operational condition, consuming 18 mg $O_3 \cdot L^{-1}$ (Fig. 5.5).

Fig. 5.6A represents the evolution of OFX, DOC and the concentration profiles for consumed and dissolved ozone during the ozonation of OFX in STP effluent. A new zone, denoted zone 0, was identified for ozone dosages lower than 16 $mg \cdot L^{-1}$. In it, OFX was only slightly oxidized, with a depletion of about 20%, whereas in synthetic water matrix for similar ozone exposures it reached 67%. On the contrary, DOC steeply decreased quickly achieving a mineralization degree of 16%. These data show the competition for ozone between the dissolved organic compounds of the water matrix and OFX, suggesting that ozone/hydroxyl radicals would preferably attack certain moieties in wastewater organic matter. This fact is in line with Katsoyiannis *et al.* (2011), who showed that kinetics of the reaction of ozone with DOC strongly affects the rate at which target compounds were transformed by ozone. Beyond this preliminary zone, a similar

profile synthetic matrix was observed. In zone 1, OFX was almost completely abated and DOC fell steadily down to 16 $mg \cdot L^{-1}$ (28%) with increasing ozone exposure up to 84 $mg \cdot L^{-1}$. In zone 2, between 84 to 124 $mg \cdot L^{-1}$, ozone consumption increased and the mineralization degree slightly rose from 28 to 33% (DOC = 15 $mg \cdot L^{-1}$). Further ozone dosage up to 124 mg $O_3 \cdot L^{-1}$, did not increase mineralization and the amount of ozone consumed remained constant at 64 $mg \cdot L^{-1}$. As a result, this value was considered the upper operational limit.

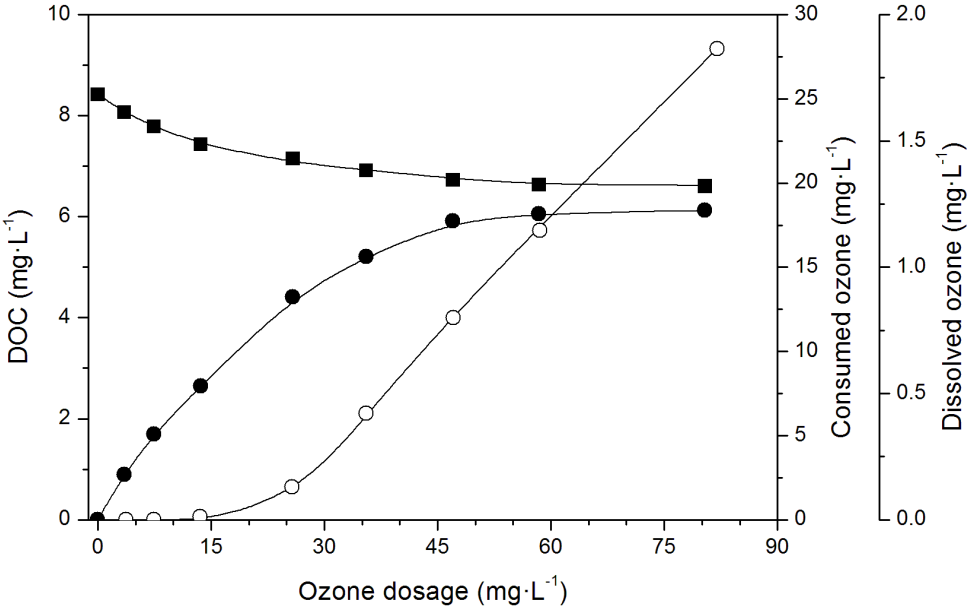


Fig. 5.5 Evolution of DOC (■), consumed (●) and dissolved ozone (○) at different ozone dosages in STP effluent.

The evolution of individual carboxylic acids and inorganic ions with ozone dosage is displayed in Fig. 5.6B. The pattern of organic acids was similar to that found in synthetic water matrix for zones 1–3. However, higher concentrations were detected at the upper operational limit due to the partial oxidation of wastewater organic matter (Liu *et al.*, 2012b). The nitrate concentration was significantly higher than that found in synthetic matrix, reasonably as a consequence of the oxidation of ammonium and nitrite present in the STP effluent. Nitrite reacts rapidly with ozone and is almost stoichiometrically oxidized to nitrate (von Sonntag and von Gunten, 2012). This reaction took place in zone 0 at low ozone dosage (Fig. 5.6B). Taking into account the nitrate from wastewater matrix, OFX nitrogen mineralization was around 25%, value close to

the observed in the synthetic water. Fluoride represented a value close to 100% of the fluoride in the structure of OFX and was not detected in zone 0 in which a low OFX depletion took place.

The total abatement of OFX was reached for an ozone dosage of 85 $mg \cdot L^{-1}$, which was considerably higher than that observed in the synthetic water matrix (60 $mg \cdot L^{-1}$).

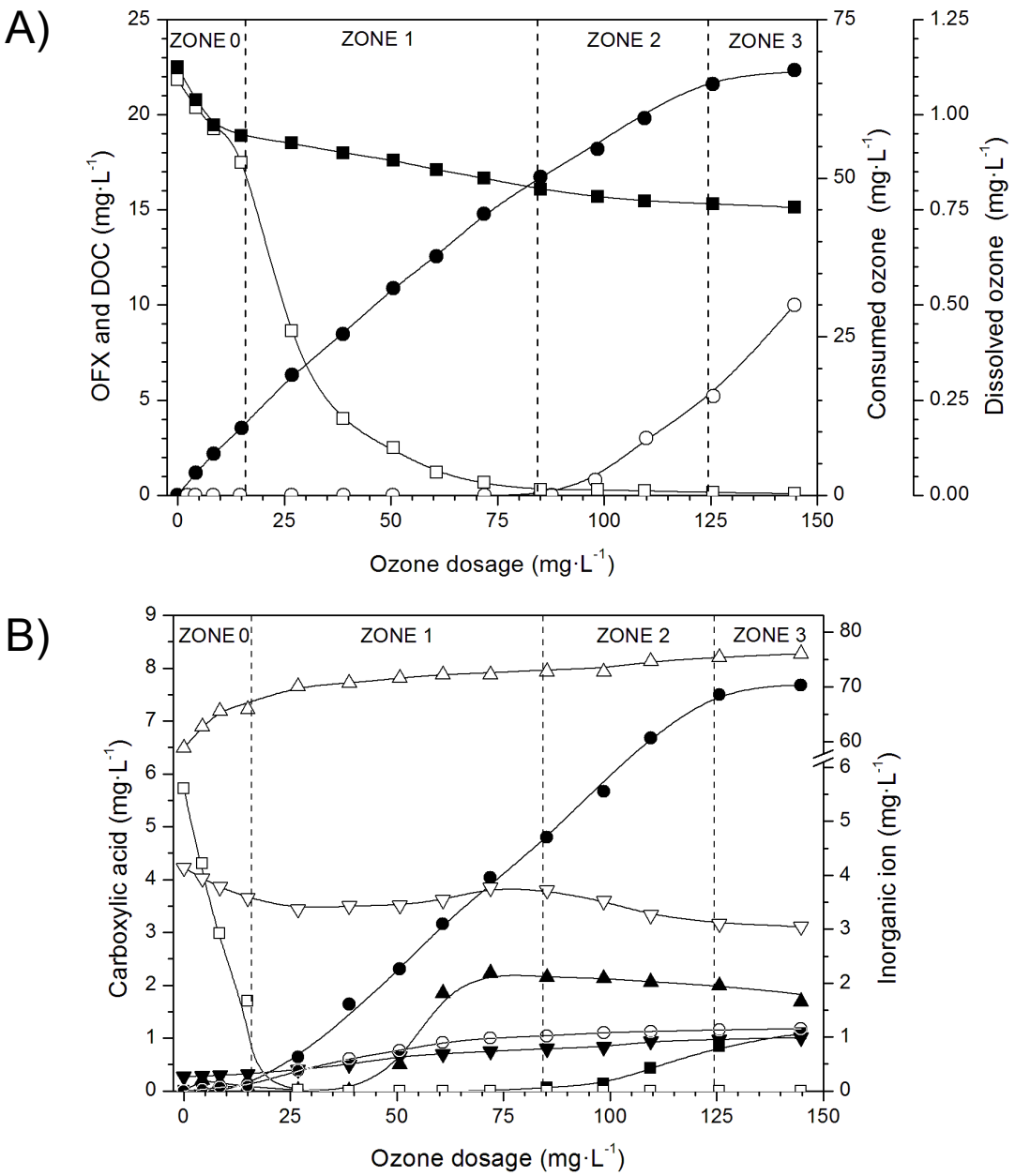


Fig. 5.6 Evolution of OFX (□), DOC (■), consumed (●) and dissolved (○) ozone (A) and mesoxalic (■), oxalic (●), acetic (▼), formic acid (▲), nitrate (Δ), nitrite (□), ammonium (▽) and fluoride (○) (B) for different ozone dosages in STP effluent.

The maximum ozone consumption was 64 $mg \cdot L^{-1}$, which was also higher than the value obtained in synthetic water (48 $mg \cdot L^{-1}$) and close to the sum of ozone consumed by the wastewater matrix, 18 $mg \cdot L^{-1}$ (Fig. 5.5), and that due to OFX abatement, 45 $mg \cdot L^{-1}$ (Fig. 5.1). The ozone dose in STP effluent was 2.95 mg $O_3 \cdot (mg\ OFX)^{-1}$, which was remarkably higher than that observed in synthetic water (2.15 mg $O_3 \cdot (mg\ OFX)^{-1}$). Fig. 5.7 displays the evolution of ozone consumption as a function of OFX removed in both matrices. In the synthetic water matrix, the amount of ozone consumed increased steadily with OFX removed (zone 1) with a sharp rise at the highest values as a consequence of the reactions of ozone with partial oxidized organic matter (*e.g.*, carboxylic acids), which are mainly occurred in zone 2. On the other hand, in the real wastewater matrix, the ozone consumed rose relatively quickly up to 12 mg $O_3 \cdot L^{-1}$, for low OFX abatement. Subsequently, the profile of consumed ozone of both water matrices runs almost in parallel. The ozone consumption gap between both matrices matches with the amount consumed by the STP effluent in the previously defined zone 0. In this preliminary zone, ozone is primarily consumed by reactions with the dissolved organic matter in wastewater (8.4 $mg \cdot L^{-1}$), part of which was easily oxidizable at low ozone dosages (Nöthe *et al.*, 2009), and the oxidation of reduced nitrogen species.

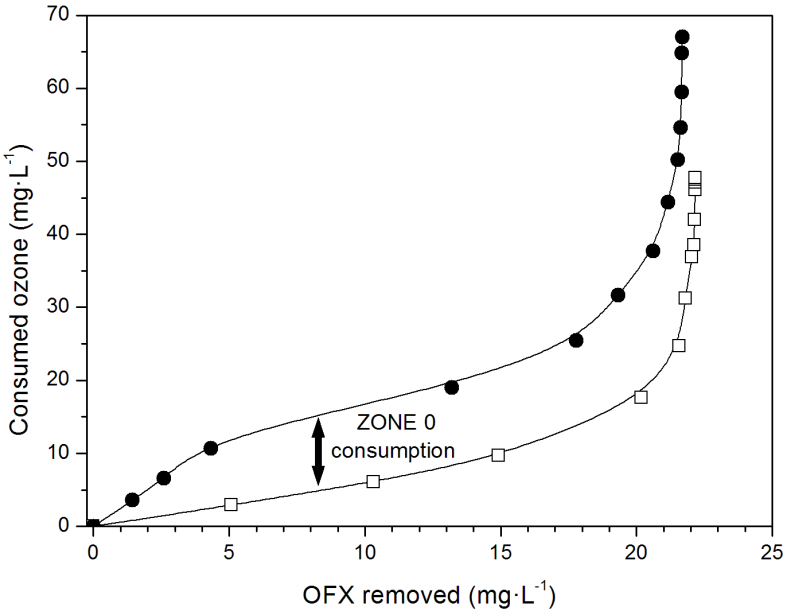


Fig. 5.7 Evolution of ozone consumption throughout OFX abatement in synthetic water matrix (□) and STP effluent (●).

Total OFX depletion did not lead to its full mineralization in the real matrix either, achieving a maximum DOC removal of 33%. Because OFX was not the only organic compound in the spiked STP effluent, and taking into account that the maximum amount of organic carbon mineralized in wastewater was 1.9 mg·L^{-1} (Fig. 5.5), the OFX mineralization degree for maximum ozone dosages was 24%, significantly less than the 41% obtained in synthetic water. These facts underline that OFX oxidation and mineralization degrees were not only influenced by the presence of naturally occurring radical scavengers (mainly carbonates and bicarbonates), but also by other inorganic and organic compounds which hamper its depletion and mineralization through indirect reactions (Nöthe *et al.*, 2009 and Katsoyiannis *et al.*, 2011).

3.4. Aquatic toxicity assessment

First, the toxicity of OFX on single species was evaluated by determining concentration-response curves (Fig. 5.8). The growth inhibition assay with *P. putida* test was the most sensitive with an EC_{50} value of 0.11 mg·L^{-1}. This is a consequence of the specific design of quinolone, which inhibits bacterial cell division (Sukul and Spiteller, 2007). *P. subcapitata* also presented a low EC_{50} value, 1.9 mg·L^{-1}, although microalgae are non-target organisms for the antibiotic. Nevertheless, it has been indicated that the presence of gyrase-like proteins makes algae sensitive to OFX and warns about the effect of quinolone on non-target organisms (Brain *et al.*, 2009). On the other hand, *T. thermophila* and *V. fischeri* have EC_{50} values >100 mg·L^{-1}. *T. thermophila*, an eukaryote, is not expected to be affected by antibiotics (Láng and Kőhidai, 2012), whereas *V. fischeri*, despite being a target organism, was not OFX sensitive due to the short incubation time of the bioassay (Backhaus *et al.*, 1997). The EC_{50} values were in good agreement with those previously reported for *V. fischeri*, *P. putida* and *P. subcapitata* (Alexy, 2003 and Isidori *et al.*, 2005). No prior data have been found for *T. thermophila*.

Fig. 5.9 displays the evolution of the toxicity of untreated and treated samples at different ozone exposures in the synthetic water matrix and STP effluent for the organisms of the bioassay battery. The aquatic toxicity of raw synthetic water

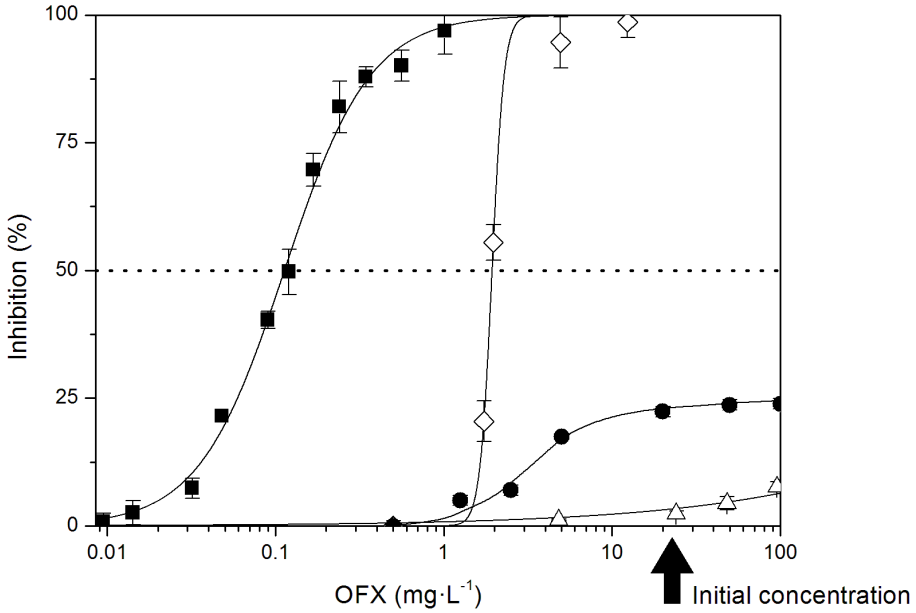


Fig. 5.8 Concentration-response curve of ofloxacin for *V. fischeri* (●), *P. putida* (■), *T. thermophila* (Δ) and *P. subcapitata* (◊) test (mean ± 95% confidence interval). Lines gives nonlinear-regression sigmoidal dose-response curve model fit and black arrow represents the initial OFX concentration in spiked waters (22 mg·L^{-1}).

(OFX = 22 mg·L^{-1}) displayed significant interspecies differences, which essentially correspond to the already described sensitivity to OFX. Accordingly, the growth of *P. putida* and *P. subcapitata* was severely inhibited as quinolone concentration was considerably higher than EC_{50} values. The lower effect on *V. fischeri* and *T. thermophila* was consistent with their lower sensitivity to OFX. A similar behaviour was observed for spiked STP effluent on all bioassays except for *T. thermophila*, whose toxicity was markedly higher. This fact is result of the toxicity of the STP effluent itself. In contrast, the wastewater matrix did not display noticeable toxicity for the rest of bioassays.

In the synthetic water matrix, the toxicity for *P. putida* and *P. subcapitata* was reduced with the increasing ozone dosage up to its total depletion. At the end of zone 1, aquatic toxicity for both microorganisms reached the same value of the non-spiked synthetic water. The toxic effects for *V. fischeri* increased with ozone exposure at low ozone dosage, whereas for *T. thermophila* no growth inhibition was observed in any case. The toxicity for *V. fischeri*, *P. putida* and *P. subcapitata* in STP effluent, decreased with increasing ozone dosage until the inhibition value of control sample was reached. The toxic effects for *T. thermophila* follow a similar trend to the non-spiked wastewater

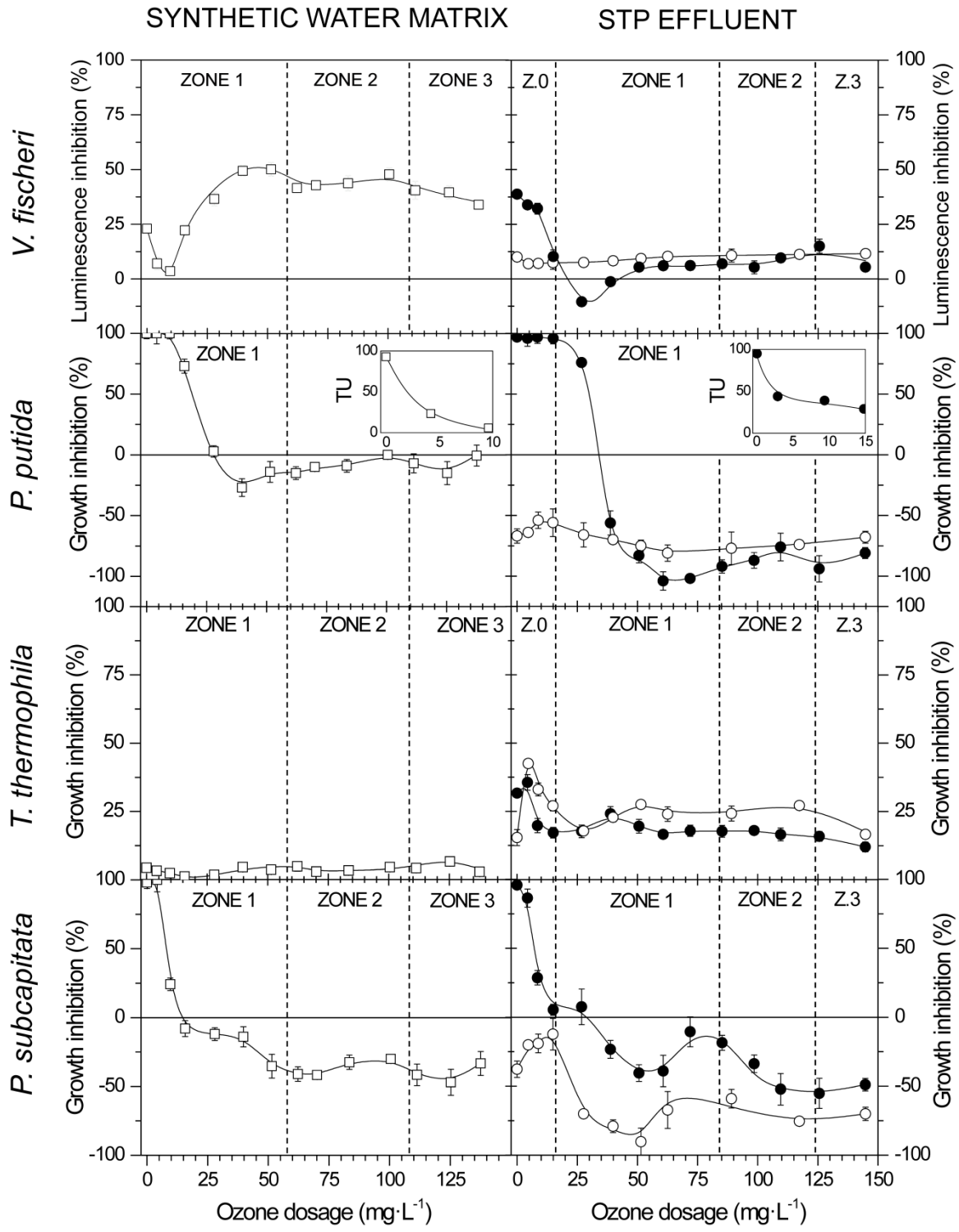


Fig. 5.9 Evolution of toxic effects of treated samples at different ozone dosages in OFX spiked synthetic matrix (□) and STP effluent (●), and non-spiked STP effluent (○) for the biotest battery. Inset plots represent the evolution of toxicity on *P. putida* as toxic units ($TU = 100/EC_{50}$) for samples with inhibition equal to 100%.

profile throughout all input ozone levels, suggesting that ozonated STP effluent appeared to be the main source of toxicity to the protozoan.

Aquatic toxicity and OFX concentration followed a similar profile with increasing ozone dosage, both being completely depleted at the end of zone 1. Toxicity decay in wastewater matrix required a higher amount of ozone with regards to the synthetic water matrix. In general, it can be observed that the toxicity did not significantly decay in zone 0 in the STP effluent where OFX depletion was slowed down by matrix effects. Fig. 5.10 shows a comparison between toxic effects of pure OFX dissolved in ultrapure water and that exerted by ozonated solutions of OFX in two different water matrices on the most sensitive organisms: *P. putida* and *P. subcapitata*. Despite treated water mixtures being notably less toxic than the single OFX, all profiles followed the same pattern. These data suggest that OFX is the main cause of aquatic toxicity and that the influence of ozonated by-products, especially those with potential biological activity (*i.e.*, TP_1-TP_6), was almost negligible. It is interesting to note that the generation of easily assimilable organic matter (Thayanukul *et al.*, 2013), bicarbonate (Luzhøft *et al.*, 1999) and/or extra amounts of nitrate and phosphate (Selivanovskaya *et al.*, 2004) are the most likely cause of the remarkable stimulation observed for *P. putida* and *P. subcapitata* growth.

Despite the toxic effects towards *V. fischeri* initially decline in parallel with remaining OFX concentration afterwards (Fig. 5.9), luminescence inhibition significantly increased at low ozone dosages (remaining OFX ≈ 11 $mg \cdot L^{-1}$). Particularly, a steep increase was observed in the synthetic water matrix, reaching 50% for an ozone dosage of 39 $mg \cdot L^{-1}$. Part of this toxicity enhancement could be attributed to the formation of formaldehyde, whose concentration in synthetic water reached 1.0 $mg \cdot L^{-1}$ for an ozone dosage of 39 $mg \cdot L^{-1}$. This value is close to the EC_{50} value of 8.4 $mg \cdot L^{-1}$ reported by Ricco *et al.* (2004). The occurrence of organic nitrogen compounds (1.85 $mg \cdot L^{-1}$ as organic nitrogen) could also represent a contribution to the total toxicity due to the high toxicity of some of them formed by the ozonation of the piperizinyl group (Calamari *et al.*, 1980). A similar toxicity trend was observed for *V. fischeri* in previous studies (Calza *et al.*, 2008, Vasquez *et al.*, 2013 and El Najjar *et al.*, 2013). Calza *et al.* (2008) also

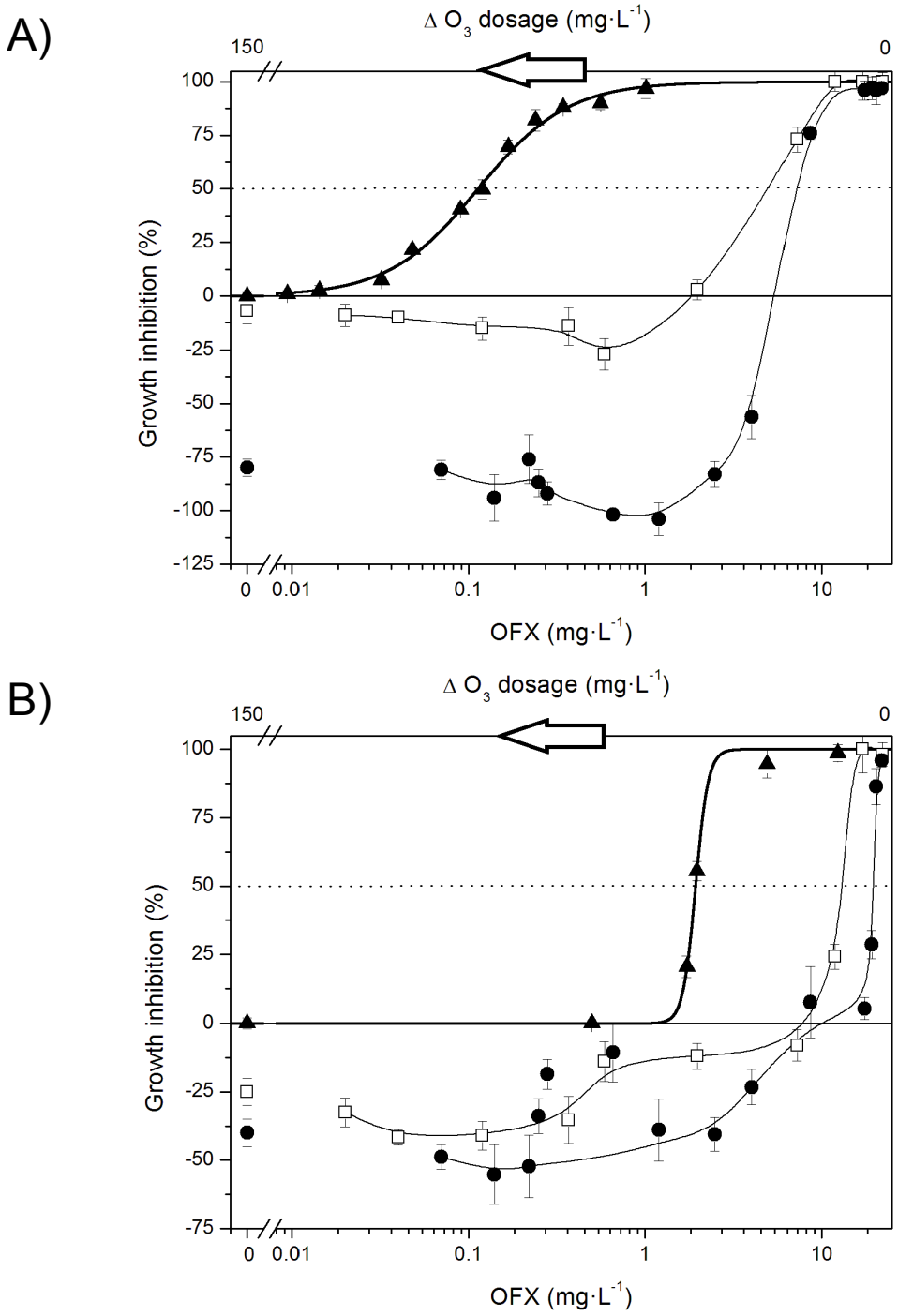


Fig. 5.10 Concentration-response curve of single OFX (▲) and evolution of the effects of treated water samples at different remaining OFX concentrations in ozonated synthetic matrix (□) and STP effluent (●) on *P. putida* (A) and *P. subcapitata* (B).

suggested that the increase in the luminescence inhibition during photocatalytic treatment of OFX was not due to the initial TPs but to secondary products, namely piperazine and its derivatives and other degradation products, not detected by LC/MS.

This fact highlighted the concern about the generation of secondary products with new and undesired new biological effects (von Sonntag and von Gunten, 2012). *V. fischeri* displayed the same toxicity pattern in the STP effluent although with lower toxicity, which is most likely due to the effect of the wastewater matrix. The presence of other chemicals in wastewater may interfere with the mechanisms of action of secondary products of OFX ozonation, minimizing the response or limiting the interaction with target bacterial receptors (Hernando *et al.*, 2007).

4. Conclusions

The continuous ozonation performances with short residence times attained total abatement of OFX in synthetic water and real STP effluent, but not totally mineralization is achieved. The water matrix has a strong influence on the ozone dose required for OFX removal and a given degree of mineralization.

The extent of mineralization was limited in both water matrices and a number of TPs appeared which suggested that reaction pathway include the oxidation of piperazinyl and quinolone moieties. The degradation of the initial TPs gave rise to the formation and accumulation of final by-products such as carboxyl acids, aldehydes, nitrogen-containing organic compounds and inorganic ions.

Although OFX is toxic both for target (*P. putida)* and non-target *(P. subcapitata)* organisms, ozonation completely removed its toxic effects. This fact implies that the generated by-products presented negligible toxicity.

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Chapter 6

Comparative toxicity assessment of an ozonated antibiotics mixture using single species and natural biofilm communities

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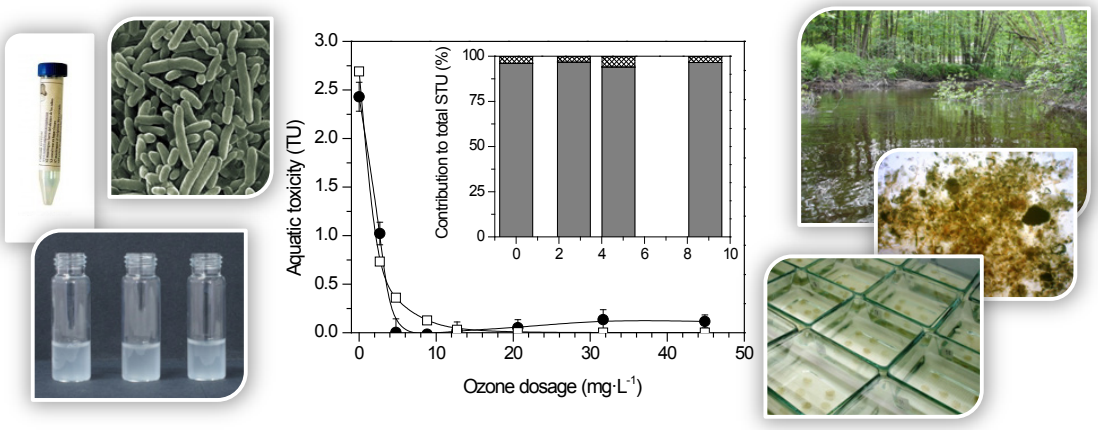
Science of the Total Environment (submitted)

Graphical Abstract



COMPARATIVE TOXICITY ASSESSMENT OF AN OZONATED ANTIBIOTIC MIXTURE USING SINGLE SPECIES AND NATURAL BIOFILM COMMUNITIES

Abstract

The continuous ozonation of a mixture of six antibiotics frequently detected in wastewater and surface water (doxycycline, erythromycin, metronidazole, ofloxacin, sulfamethoxazole and trimethoprim) has been performed in a sewage treatment plant (STP) effluent. The study aims to evaluate ozonation as tertiary wastewater treatment with particular emphasis on the aquatic toxicity of ozonated STP effluent. The antibiotics were significantly degraded (≥98%) at ozone dosage of 1.2 mg O_3·(mg DOC)$^{-1}$ except metronidazole, which required 4.3 mg O_3·(mg DOC)$^{-1}$ to achieve similar removal efficiency. The degradation of the antibiotics did not imply their complete mineralization (in all cases <30%) as a consequence of the accumulation of transformation products (TPs). In order to evaluate the toxic effects of TPs, aquatic toxicity assessment based on effect-driven approach was performed using the single species toxicity to the bacterium *Pseudomonas putida* and the alga *Pseudokirchneriella subcapitata* and the impact on natural biofilm communities, in which the effect on the heterotrophic and phototrophic part of a limnic periphyton was studied. The ozonation avoided the toxicity to the growth rate of *P. putida* and *P. subcapitata*, and to the metabolic activity of periphytic bacterial communities. The comparison between the evolution of toxic effects based on a whole mixture approach and antibiotic degradation profiles indicates that aquatic toxicity is caused predominantly by the parent compounds whereas the formation of TPs did not significantly contribute to the mixture toxicity. The prediction of aquatic toxicity of ozonated wastewater by component-based approach, primarily based on Concentration Addition (CA) concept, was slightly overprotective with a relatively small likelihood of overestimating the toxic effects of treated STP effluent. Ozonated wastewater samples induced changes on the bacterial structure of natural biofilm communities even at ozone dosages in which antibiotics were significantly removed.

1. Introduction

Large quantities of antibiotics are consumed by humans and animals to treat diseases and infections (Khetan and Collins, 2007). As a consequence of their poor metabolization and their incomplete removal in conventional sewage treatment plants (STPs), antibiotics are continuously discharged into the aquatic environment (Kümmerer, 2009a, Verlicchi *et al.*, 2012 and Michael *et al.*, 2013). Their occurrence in surface water is of concern as antibiotics could impair microbial ecology, increase the proliferation of antibiotic resistant pathogens, and pose threats to human health (Khetan and Collins, 2007, Segura *et al.*, 2009 and Rizzo *et al.*, 2014).

Ozonation of secondary STP effluents as an end-of-pipe polishing step is an effective process for the degradation of a wide range of micropollutants including antibiotics (Ikehata *et al.*, 2006, Michael *et al.*, 2013 and Hübner *et al.*, 2015). A further advantage of the ozonation is its disinfection potential, which is able to deactivate antibiotic resistance genes (ARGs) and antibiotic resistant bacteria (ARB), preventing the dissemination of antibiotic resistance (Dodd, 2012). Given these facts, ozonation is generally recognized as a suitable technology for the tertiary treatment of wastewater in order to reduce the contamination in the aquatic environment. However, the mineralization of micropollutants during ozonation is typically low and most target compounds are only transformed into more oxidized by-products (Hübner *et al.*, 2015). There is a growing concern on whether transformation products (TPs) keep or not the biological effects of the parent compounds (Dodd *et al.*, 2009), or whether or not new and undesired biological effects are developed (Li *et al*., 2008, Dodd *et al.*, 2010, Gómez-Ramos, *et al.*, 2011 and El Najjar *et al.*, 2013). Therefore, a thorough aquatic toxicological evaluation of treated wastewater is essential for the optimization of ozonation treatment (Escher and Fenner, 2011).

The most straightforward approach is to test ozonated STP effluent with the aim of providing an experimental estimation of its hazard (Petala *et al.*, 2008, Stalter *et al.*, 2010 and Magdeburg *et al.*, 2012). According to the effect-driven approach defined by Escher and Fenner (2011), the toxic effects should be compared to the concentration

profiles of parent compounds in order to assess the toxicity of TPs. If the decrease in toxicity parallels the decrease of concentration of parent compounds, the TPs are considered to be irrelevant and toxicity could be estimated based on the information of identified parent compounds only (*i.e.*, the toxicities of the individual antibiotics and their concentrations in the mixture) (Tang *et al.*, 2014). Component-based approach is a predictive approach that has been widely based on the mathematical concepts of Concentration Addition (CA) and Independent Action (IA) (Altenburger *et al.*, 2004 and Kortenkamp *et al.*, 2009). In the field of ecotoxicology, current scientific evidence seems to support the choice of CA as a first, pragmatic default approach for predicting the joint action of chemicals (Belden *et al.*, 2007, Kortenkamp *et al.*, 2009, Coors and Frische, 2011, Tang *et al.*, 2013 and Tang *et al.*, 2014).

In order to a achieve a comprehensive hazard assessment of treated effluents, biotests of species representative of the different trophic levels present in the receiving water body are required (Kortenkamp *et al.*, 2009 and Escher and Fenner, 2011). Standardized single-species tests are fast, simple to perform, cost-effective and reliable. However, they have significant shortcomings such as not taking into account the interaction among species, the use of species that are not indigenous to recipient streams, their genetically homogeneous population and the fact that test procedures are usually conducted under experimental conditions very different from those of the receiving water body (Proia *et al.*, 2013). Therefore, aquatic toxicity assessment should also be monitored using natural communities in order to complement the toxicological information obtained with single-species tests and provide a better indication of the effects of effluents on exposed ecosystems (Geiszinger *et al.*, 2009).

Periphyton is an aquatic biofilm-forming community that comprises a broad range of heterotrophic (*e.g.*, bacteria, protozoa and fungi) and autotrophic species (*e.g.*, green algae, diatoms and cyanobacteria) embedded in an extracellular polymeric matrix. They develop on submerged surfaces and constitute the major component for the uptake, storage and cycling of carbon, nutrients (Pusch *et al.*, 1998 and Battin *et al.*, 1999) and anthropogenic contaminants in many river sections (Sabater *et al.*, 2007). The mutual benefits and the close spatial relationships between organisms with distinct life-

strategies closely displays the quality of the surrounding flowing water, generating a complex micro-ecosystems in which specific metabolic process and interactions may occur (Proia *et al.*, 2013). Species-dependent changes in ecological fitness due to exposure to toxic compounds do not only change the overall physiological activity of the biofilm species, but also affect its biodiversity (Blanck, 2002).

The study assesses ozonation as technology for tertiary treatment of wastewater spiked with six antibiotics frequently detected in STP effluents. The aquatic toxicological assessment was based on the effect-driven approach performed with two levels of biological complexity: single species (the bacterium *Pseudomonas putida* and the alga *Pseudokirchneriella subcapitata*) and natural biofilm communities. The predictive power of component-based approach, primarily based on CA concept, was studied in order to provide reliable estimates of the toxicity of treated STP effluents.

2. Materials and methods

2.1. Materials

The antibiotics used in this study belong to different classes and were selected based on their occurrence in wastewater and freshwater (see Table 4.1 and Supplementary data of Chapter 4). The following six antibiotics were selected: doxycycline (DXY), erythromycin (ERY), ofloxacin (OFX), and trimethoprim (TMP) purchased from Sigma-Aldrich, and sulfamethoxazole (SMX) and metronidazole (MNZ) purchased from Fluka. Wastewater was collected from the effluent of the secondary clarifier of a STP located in Alcalá de Henares (Spain). The plant treats domestic wastewater with a minor contribution of industrial effluents from facilities located near the city (374 000 population equivalent) and has a nominal capacity of 3 000 $m^3 \cdot h^{-1}$. Details on STP effluent characterization are included in Table 6.1. Wastewater was spiked with the selected antibiotics before ozonation runs (DXY: 42 $\mu g \cdot L^{-1}$; ERY: 491 $\mu g \cdot L^{-1}$; MNZ: 117 $\mu g \cdot L^{-1}$; OFX: 2 920 $\mu g \cdot L^{-1}$; SMX: 320 $\mu g \cdot L^{-1}$; TMP: 140 $\mu g \cdot L^{-1}$), keeping constant the mixture ratio according to their maximum detected values in an effluent of Alcalá STP (Rosal *et al.*, 2010).

Table 6.1 Main physico-chemical parameters of STP effluent.

pH	7.84	Na^+ ($mg \cdot L^{-1}$)	70.6
Conductivity ($\mu S \cdot cm^{-1}$)	763	NH_4^+ ($mg \cdot L^{-1}$)	2.28
TSS ($mg \cdot L^{-1}$)	4.22	K^+ ($mg \cdot L^{-1}$)	16.1
Turbidity (NTU)	3.2	Mg^{2+} ($mg \cdot L^{-1}$)	14.5
COD ($mg \cdot L^{-1}$)	24.5	Ca^{2+} ($mg \cdot L^{-1}$)	43.7
DOC ($mg \cdot L^{-1}$)	8.25	Cl^- ($mg \cdot L^{-1}$)	98.1
BOD_5 ($mg \cdot L^{-1}$)	3.00	NO_2^- ($mg \cdot L^{-1}$)	1.89
BOD_5/COD	0.12	NO_3^- ($mg \cdot L^{-1}$)	41.5
$SUVA_{254}$* ($L \cdot mg\ C^{-1}\ m^{-1}$)	2.36	PO_4^{3-} ($mg \cdot L^{-1}$)	1.74
Alkalinity (mg $CaCO_3 \cdot L^{-1}$)	112	SO_4^{2-} ($mg \cdot L^{-1}$)	62.2
Cr ($\mu g \cdot L^{-1}$)	0.36	Doxycycline ($ng \cdot L^{-1}$)	<LOQ
Ni ($\mu g \cdot L^{-1}$)	12.2	Erythromycin ($ng \cdot L^{-1}$)	670
Cu ($\mu g \cdot L^{-1}$)	11.9	Metronidazole ($ng \cdot L^{-1}$)	330
Zn ($\mu g \cdot L^{-1}$)	62.7	Ofloxacin ($ng \cdot L^{-1}$)	4 700
As ($\mu g \cdot L^{-1}$)	9.16	Sulfamethoxazole ($ng \cdot L^{-1}$)	670
Se ($\mu g \cdot L^{-1}$)	0.29	Trimethoprim ($ng \cdot L^{-1}$)	430
Cd ($\mu g \cdot L^{-1}$)	ND		
Sn ($\mu g \cdot L^{-1}$)	3.91		
Hg ($\mu g \cdot L^{-1}$)	ND		
Pb ($\mu g \cdot L^{-1}$)	ND		

*Specific ultraviolet absorption at 254 nm;
LOQ: limit of quantification
ND: not detected

2.2. Ozonation process

The experiments were carried out in a cylindrical reactor with a total working volume of 1.44 L (internal diameter of 6.0 cm and working height of 51 cm), which operated in continuous co-current mode. The retention time distribution curve yielded an average liquid retention time of 10.3 min. The reactor modelling using the continuous stirred tank reactor (CSTR) in series model determined an equivalent value of 1.13 tanks, indicating that the bubble column can reasonably approach a perfect CSTR (Asenjo and Merchuck, 1995). Water and gas flow rates were 142 and 390 $mL \cdot min^{-1}$ respectively, with different inlet ozone concentrations. During the runs, the inlet ozone dosage was stepwise increased from 2.5 to 50 milligrams of ozone per liter of wastewater ($mg \cdot L^{-1}$). For the different ozone dosages, samples were withdrawn for analysis at the column outlet once the stationary state was reached. This was ensured by circulating four times the hydraulic retention volume after constant ozone concentration was obtained both in

the liquid and gas phases at the column outlet. Details are given elsewhere in Chapter 3 (section 2.2).

2.3. Analytical methods

A solid phase extraction (SPE) procedure was applied for simultaneous clean-up and/or concentration of studied antibiotics from wastewater. SPE was performed using commercial Oasis^{TM} HLB (divinylbenzene/N-vinylpyrrolidone copolymer) cartridges (200 mg, 6 cm^3) from Waters (Mildford, MA, USA). The method is described in Gómez *et al*. (2006) and was extensively used for the determination of a wide range of pharmaceutical compounds in waters offering high rates of recovery for the studied antibiotics (Gómez *et al.*, 2007, Gros *et al.*, 2009 and Martínez-Bueno *et al.*, 2010).

All extract samples were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using a triple-stage quadrupole mass spectrometer (TSQ Quantum; Thermo Scientific Inc., San José, CA, USA), working in selected reaction monitoring (SRM) mode with positive electrospray ionization (ESI). The separation of the analytes was performed using an HPLC (Agilent 1200 Series; Agilent Technologies, Palo Alto, CA, USA) equipped with a reversed-phase C-18 analytical column (Agilent Eclipse XDB, 150 × 4.6 mm, 5 µm). Acetonitrile (mobile phase [A]) and HPLC-grade water (mobile phase [B]) with 0.1% formic acid were used as mobile phase at a flow rate of 0.4 $mL \cdot min^{-1}$ for gradient elution (gradient curve: 0–1 min, 10% [A]; 1–23 min, linear change from 10 to 95% [A]; 23–29 min, 95% [A]; 29–30 min, linear change from 95 to 10% [A]; post run-time, 30–35 min). Mass spectra of the column elutes were recorded in MS/MS using the following conditions: the spray needle voltage was 3.0 kV, heated capillary temperature 350ºC, sheath gas pressure 40, and auxiliary gas setting 2. Both the sheath gas and auxiliary gas used were nitrogen. The collision gas was argon at a pressure of 200 Pa for all studies. Quantitative analyses were done in SRM mode. The confirmation of each antibiotic was performed by means of two SRM transitions at the correct retention time and by monitoring the SRM ratio (Table 6.2), in accordance with EU guidelines for LC-MS/MS analysis (Commission Decision, 2002/657/EC). All

determinations were performed in duplicate. Data acquisition and processing were carried out using Thermo Xcalibur software (v. 2.2, Thermo Fisher Scientific Inc., San José, CA, USA).

Table 6.2 Values of the parameters optimized with the developed method by LC-TQS-MS/MS to antibiotic analysis.*

Antibiotic	Rt (min)	Precursor ion (m/z)	SRM1	CE1	SRM2	CE2	SRM3	CE3	[SRM2]/[SRM1] (%RSD)
Doxycycline	13.2	445	428	18	201	38	267	35	0.05
Erythromycin	14.1	734	158	30	576	17	558	18	0.7
Metronidazole	9.1	172	128	14	82	24	111	24	0.4
Ofloxacin	10.8	362	318	18	261	26	205	41	0.9
Sulfamethoxazole	15.2	254	108	24	156	16	92	28	1.0
Trimethoprim	10.6	291	230	23	123	25	110	33	0.5

* Rt: retention time; CE: collision energy (eV); SRM 1: quantitation; SRM 2–3: confirmation.

Dissolved Organic Carbon (DOC) was determined using a TOC-V_{CSH} Shimadzu TOC analyzer. Oxalic and mesoxalic acid concentration were measured by IonPac AS9-HC analytical column (4 × 250 mm) with ASRS-Ultra suppressor, whereas acetic and formic acid concentrations were determined using an IonPac ICE analytical column (9 × 250 mm) with AMMS-ICE II suppressor.

2.4. Procedures for aquatic toxicity tests

Aquatic toxicity assessment of wastewater samples was performed by means of tests with two levels of biological complexity: single species and natural biofilm communities. Taking into account the mean dilution factor of the STP effluent in the recipient river (Henares River; mean annual flow rate of 10.7 $m^3 \cdot s^{-1}$, CEDEX, 2015), wastewater samples were 10 times diluted in the test medium of each bioassay.

2.4.1. Single-species tests

P. putida test determines the inhibitory effect of a substance on the bacteria (*P. putida*, NCIB 9494 from CECT, Spain) by means of cell growth inhibition. The bioassay was performed according to the ISO guideline 10712 (ISO, 1995). Bacterial cultures were exposed to test solutions (10% of raw or ozone treated wastewater in test medium) at 23 ± 1ºC for 16 h in 10 mL glass incubation vials, which were constantly shaken in the dark. Cell growth was determined by optical density (λ 600 nm) in 96-well clear polypropylene microplate using a Rayto RT-2100C microplate reader. Algal growth inhibition test was performed following the procedure described in the European Guideline OECD TG (Guideline) 201, using *P. subcapitata* open system (OECD, 2011). The algal stock culture for inoculation was taken from the commercial test system Algaltoxkit F^{TM} (MicroBioTest Inc., Belgium). The cells of *P. subcapitata* were exposed to water samples (10% of raw or ozonated wastewater in test medium) at 23 ± 1ºC for 72 h in 10 mL glass incubation vials which were constantly shaken and illuminated in a chamber (~100 µmol foton·m^{-2}·s^{-1}). Algal biomass was measured daily using chlorophyll-a content, the extraction of which was carried out as follows: 50 µL samples of cultures were transferred to a 96-well black polypropylene microplate, 200 µL of ethanol was added to each well and the plate was shaken for 3 h in the dark. Thereafter, the fluorescence was measured using a Fluoroskan Ascent FL microplate fluorometer (Excitation 450 nm, Emission 672 nm) from Thermo Scientific.

3,5-dichlorophenol for *P. putida* and potassium dichromate for *P. subcapitata* were used as reference substances in order to check test procedures. Three independent experiments with duplicate samples were carried out to ensure reproducibility. All aquatic toxicity data are expressed as mean ± 95% confidence interval and data analysis was carried out using a nonlinear-regression sigmoidal concentration-response curve model provided in the GraphPad Prism software (v. 6.0, GraphPad Inc., San Diego, USA).

2.4.2. Natural biofilm community assay

Tests with natural biofilm communities were performed according to a slightly modified version of the semi-static SWIFT periphyton test, described by Porsbring *et al.* (2007). Biofilms were sampled in Mölndalsån (N 57º 40´ 59´´ E 12º 13´ 7´´), a small river near Gothenburg (Sweden), which is neither recipient of STP effluent nor subjected to run offs from agricultural areas, and is hence considered free from antibiotic contamination. Biofilms were established on submerged glass discs (1.5 cm^2) that were mounted on polyethylene racks (Blanck and Wangberg, 1988) over seven days at an approximate depth of 0.5 m and then transferred to the laboratory.

Eight colonized discs were placed in glass beaker (10 × 15 × 5 cm), then adding 200 mL test solution. Test solution is composed by 10% of raw or ozone treated wastewater in filtered Mölndalsån river water (GF/F, Whatman, pore size 0.7 µm) amended with nutrients (Z8 medium, Scandinavian Culture Collection for Algae and Protozoa). Periphyton communities were incubated under constantly shaken in a thermo constant room at river temperature (16–17ºC) with a day-light cycle illumination (16 h light of ∼125 µmol foton·m^{-2}·s^{-1}, 8 h darkness).

In order to assess effects on bacteria, three glass discs were sampled after 72 h from each test vessel. The discs were transferred to glass scintillation vials containing 20 mL test solutions. Scintillation vials were sonicated three times over 15 seconds followed by vigorous shaking over 15 seconds with the aim of detaching the periphyton biolfim from the discs. Afterwards, the suspension was filtered through sterile paper (Kimcare, Kimberly-Clark Professional) into sterile plastic Petri dish. 150 µL of filtered suspension was pipetted into each well of a Biolog EcoPlates™ (from now on only referred to as EcoPlates) which was purchased from Dorte Egelung ApS, Roskilde (Denmark). These 96-well plates, pre-loaded with 31 different carbon-sources and a tetrazolium dye (Table 4.4 in Chapter 4), provide information on total metabolic activity and functional diversity of the bacteria growing in them. Optical densities were measured over 96 h (24, 42, 48, 66, 72, 86 and 96 h) at 595 nm (absorbance of the

oxidized tetrazolium dye) and 700 nm (in order to correct for turbidity) using a microplate spectrophotometer (μ Quant™, Bio-Tek Instruments).

The recorded OD was corrected first for turbidity by subtracting the absorbance at 700 nm from the absorbance at 595 nm for each well. The resulting OD was subsequently corrected for any unspecific colour formation by subtracting the median absorbance of the three wells without any added carbon source per plate (blanks) to yield the final correct absorbance for each carbon source (OD_{corr}) and exposure time. Negative values for OD_{corr} were set to zero for the following calculations. Average well colour (AWC) was then determined for each plate and exposure time by calculating the arithmetic mean of the OD_{corr} of carbon source wells. The inhibition of AWC for each treatment was finally calculated in relation to the average AWC of the control plates as an indicator of general metabolic activity response of the whole bacterial communities. For this purpose, the data recorded after 66 h incubation was selected because evident colour development was visible in most wells, but still not exceeding the linear detection range.

Curves describing the bacterial activity (colour development) of each carbon source over incubation time from 0 to 96 h were determined by fitting a Weilbull model as follows:

$$OD_{corr} = 1 - \exp\left(-\exp\left(\theta_1 + \theta_2 * log_{10}(time)\right)\right) \quad (6.1)$$

Then, area under the curve (AUC) was measured using the classical simplex method as indicator of the bacterial community structure. The ordination of the data was done via nonmetric multidimensional scaling (nMDS), an ordering method that reduces the multidimensional data structure into a 2-dimensional plot in which the distances between individual samples reflect the multivariate dissimilarity between them (Clarke and Warwick, 2001). Manhattan Distances (City-Block Metric) (Eq. 6.2) between all pairs of samples j, k were used as input data for a similarity matrix. Calculations were implemented using PROXSCAL algorithm in SPSS software (v. 22, IBM SPSS).

$$MD = \sum_{i=2}^{31} \left| OD_{corr\,j,i} - OD_{corr\,k,i} \right| \quad (6.2)$$

Total content and relative fractions of photosynthetic pigments were used as a measure of algal biomass and community structure. Five discs glass were collected after 96 h of incubation and were transferred into scintillation vials containing 2 mL of ice-cold extraction media (30% methanol, 30% acetone, 30% dimethyl sulfoxide (all HPLC grade) and 10% of ultrapure water). The samples were shielded from light and stored -18ºC until analysis. Prior to analyses, scintillation vials were sonicated over 15 seconds followed by vigorous shaking over 15 seconds and finally the samples were filtered through 0.45 µm filters into HPLC vials.

Pigments were determined by means of a HPLC (ThermoQuest, Thermo Scientific) equipped with a C18-column (Genete Kinetex™, 150 × 3.0 mm, 2.6 µm). Analytes were separated using a gradient following the method described by Porsbring *et al.* (2007) (Table 6.3) and detected with a diode array detector (TSP UV6000LP) at 436 nm. The chromatograms were finally analyzed using LaChrome software (v. 4.0, Thermo Finnigan, Thermo Fisher Scientific). Eleven pigments were detected and four of them were identified: fucoxanthin, diadinoxanthin, chlorophyll-a and β-carotene. The effects on pigment content were expressed as percent inhibition compared to the arithmetic mean of controls an indicator of general response of the whole algal community.

Table 6.3 Mobile phase gradient used in HPLC pigment analysis. [A] methanol:ammonium acetate buffer 85:15 (buffer 0.5 M ammonium acetate pH 7.2); [B] acetonitrile:water 90:10; [C] ethyl acetate.

Time (min)	A (%)	B (%)	C(%)
0	100	0	0
8	0	100	0
8.6	0	90	10
13.1	0	65	35
21	0	31	69
25	0	31	69
27	0	100	0
28	100	0	0
30	100	0	0

2.5. Data treatment for assessing the aquatic toxicity

Component-based approach estimates the expectable total toxicity of a mixture of selected pure compounds in terms of their individual effects (Kortenkamp *et al.*, 2009). Aquatic toxicity was calculated as the sum of toxic units (STUs) of single antibiotics within each scenario following the strategy proposed by Backhaus and Faust (2012):

$$STUs = \sum_{i=1}^{n} \left(\frac{EnvConc_i}{EC_{50_i}} \right) \quad (6.3)$$

where $EnvConc_i$ are the actual concentrations of the individual substances in a mixture and EC_{50_i} denote the concentration of these substances that cause 50% inhibition if present singly. The quotients $EnvConc_i/EC_{50_i}$ are termed Toxic Units (TUs), which were calculated for each ozone dosage using the remaining concentration of antibiotics in wastewater and their single EC_{50} values (Table 6.4). This strategy is primarily based on the classical mixture toxicity concept of CA, which assumes similar mode or mechanisms of action for all toxicants. However, the antibiotics comprising the mixture have different mechanisms of action and consequently, it could be argued that the competing Independent Action (IA) concept should be the more appropriate model.

Table 6.4 EC_{50} values of studied antibiotic for single species and natural biofilm communities. Values are expressed in µg·L^{-1}.

	Single species		Natural biofilm communities	
	P. putida	*P. subcapitatata*	Bacterial	Algal
Doxycycline	40[a]	62[e]	94[a]	445[a]
Erythromycin	54 500[b]	20[f]	>7 340[a]	>7 340[a]
Metronidazole	>800 000[b]	39 100[g]	>1 710[a]	>1 710[a]
Ofloxacin	113[c]	1 440[f]	117[a]	>3 040[a]
Sulfamethoxazole	12 700[d]	146[h]	2 520[a]	>2 530[a]
Trimethoprim	75 500[d]	40 000[i]	>2 900[a]	>2 900[a]

[a] Present study; [b] Alexy, 2003; [c] Carbajo *et al.*, 2015; [d] Akhyany, 2013; [e] Suda *et al.*, 2012; [f] Isidori *et al.*, 2005; [g] Lanzky and Halling-Sørensen, 1997; [h] Ferrari *et al.*, 2004 ; [i] Yang *et al.*, 2008.

The acceptability of the component-based approach might depend on the quantitative error that can possibly occur when using CA for mixtures in which the components are not strictly similarly acting. Under the assumption of no interaction between the mixture components, this error may equal the quantitative difference between CA and IA predictions in accordance with Junghans *et al.* (2006):

$$\frac{EC_{50}^{IA}}{EC_{50}^{CA}} \leq \frac{\sum_{i=1}^{n} \frac{c_i}{EC_{50_i}}}{\underset{i \in (1 \ldots n)}{max} \left(\frac{c_i}{EC_{50_i}}\right)} \tag{6.4}$$

Under these circumstances a maximum possible ratio by which CA may predict higher mixture toxicity than IA equals the number of mixture components (n). Given the uncertainty of the hazard and exposure estimates of the individual antibiotics (*i.e.*, quality, quantity and spread of the individual toxicity data and the expectable fluctuations of the concentration of individual antibiotics), a maximum possible ratio of less than 2 might be considered acceptable (Backhaus and Karlsson, 2014).

In order to allow comparing predictive and experimental toxicity data, the toxic-effects obtained from whole mixture approach were transformed into TUs, which can also be defined as the reciprocal of the wastewater dilution (expressed in percentage) need to achieve 50% inhibition (EC_{50}) (Sprague and Ramsay, 1965):

$$TU = \frac{100}{EC_{50}} \tag{6.5}$$

TUs of non-diluted samples whose effect percentage observed was higher than controls but below 50% (<1 TU) were estimated using the approach proposed by Persoone *et al.* (2003) ($TU = inh/50$, in which inh is the percentage of inhibition).

3. Results and discussion

3.1. Ozonation

The continuous ozonation process of the spiked STP effluent was studied using different ozone dosages in order to achieve the maximum degradation degree for the selected antibiotics. Fig. 6.1 represents the evolution of each antibiotic as a function of the amount of ozone supplied during the ozonation. Antibiotics declined with ozone dosage up to achieve a significant abatement for each target pollutant (≥98%). DXY, ERY, TMP and SMX were totally depleted (*i.e.*, concentrations below quantification limit). Nonetheless, the ozone dosage required for depletion was different for MNZ than for the other antibiotics. DXY, ERY, OFX, TMP and SMX were removed ≥98% for ozone exposure of 13 $mg \cdot L^{-1}$ (1.2 mg $O_3 \cdot (mg\ DOC)^{-1}$), whereas MNZ required more than threefold ozone (46 $mg \cdot L^{-1}$, that is 4.3 mg $O_3 \cdot (mg\ DOC)^{-1}$) to reach similar degradation efficiency. These results are consistent with the direct ozonation rate constant of each antibiotic: MNZ reacts with ozone two orders of magnitude slower ($3.1\ 10^3\ M^{-1} \cdot s^{-1}$ in Rosal *et al.*, 2010) than the rest of the studied antibiotics ($\geq 10^5\ M^{-1} \cdot s^{-1}$ in Huber *et al.*, 2003, Dodd *et al.,* 2006, Rivas *et al.*, 2010, Huang, 2011 and Márquez *et al.*, 2013).

The evolution of DOC, $SUVA_{254}$ and carboxylic acids was also monitored during ozonation as shown in Fig. 6.2. DOC decreased steadily with increasing ozone supply, achieving a mineralization of 13% for 32 mg $O_3 \cdot L^{-1}$. An additional ozone exposure of 13 $mg \cdot L^{-1}$ more led only to an extra 2% organic carbon depletion. Despite the limited extent of mineralization (15%), partial oxidation reactions of aromatic compounds took place as indicated by the strong reduction of 65% in $SUVA_{254}$ (*i.e.*, the specific UV absorbance of the effluent at 254 nm). It is well known that ozone has the ability to cleave ultraviolet absorbing moieties in organic molecules (Wert *et al.*, 2009). The reaction of ozone with unsaturated bonds or aromatic rings leads to oxygenated saturated functional groups, such as aldehydic, ketonic and especially carboxylic groups (van Geluwe *et al.* 2011). In fact, the concentration of carboxylic acids (mesoxalic, oxalic, acetic and formic acids) increased with ozone dosage up to 3.6 mg $C \cdot L^{-1}$, which corresponds to 40% of the remaining DOC. Oxalic acid, formed mainly by the destruction

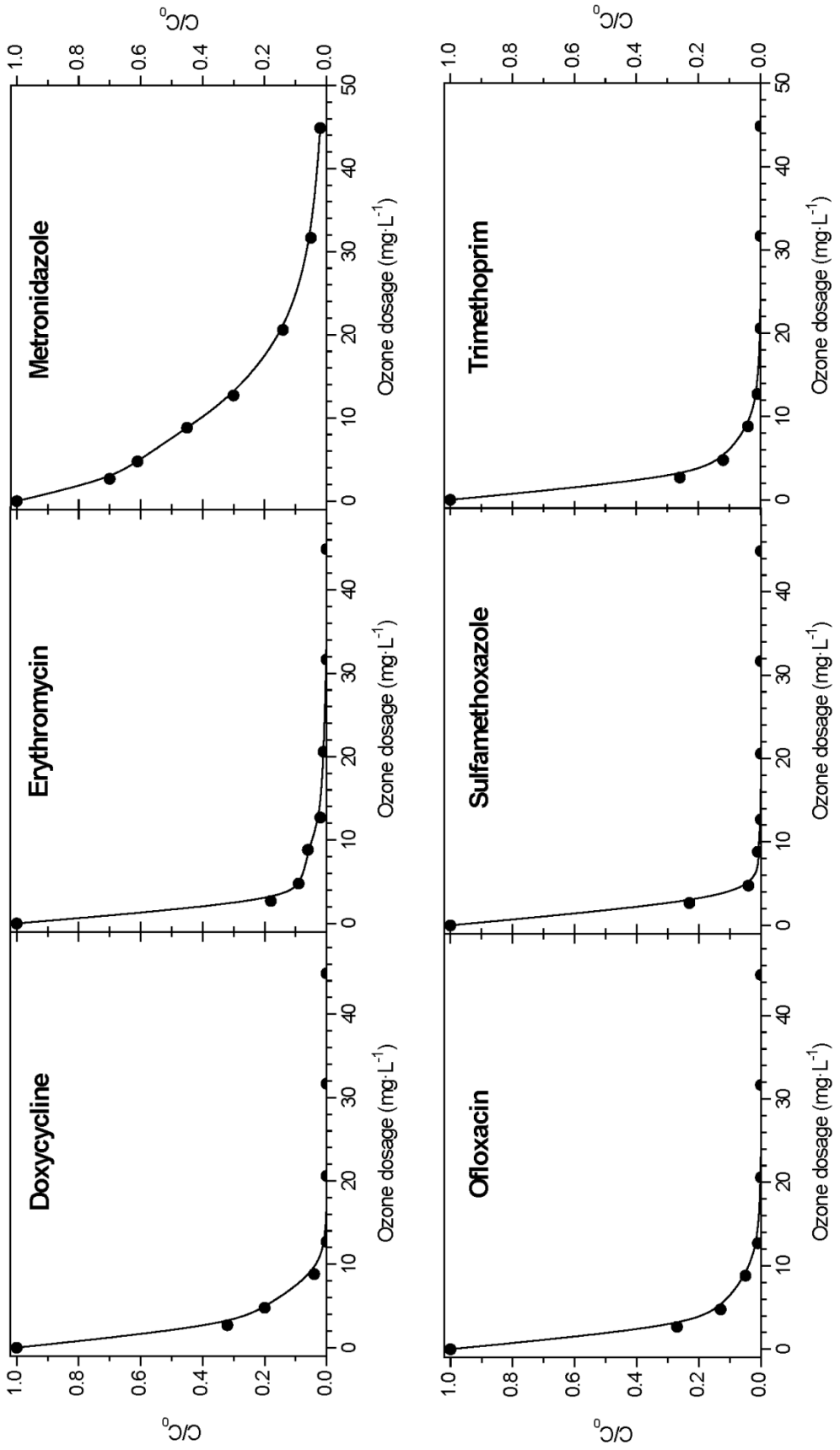


Fig. 6.1 Evolution of dimensionless antibiotic concentration (C/C_0) for different ozone dosages.

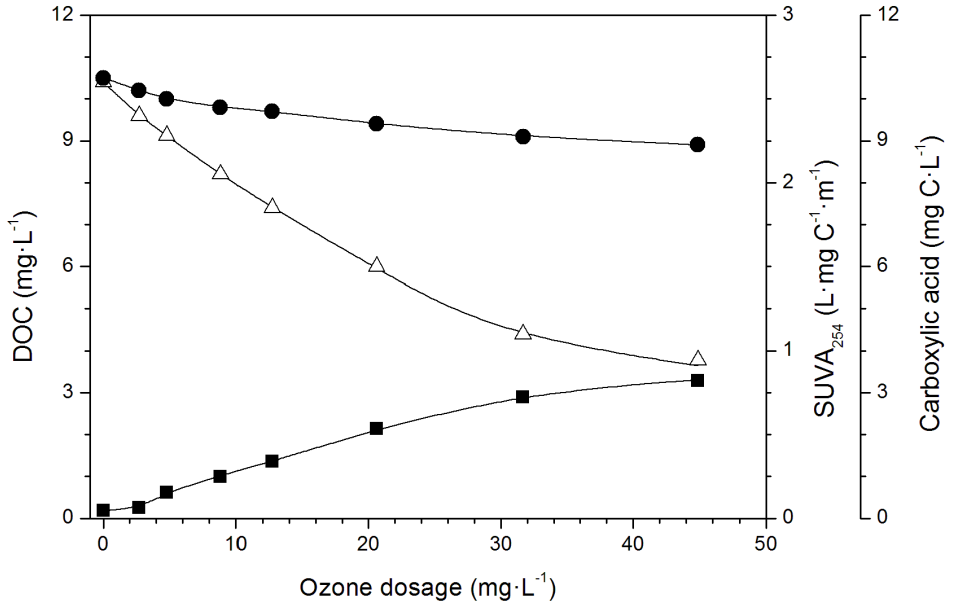


Fig. 6.2 Evolution of DOC (●), $SUVA_{254}$ (Δ) and carboxylic acids (■, sum of mesoxalic, oxalic, acetic and formic acids) for different ozone dosages.

of aromatic rings by ozone (van Geluwe *et al.* 2011), was by far the predominant carboxylic acid (maximum concentration of oxalic acid: 7.6 $mg \cdot L^{-1}$). Due to the fact that the spiked antibiotics were not the only organic compounds in the STP effluent, and taking into account that the maximum amount of organic carbon mineralized in raw wastewater was 1 $mg \cdot L^{-1}$, the highest mineralization degree of the antibiotic mixture could be estimated as 26%. This value represented close to twice the overall mineralization extent of the effluent. Mineralization is not a single chemical process and represents a series of reactions that are slow for highly oxidized molecules such as carboxylic acids (Rosal *et al.*, 2008 and Petre *et al.*, 2015). It has been stated that the mineralization that takes place during ozonation is mainly due to the reaction of high molecular weight compounds rather than to the depletion of the lighter carboxylic acids (van Geluwe *et al.*, 2011). This fact is in good agreement with the well-known refractory character of the ozone by-products detected and the reason why their concentrations increased in the reaction mixture (von Sonntag and von Gunten, 2012 and Hübner *et al.*, 2015).

3.2. Aquatic toxicity assessment

3.2.1. Single-species tests

The results of aquatic toxic tests for *P. putida* and *P. subcapitata* in spiked STP effluent and its evolution throughout different ozone dosages are shown in Figs. 6.3A and B. Raw wastewater, before spiking, displayed no toxicity for both standardized single-species tests. On the contrary, both *P. putida* and *P. subcapitata* were sensitive to the presence of antibiotics, with growth inhibition of 87 and 60%, respectively. These toxic effects of whole-mixture were sharply reduced with ozone up to its total depletion for a dosage of 4.8 mg $O_3 \cdot L^{-1}$, in spite of certain remaining concentration of antibiotics in the tested sample (Table 6.5). Above 4.8 mg $O_3 \cdot L^{-1}$, the growth rate for both microorganisms was not significantly different than that of the controls. The toxicity decreased with increasing ozone dosage following a similar profile as antibiotics concentration. This fact supports the prediction of mixture toxicity of treated STP effluent using component-based approach.

Figs. 6.3C and D represent aquatic toxicity of predicted mixture toxicity values by component-based approach and whole mixture experimental data. The component-based approach is based on Concentration Addition (CA) concept, which has been proposed as a precautionary first tier in the environmental risk assessment of mixture (Backhaus and Faust, 2012), was calculated for each ozone dosage using the remaining concentration of antibiotics (Table 6.5) and their single EC_{50} values (Table 6.4). The application of CA can be criticized for violating the assumption of similar mode of action for the antibiotics used in this work. As explained before, the maximum departure from CA on benefit of IA was estimated according to Eq. (6.4) (Junghans *et al.*, 2006). The results showed that the maximal factor by which CA may predict a lower EC_{50} than IA can be between 1.0 (*P. putida* from 2.7 mg $O_3 \cdot L^{-1}$) and 1.4 (*P. subcapitata* at 2.7 mg $O_3 \cdot L^{-1}$), which are significantly lower than 2. Assuming, that IA and CA indeed describe the two extreme situations of expectable mixture toxicity (*i.e.*, whenever the mixture components do not interact), the maximum error that might occur from the sole use of CA can be considered acceptable (Backhaus and Karlsson, 2014). Thus, CA seems

to be a reasonable approach for the prediction of mixture toxicity on the basis of individual EC_{50} values in all analysed scenario (Junghans *et al.*, 2006). Experimental and predicted toxicity values followed the same toxicity pattern for increasing ozone dosages. This suggests that the antibiotic parent compounds were the main source of toxicity, whereas the formation of TPs did not significantly contribute to mixture toxicity. Similar behaviour was observed on the toxicity to *P. putida* and *P. subcapitata* of ozonated STP effluent spiked with OFX (Carbajo *et al.*, 2015).

Table 6.5 Antibiotics concentration ($\mu g \cdot L^{-1}$) of water samples, whose toxicity has been performed using single-species test and natural communities biofilms (wastewaters were 10 times diluted in the test medium of each bioassay to obtain the concentration shown below).

Antibiotic	Ozone dosage ($mg \cdot L^{-1}$)							
	0	2.7	4.8	8.8	13	21	32	46
Doxycycline	4.2	0.91	0.84	0.17	<LOQ	<LOQ	<LOQ	<LOQ
Erythromycin	49.1	9.0	4.6	2.8	1.1	0.46	<LOQ	<LOQ
Metronidazole	11.7	8.2	7.1	5.3	3.5	1.7	0.59	0.19
Ofloxacin	292	80.0	38.4	13.7	3.5	0.58	0.17	0.13
Sulfamethoxazole	32.0	18.0	6.0	2.2	0.60	0.10	<LOQ	<LOQ
Trimethoprim	14.0	3.7	1.7	0.58	0.14	<LOQ	<LOQ	<LOQ

Taking for granted that parent compounds were the principal source of toxicity, it was possible to quantify the fraction of effect explained by each one and whether or not some of them dominated the mixture effects (Tang *et al.*, 2014). The distribution of the relative toxic units according to the component-based approach is shown in the inset of Figs. 6.3C and D for ozonated STP effluents with significant toxicity values (*i.e.*, >0.1 TUs). The plots allow an easy identification of the relative importance of individual antibiotics for treated wastewater at ozone dosages lower than 10 $mg \cdot L^{-1}$ and for each microorganism (Backhaus and Karlsson, 2014). The figure clearly shows that only one antibiotic was responsible to most to the joint predicted toxic effects, with many of the rest had a negligible contribution to STUs. The data revealed that OFX for *P. putida* (contribution >95% in all scenarios) and ERY for *P. subcapitata* (contribution >70% in all scenarios) were the toxicological drivers for the toxic ozonated effluents (oxidized mixture).

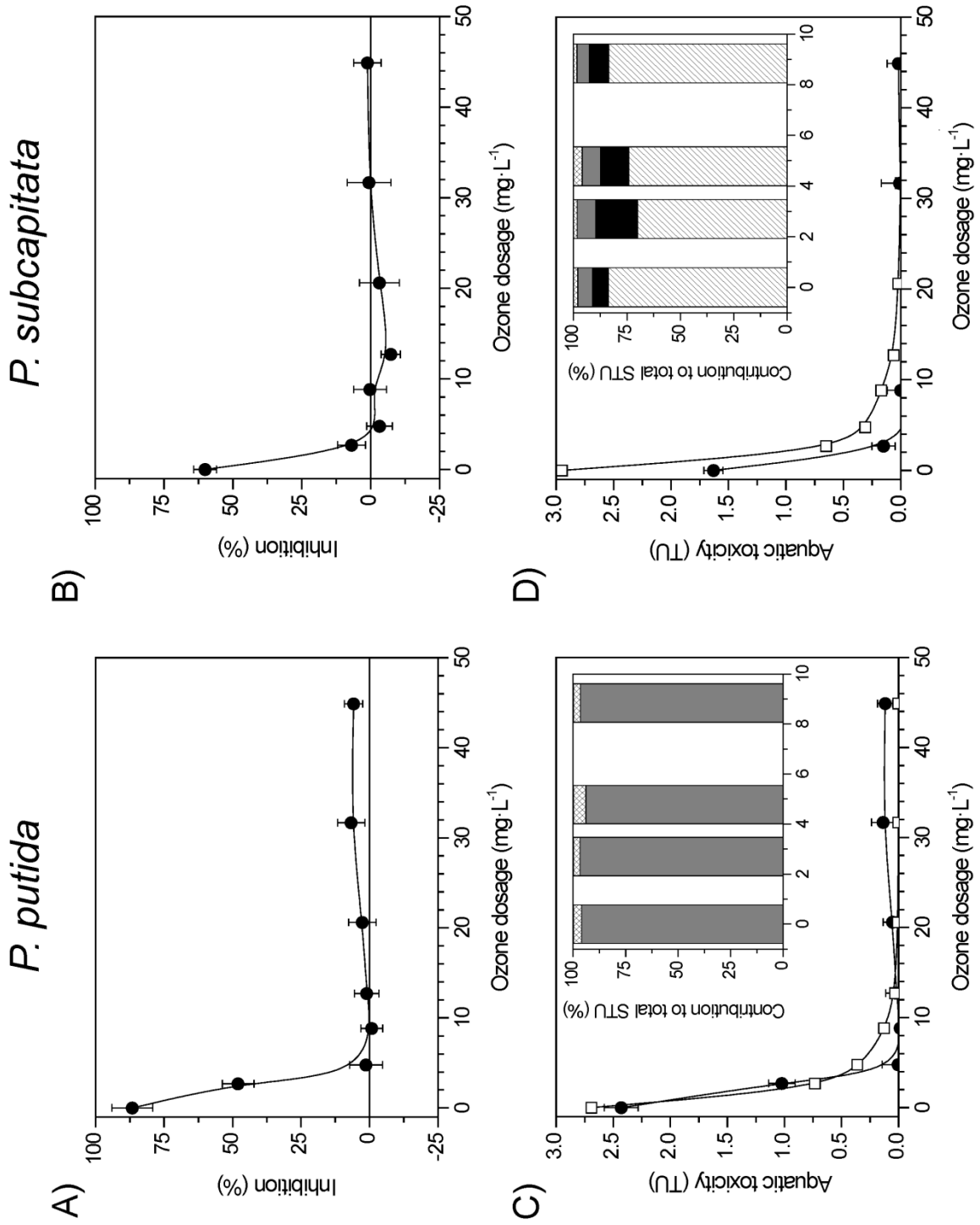


Fig. 6.3 Evolution of experimental (●, mean ± 95% confidence interval) and CA-predicted (□) toxicity of treated wastewater for different ozone dosages to single-species tests. Insets (C,D) represent the contribution to STU according to Concentration Addition. Doxycycline (▨), erythromycin (▨), metronidazole (▨), ofloxacin (▬), sulfamethoxazole (■) and trimethoprim (□).

3.2.2. Natural biofilm community assay

The aquatic toxicity of wastewater samples was also assessed using the heterotrophic and the phototrophic part of natural biofilm communities. Figs. 6.4A and B show the toxicity evolution of ozonated STP effluent for different ozone dosages. The effects on the periphytic algal communities were described by means of the total pigment content as first overall indicator for biomass (Porsbring *et al.*, 2007). Non-toxic effects were observed in raw wastewater or spiked STP effluent in line with the corresponding EC_{50} values of the studied antibiotics (Table 6.4). Ozonation caused a slight stimulation on the natural algal communities for all ozone dosages (Fig. 6.4B). Non-remarkable changes in algal community structure were observed according to the analysis of community pigment profiles.

The general response of bacterial communities as a function of the ozone dosage was also assessed using the average well colour (AWC) (Fig. 6.4A). Raw wastewater caused a slight stimulation effect on the metabolic activity of the heterotrophic part of the natural biofilm (-7%), while spiked STP effluent provoked 61% inhibition with respect to the controls. The toxic effects steadily reduced with increasing ozone dosage up to their total depletion at 4.8 mg $O_3 \cdot L^{-1}$. Higher ozone exposures stimulated the metabolic activity of bacterial communities. Stimulation of treated STP effluent for ozone dosage higher than 8.0 mg$\cdot L^{-1}$ reached values near 25%. Fig. 6.4C represents the aquatic toxicity from whole mixture experimental data and the predicted toxicity from component-based approach. In spite of CA predicted higher toxicity than the experimental data in line with the results obtained in Chapter 4, prospective and experimental profiles displayed the same pattern for ozone dosages lower than 7 mg$\cdot L^{-1}$. According to component-based approach, OFX was the driver of treated STP effluent for low ozone dosages (contribution >95% in all scenarios, inset Fig. 6.4C). The result was coincident the findings reported before for *P. putida*, which was also a prokaryotic-based test.

For ozone dosage higher than 7 mg$\cdot L^{-1}$, the significant stimulating effects observed on natural bacterial communities can be attributed to the formation and accumulation of refractory ozonation products (Hammes *et al.*, 2006 and Hübner *et al.*

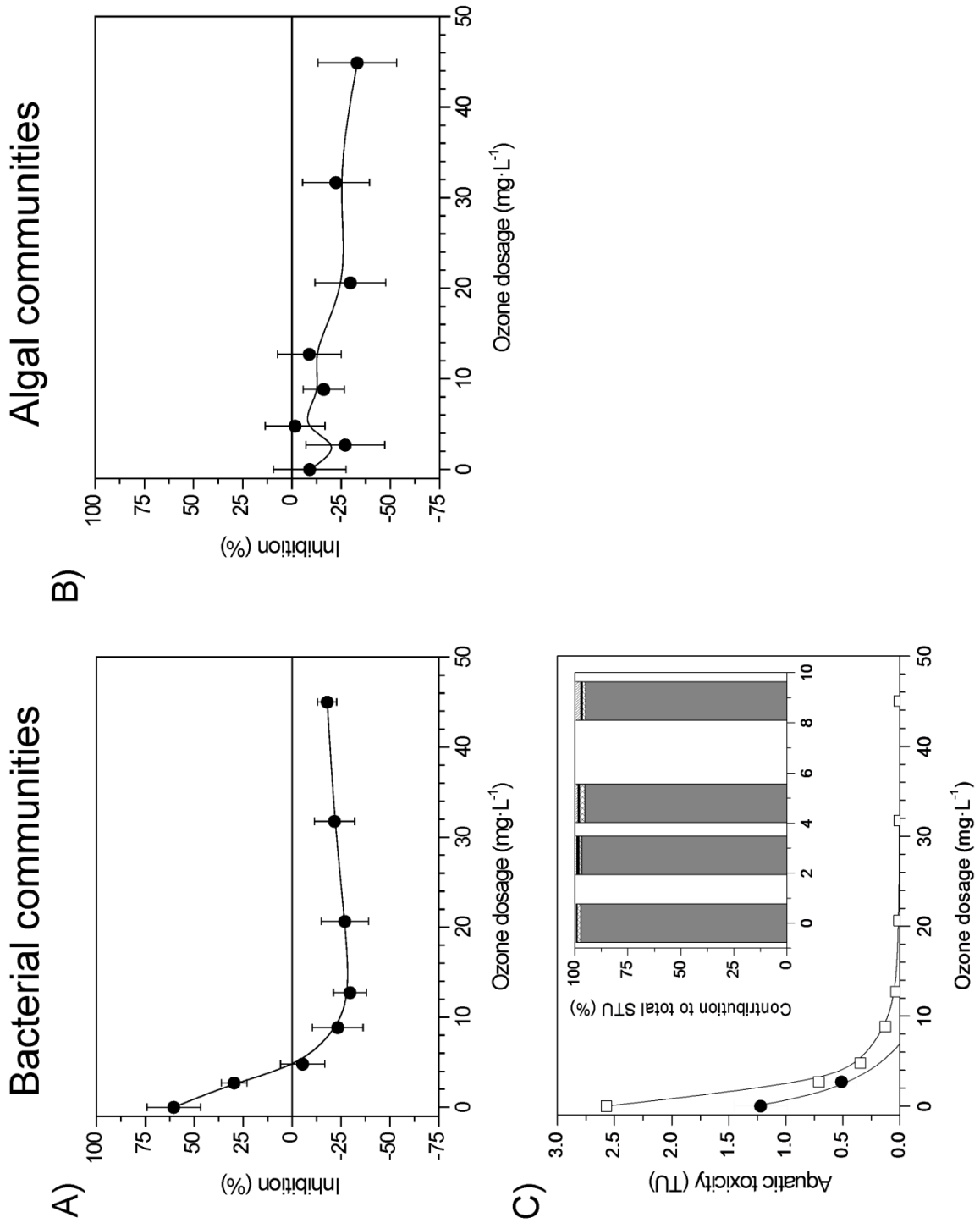


Fig. 6.4 Evolution of experimental (●, mean ± 95% confidence interval) and CA-predicted (□) toxicity of treated wastewater for different ozone dosages to natural biofilm communities. Inset plot C represents the contribution to STU according to Concentration Addition. Doxycycline ( ), erythromycin ( ), metronidazole ( ), ofloxacin ( ), sulfamethoxazole ( ) and trimethoprim ( ).

2015). Ozone degrades macromolecular organic compounds (low O/C and low H/C ratio) into smaller compounds (high O/C and high H/C ratio) such as aldehydes, ketones and especially carboxylic acids, whose concentration explains up to 40% of DOC remaining in ozonated wastewater (Fig. 6.2). These ozone-refractory compounds are generally biodegradable organic matter (Hammes *et al.*, 2006). They are the reason why ozonation is able to increase assimilable organic carbon (AOC) up to a factor of 6 in treated wastewater compared to raw effluents (Zimmermann *et al.*, 2011). AOC refers to carbonaceous compounds that are rapidly metabolized by microorganisms leading to an increase in biomass: 1 µg of consumed AOC equivalent to an increase of 10^7 cells of natural microbial consortium used as inoculums (Hammes and Egli, 2005). Readily biodegradable DOC enrichment has been shown to enhance biomass and the metabolic activity of natural microbial communities (Olapade and Leff, 2006 and Johanson *et al.*, 2012). Sun *et al.* (1997) also suggested that aliphatic carbon is the principal form of carbon being utilized by bacteria in fluvial ecosystems and that their ability to use DOM increases as the aliphatic carbon content (H/C ratio) of DOM increases. However, highly oxidized DOM (high O/C ratio), with higher carboxylic content, was found to decrease the bioavailability of aliphatic DOC (Sun *et al.*, 1997).

In order to gain further insight into the characterization of the change induced on bacterial communities by ozonated wastewater and to determine the potential impact on their biodiversity, we also analysed the responses of each individual carbon source by calculating the area under the curve (AUC). AUC gives a measure of time-integrated effects on the metabolization of each individual carbon source. The results showed that the metabolic activity was unevenly distributed between the 31 carbon sources present on the EcoPlates (Table 4.4 in Chapter 4), and that not all carbon sources were used by the heterotrophic communities. Three carbon sources never reached a corrected absorbance of 0.05 or lower (C11 (*i*-erythritol), C19 (2-hydroxy benzoic acid), C21 (γ-hydroxybutyric acid) and C30 (glycyl-L-glutamic acid)) and were classified as inactive.

The AUC values of individual carbon sources allow for a multivariate data exploration and the corresponding nonmetric multidimensional scaling (nMDS) plot shown in Fig. 6.5. Raw wastewater, the antibiotic mixture and the spiked STP effluent

gave rise to changes in bacterial communities with respect to the controls as demonstrated by their distance in the graph. Concerning ozonated wastewater samples, the main trend was the general movement from the right-up to the left-down side of the plot for increasing ozone dosage. This trajectory can be explained by the removal of antibiotics, particularly OFX, which was the main source of toxicity towards periphytic bacterial communities. As previously shown in Fig. 6.1, a notable abatement of the antibiotic mixture was observed for ozone exposure of 4.8 $mg \cdot L^{-1}$ (84% for the mixture and 87% for OFX). This fact can also be observed in Fig. 6.6, which represents the relative AUC of individual carbon sources and the corresponding AWC inhibition with increasing ozone dosage. For ozone dosage higher than 4.8 $mg \cdot L^{-1}$, no major re-arrangement of carbon source utilization was observed by bacterial communities. Under the assumption that differences in relative carbon source utilization are indicative of changes in community biodiversity (species composition and physiological activity of each species), it can be concluded that increased concentration of AOC without significant occurrence of toxic compounds (*i.e.*, ozone dosage higher than 7 $mg \cdot L^{-1}$) may

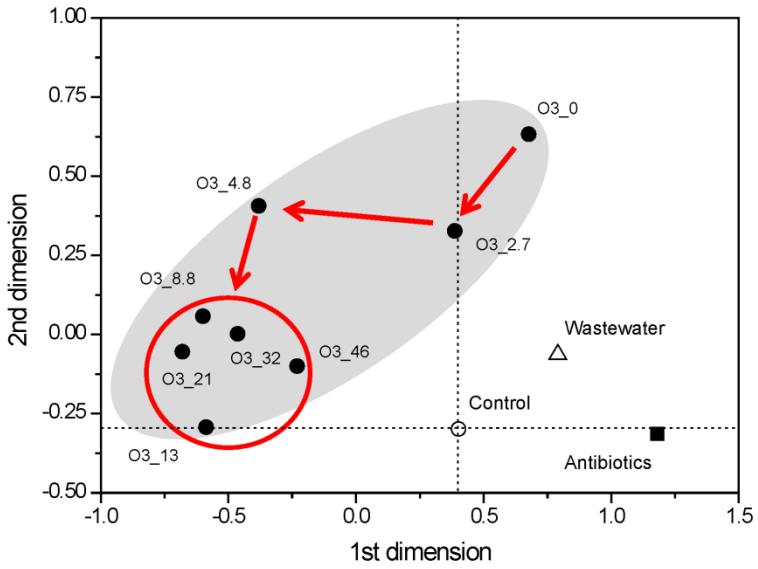


Fig. 6.5 Nonmetric multidimensional scaling (nMDS) showing effects on metabolic activity (analysis under the curve, AUC) differences (Manhattan Distances) for the bacterial part of natural biofilm communities using PROXSCAL algorithm. O3_X: Treated STP effluent with ozone dosage X $mg \cdot L^{-1}$ (PROXSCAL algorithm: Normalized Raw Stress = 0.0169; Stress-I = 0.0000 and Stress-II = 0.0000 with optimal scaling factor = 1.017; S-Stress = 0.0000 with optimal scaling factor = 0.983; Dispersion Accounted For = 0.0000 and Tucker's Coefficient of Congruence = 0.0000).

have an influence on the bacterial biodiversity of the natural biofilm communities. This fact is consistent with Judd *et al.* (2006), who demonstrated that the bacterial community composition is controlled by the nature of the organic matter available. It is also interesting to note that all of ozonated wastewater samples induced changes on the biodiversity of bacterial communities and consequently, any ozone dosage contributed to the preservation of the original state of natural communities, even for ozone dosages for which antibiotics were not significantly removed.

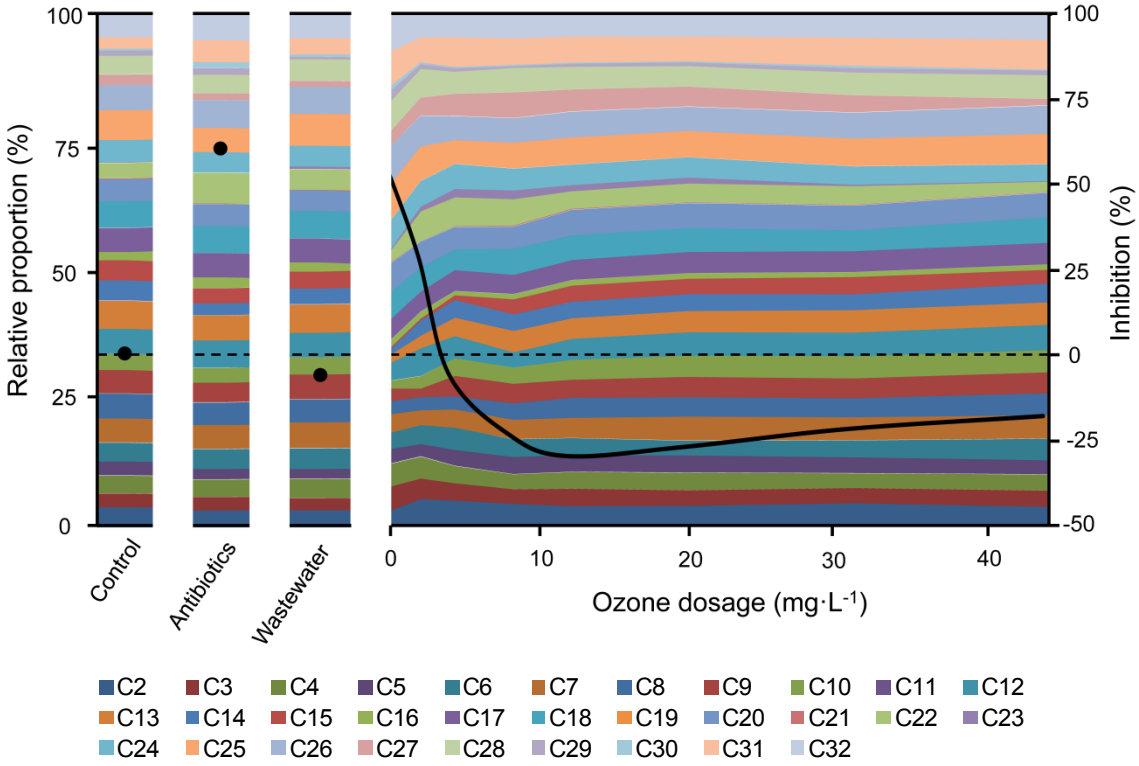


Fig. 6.6 Evolution of analysis under the curve (AUC) of individual carbon sources and the corresponding inhibition of average well colour (AWC) for the bacterial part of natural biofilm communities at different ozone dosages.

When comparing the toxicity of STP effluents to natural biofilm communities, to the bacterium *P. putida* and to the alga *P. subcapitata*, it becomes evident that untreated and ozone treated wastewater showed a comparatively lower toxicity towards limnic periphyton. Although the differences might be at least partly caused by the different endpoints employed, the higher tolerance of natural communities fits expectation considering their intrinsic biodiversity (Kümmerer, 2009b), and the numerous mechanisms of resistance to antibiotics displayed by biofilms (Russell, 2003).

4. Conclusions

The continuous ozonation is a suitable technology for the abatement (≥98%) of six antibiotics frequently detected in an STP effluents for ozone dosages below 4.3 $mg \cdot L^{-1}$. However, the limited extent of mineralization (<30%) demanded an in-depth aquatic toxicological assessment of ozone treated wastewater.

The toxicological evaluation showed that ozonation totally removed whole-mixture toxic effects measured by the growth rate of *P. putida* and *P. subcapitata* and the metabolic activity of natural bacterial communities. The toxic effects were caused predominantly by antibiotic parent compounds, whereas the formation of degradation products did not significantly contribute to mixture toxicity. This fact allows predicting mixture toxicity by means of a component-based approach. A predictive approach, based on CA, showed a slight overestimation of the toxic effects of ozone treated STP effluent.

Ozonated wastewater samples induced changes on the biodiversity of bacterial communities. This finding suggests the need to introduce structural endpoints such as microbial population composition, in the evaluation of wastewater treatment technologies, which would allow identifying effects overlooked otherwise.

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Chapter 7

Influence of water matrix on copper-catalysed ozonation and related ecotoxicity

Alice L. Petre, Jose B. Carbajo, Roberto Rosal, Eloy García-Calvo, Pedro Letón and Jose A. Perdigón-Melón

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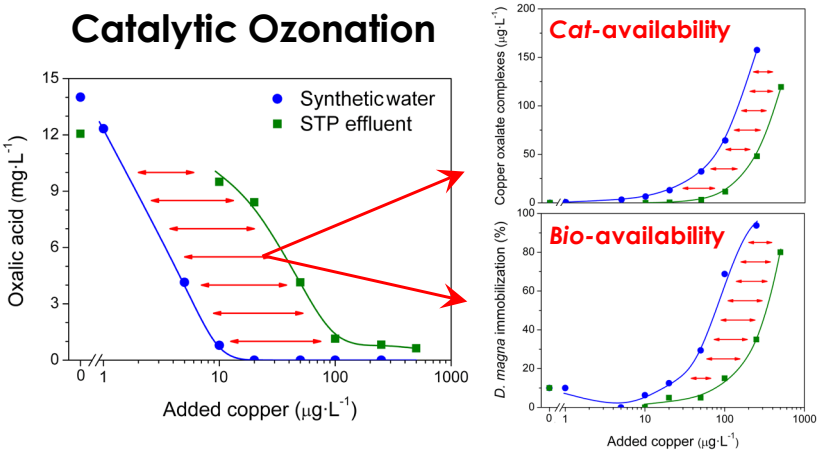
Graphical Abstract



INFLUENCE OF WATER MATRIX ON COPPER-CATALYSED OZONATION AND RELATED ECOTOXICITY

Abstract

The continuous ozonation of a mixture of carboxylic acids (formic, acetic, oxalic and maleic) has been performed under non-catalytic and copper-catalysed ozonation using a synthetic water matrix and a real sewage treatment plant (STP) effluent. The aim was to study the effect of water matrix on catalytic performance, particularly considering the toxicity of treated water to aquatic organisms. The non-catalytic ozonation of carboxylic acids in synthetic water resulted in a low reduction (36%) of the total organic carbon (TOC), the main feature being the accumulation oxalic acid due to the partial oxidation of maleic acid. Catalytic ozonation, adding copper concentration of 20 $\mu g \cdot L^{-1}$, achieved a TOC reduction of 75%, mainly due to the total depletion of oxalic acid. In wastewater effluent, the same general pattern was found with oxalic acid as the main by-product and its almost complete removal in catalytic ozonation. However, to attain the latter it was necessary to use copper concentrations as high as 100 $\mu g \cdot L^{-1}$. Copper proved to be a good catalyst for the oxidation of oxalic at near neutral pH, with short reaction times and matrix with high scavenging rate. The aquatic toxicity of treated mixtures was studied by means of five single species placed on different trophic levels: *Vibrio fischeri*, *Pseudomonas putida*, *Tetrahymena thermophila*, *Pseudokirchneriella subcapitata* and *Daphnia magna*. The results showed that copper in STP effluent was less toxic than in synthetic water, an effect attributed to copper complexation with organic and inorganic compounds present in the matrix. The reduced biological availability could also explain the lower catalytic effect observed in real wastewater.

1. Introduction

Ozone is widely used in drinking water and wastewater reclamation treatments due to its high disinfection power and oxidation potential (von Sonntag and von Gunten, 2012). The direct ozonation of organic compounds results in many refractory oxidation by-products, particularly carboxylic acids (von Gunten, 2003). Different ozone-based processes have been developed to improve ozone oxidation performance in order to increase the degree of mineralization. These technologies include O_3/OH^-, O_3/H_2O_2, and O_3/UV and belong to the group of advanced oxidation process (AOP) based on the generation of hydroxyl radicals ($OH^\bullet$). Contrary to ozone, $OH^\bullet$ reactions are not selective, but their concentration depends on the scavenging rate of the water matrix (Kasprzyk-Hordern *et al.*, 2003, Katsoyiannis *et al.*, 2011 and Zhang *et al.*, 2012).

Catalytic ozonation has also been proposed to increase the degree of mineralization and reduce ozone consumption (Centi and Perathoner, 2005 and Nawrocki and Kasprzyk-Horden, 2010). Different transition metals and oxides have been studied as ozonation catalysts (Kasprzyk-Hordern *et al.*, 2003). Among them, copper has shown a significant catalytic effect in the degradation of carboxylic acids (Pines and Reckhow, 2002, Pi *et al.*, 2003, El-Raady and Nakajima, 2005, Beltrán *et al.*, 2005, Zhang *et al.*, 2012 and Petre *et al.*, 2013). It has been noted that the performance of catalytic ozonation strongly depends not only on the catalyst itself, but on the composition of water matrix (von Gunten, 2003 and Petre *et al.*, 2013). Moreover, most catalytic ozonation studies have been carried out in batch or semi-batch conditions, but more relevant data would be obtained from continuous ozonation devices. Contrary to batch processes in which a well-defined reaction time is established, continuous treatments display a statistical distribution of residence times (Beltrán, 2004).

Catalytic ozonation is able to remove certain pollutants, but can generate new compounds as oxidation by-products and from the leaching of catalyst active phases, which may be more hazardous than the original mixture (Centi and Perathoner, 2005 and Petala *et al.*, 2008). Treated water is a complex mixture of organic and inorganic compounds, whose ecotoxicological impact cannot be predicted by simple chemical

determinations due to the potential interactions among pollutants (Petala *et al.*, 2008). The chemical analyses in which regulations are based identify and quantify trace metals in an aquatic environment. However, they do not provide direct indication of the potential effects of the metals on the biota (Rodríguez-Mozaz *et al.*, 2006). Thus, ecotoxicological bioassays are required to provide a holistic direct estimation of the environmental hazard of a given mixture. In particular, metal ecotoxicity is directly affected by physico-chemical parameters such as pH, alkalinity, hardness and dissolved organic and suspended matter, which alter its speciation and bioavailability (Girling *et al.*, 2000 and Wilde *et al.*, 2006), and, indirectly, through synergistic or antagonistic effects (Mowat and Bundya, 2002 and Gallego *et al.*, 2007). Therefore, aquatic toxicity assessment should include a battery of different species representative of the different taxa in the trophic chain (Okamura *et al.*, 2000), with emphasis on organisms placed at the bottom, like phytoplankton and zooplankton, where damage caused by metals primarily occur (SEPA, 2000). Many ozonation catalytic studies have been carried out in ultrapure water neglecting the effects on catalyst performance of the organic and inorganic species present in real matrices. Similarly to the influence of water matrix composition over metal ecotoxicity through the bioavailability concept, the same behaviour could be applied to the influence of water matrix on copper catalytic availability.

The aim of this study was to explore the effect of the water matrix on the non-catalytic and copper-catalysed continuous ozonation of a mixture of carboxylic acids (formic, acetic, oxalic and maleic acid). These compounds are present in ozonated water as reaction intermediates or final ozone-refractory by-products. It was used homogeneous catalyst due to simplicity of application in continuous processes, but in view of the low concentration used, the results could be extrapolated to the effect of active phase leaching in heterogeneous catalysis. The ecotoxicity of ozonated water was tracked using a battery of bioassays composed of five single-species tests: *Vibrio fischeri*, *Pseudomonas putida*, *Tetrahymena thermophila*, *Pseudokirchneriella subcapitata* and *Daphnia magna*.

2. Materials and methods

2.1. Materials

Formic, acetic, oxalic and maleic acid and copper ($Cu(NO_3)_2 \cdot 3H_2O$) of analytical degree were purchased from Fluka. The initial carboxylic acid mixtures were prepared with a concentration of 7 $mg \cdot L^{-1}$ each. These organic acids and concentrations have been chosen because they have been previously identified and quantified as the main final ozonation by-products in a previous work dealing with the ozonation of pharmaceutical and personal care products in the same STP effluent (Rosal *et al.*, 2008). These acids were the main responsible of the relative low mineralization degree achieved in direct ozonation runs.

In order to study the effect of the water matrix over the ozonation performance, two different matrices were used: a synthetic matrix and wastewater from the effluent of a sewage treatment plant (STP) located in Alcalá de Henares (Spain). Synthetic water was prepared with the required amount of sodium bicarbonate in ultrapure water to equal the alkalinity and pH values of the STP effluent. Ultrapure water was obtained from a Millipore Milli-Q system with a resistivity of at least 18 MΩ·cm at 25ºC. The STP treats a mixture of domestic and industrial wastewater from facilities located near the city (374 000 population equivalent) and has a nominal capacity of 3 000 $m^3 \cdot h^{-1}$ of raw wastewater. Details on wastewater characterization are included in Table 7.1.

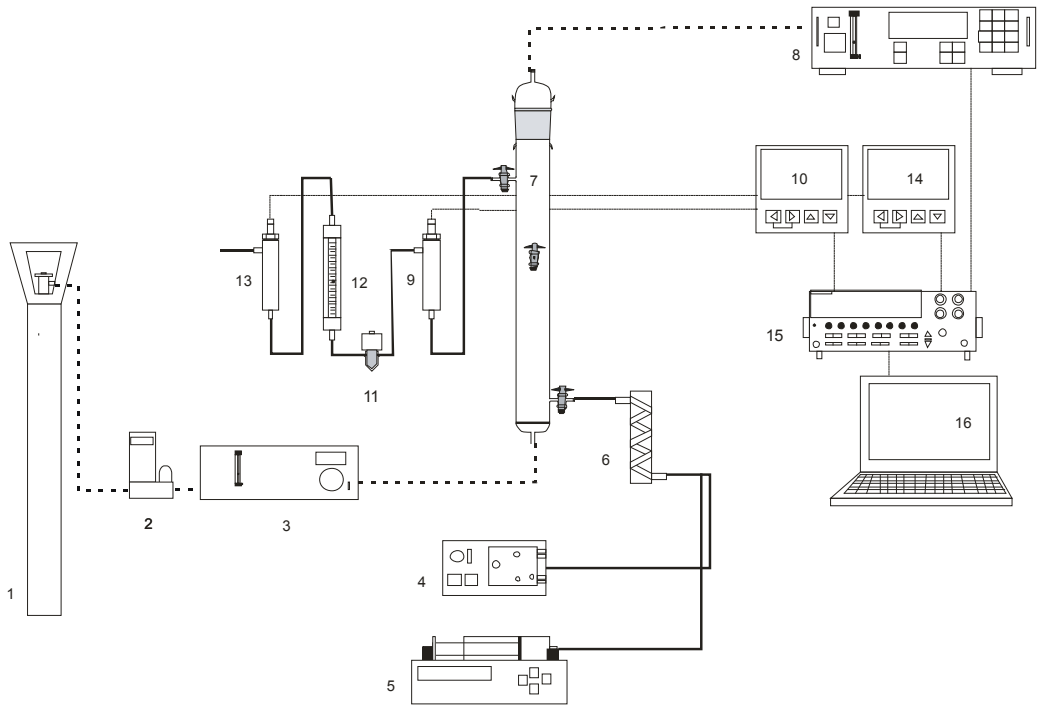
Table 7.1 Main physico-chemical parameters of STP effluent.

pH	7.24	Na^+ ($mg \cdot L^{-1}$)	67.5	Al ($\mu g \cdot L^{-1}$)	20.5
Conductivity ($\mu S \cdot cm^{-1}$)	800	NH_4^+ ($mg \cdot L^{-1}$)	0.34	Cr ($\mu g \cdot L^{-1}$)	1.1
TSS ($mg \cdot L^{-1}$)	30	K^+ ($mg \cdot L^{-1}$)	13.9	Mn ($\mu g \cdot L^{-1}$)	101
Turbidity (NTU)	0.40	Mg^{2+} ($mg \cdot L^{-1}$)	20.1	Fe ($\mu g \cdot L^{-1}$)	453
COD ($mg \cdot L^{-1}$)	14.3	Ca^{2+} ($mg \cdot L^{-1}$)	52.8	Co ($\mu g \cdot L^{-1}$)	1.3
TOC ($mg \cdot L^{-1}$)	5.12	Cl^- ($mg \cdot L^{-1}$)	88.2	Ni ($\mu g \cdot L^{-1}$)	15.4
BOD_5 ($mg \cdot L^{-1}$)	2.31	NO_2^- ($mg \cdot L^{-1}$)	0.49	Cu ($\mu g \cdot L^{-1}$)	1.2
BOD_5/COD	0.16	NO_3^- ($mg \cdot L^{-1}$)	36.8	Zn ($\mu g \cdot L^{-1}$)	48.2
Phenols ($mg \cdot L^{-1}$)	0.08	PO_4^{3-} ($mg \cdot L^{-1}$)	3.31	Cd ($\mu g \cdot L^{-1}$)	0.10
$SUVA_{254}$* ($L \cdot mg\ C^{-1} \cdot m^{-1}$)	2.06	SO_4^{2-} ($mg \cdot L^{-1}$)	68.8	Hg ($\mu g \cdot L^{-1}$)	0.15
Alkalinity ($mgCaCO_3 \cdot L^{-1}$)	155	HCO_3^- ($mg \cdot L^{-1}$)	189	Pb ($\mu g \cdot L^{-1}$)	0.24

*Specific ultraviolet absorption at 254 nm

2.2. Experimental procedure

The experiments were carried out in continuous mode in a cylindrical reactor made of Pyrex (internal diameter of 6 cm and working height of 51 cm) with a total working volume of 1.44 L operated in co-current mode (Scheme 7.1). Water flow rate was 142 mL·min^{-1} (Gilmont rotameter) and gas flow was 390 mL·min^{-1} (Aalborg mass flow controller) with different inlet ozone concentrations (Anseros ozone generator COM-AD-02). Inlet and outlet ozone gas concentration (Anseros ozone GM-PRO analyser), dissolved ozone in the reactor exit (Mettler Toledo-Thomton dissolved ozone sensor), pH and temperature (Easyferm Plus VP 120 Hamilton pH sensor) were constantly monitored and recorded (Keithley 2700 Data Acquisition System). Copper solution was continuously added to the inlet stream at different flows (Harvard 11 plus infusion pump) to achieve the desired final concentration. In order to ensure homogeneity a nine-loop glass coiled pipe was used. The dilution ratio was always lower than 1%.



Scheme 7.1 Experimental set-up. (1) oxygen cylinder, (2) mass flow controller, (3) ozone generator, (4) peristaltic pump, (5) syringe pump, (6) nine-loop coil, (7) bubble column, (8) ozone gas analyser, (9) dissolved ozone sensor, (10) dissolved ozone transmitter, (11) needle valve, (12) rotameter, (13) pH sensor, (14) pH transmitter, (15) data acquisition system, (16) computer. Water line is represented as solid line, gas line as dashed line and electrical wiring as dotted line.

For every set of working conditions, samples were withdrawn for analysis at the column outlet once the stationary state was reached. This was accomplished after circulating four times the hydraulic retention time after a constant ozone value was obtained both in liquid and gas phases at the column outlet. The retention time distribution curve yielded an average retention time of 10.3 min and was analysed using the continuous stirred tank reactor (CSTR) in series model according to the procedure described in the literature (Burrows *et al.*, 1999). The equivalent value of 1.13 tanks obtained indicated that the column can be approached to a perfect CSTR. It is generally accepted that short columns with intense gas phase hydrodynamics can be assimilated to a CSTR due to the bubble back mixing (Asenjo and Merchuck, 1995). Assuming CSTR behaviour, the amount of ozone consumption at the stationary state ($dC_{O_3}^{liq}/dt = 0$) can be obtained from the following mass balance (Eq. (7.1)):

$$Consumed\ O_3 = F_{O_3}^{gas,in} - F_{O_3}^{gas,out} - F_{O_3}^{liq,out} \quad (7.1)$$

in which F_{O_3} is the rate of ozone entering the system in the gas phase (gas, in) or existing either in the exhaust gases (gas, out) or dissolved in water (liq, out). Further details about experimental set-up are given in Chapter 3 (section 2.2).

2.3. Analytical methods

The concentration of organic acids was measured using a Dionex DX120 Ion Chromatograph (IC) with conductivity detector. Oxalic and maleic acid concentrations were determined using an IonPac AS9-HC analytical column (4 × 250 mm) with ASRS-Ultra suppressor, whereas acetic, glyoxalic and formic acids were measured with an IonPac ICE-AS6 analytical column (9 × 250 mm) with AMMS ICE II suppressor. Total organic carbon (TOC) analyses were performed on a Shimadzu TOC-V_{CSH} total carbon organic analyser equipped with an ASI-V autosampler. The concentration of copper was determined by Agilent 7700× ICP-MS operating at 3 MHz in helium cell gas mode.

2.4. Procedures for aquatic toxicity tests

The ecotoxicity of water samples was assessed by means of five bioassays using *V. fischeri*, *P. putida*, *T. thermophila*, *P. subcapitata* and *D. magna*. The battery of tests allowed the combination of acute and chronic assays and the combined use of prokaryotes and eukaryotes at several trophic levels.

Bacterial toxicity assessment was performed with *V. fischeri* and *P. putida*. *V. fischeri* acute test measure the decrease in bioluminescence induced in the cell metabolism due to the presence of a toxic substance. The bacterial reagent (*V. fischeri* NRRL-B 11177, a commercially available BioFix®Lumi test from Macherey-Nagel, Germany) is supplied freeze-dried and was reconstituted and stored at 3ºC for an interim period of 5 min before use. Water samples were prepared according to ISO 11348-3 standard protocol (ISO, 2007). The bioassay was carried out in 96-well white polypropylene microplate. 100 µL of test solution was pipetted into each well, which were supplemented with 100 µL of bacterial suspension. Light was measured at 15 ± 1ºC after 30 min by means of a Fluoroskan Ascent FL microplate luminometer (Thermo Scientific). *P. putida* test determines the inhibitory effect of a substance on the bacteria (*P. putida* NCIB 9494 from CECT, Spain) by means of cell growth inhibition. This bioassay was performed according to ISO guideline 10712 (ISO, 1995). Bacterial cultures were exposed to test solutions at 23 ± 1ºC for 16 h in 10 mL glass incubation vials which were constantly shaken in the dark. The cell growth was determined by optical density (λ 600 nm) in 96-well clear microplate (200 µL test suspension per well) using a Rayto RT-2100C microplate reader.

Chronic growth inhibition assay with the ciliate protozoan *T. thermophila* was performed according to the Standard Operational Procedure Guideline of Protoxkit F™ (1998). The test is based on the turnover of substrate into ciliate biomass. Substrate and reconstitution medium were purchased from MicroBioTest Inc. (Belgium) whereas *T. thermophila* (SB 210) was kindly supplied by D. Cassidy-Hanley (Tetrahymena Stock Center, USA). Ciliates were incubated in test vessels, with water samples and food suspension, at 30 ± 1ºC for 24 h in the dark. All assays were carried out with initial cell

concentration of 100 cell·mL^{-1}, which was quantified by use of Coulter Z2 particle counter using Isotone® as dilution medium. Growth inhibition was determined on the basis of turbidity changes (OD at λ 440 nm), at the beginning and at the end of the test.

The algal growth inhibition test was carried following the procedure described in the European Guideline OECD TG (Guideline) 201 open system, using *P. subcapitata* (OECD, 2011). The algal stock culture for inoculation was taken from commercial test kit Algaltoxkit F™ (MicroBioTest Inc., Belgium). Microalgae cells were exposed to water samples at 23 ± 1ºC for 72 h in 10 mL glass incubation vials, which were constantly shaken and illuminated in a chamber (∼100 µmol foton·m^{-2}·s^{-1}) to ensure exponential algal growth. All assays were performed with initial algal cell concentration of 10 000 cell mL^{-1} and algal biomass was measured daily by chlorophyll *a* fluorescence. Chlorophyll extraction was carried out as following: 50 µL culture samples were transferred to a 96-well black polypropylene microplate, 200 µL of ethanol was added to each well and the plate was shaken for 3 h in the dark. Thereafter the fluorescence was measured using a Fluoroskan Ascent FL microplate fluorometer (Excitation 450 nm, Emission 672 nm) from Thermo Scientific.

Finally, acute toxicity tests with the crustacean *D. magna* were carried out according to the standard protocol described in the European Guideline OECD TG 202 (OECD, 2004), using the commercially available kit format Daphtoxkit F™ (MicroBioTest Inc., Belgium). Test plates with *D. magna* neonates were incubated for 48 h at 20 ± 1ºC in the dark and immobilization of the organism was used as the toxicity endpoint.

$ZnSO_4 \cdot 7H_2O$ for *V. fischeri* test, 3,5-dichlorophenol for *P. putida* and $K_2Cr_2O_7$ for the rest of bioassays were used as reference substances in order to check each test procedures. Three independent experiments with duplicate samples were carried out to ensure reproducibility. All aquatic toxicity data are expressed as mean ± 95% confidence intervals and data analysis were performed using a nonlinear-regression sigmoidal dose-response curve model provided in the GraphPad Prism 6.0 software (GraphPad software Inc., San Diego, USA).

3. Results and discussion

3.1. Synthetic water

3.1.1. Non-catalytic ozonation

The non-catalytic ozonation of carboxylic acids in the synthetic matrix was studied by keeping a constant flow of water and ozonating gas and changing the concentration of ozone. The amount of ozone per litre of water introduced varied with the purpose of determining the efficiency of ozone usage from 4.5 $mg \cdot L^{-1}$ (0.44 $g\ O_3 \cdot g\ TOC^{-1}$), a low concentration at which ozone acts as a limiting reagent, to 93 $mg\ L^{-1}$ (9.0 $g\ O_3 \cdot g\ TOC^{-1}$). Fig. 7.1A represents the evolution of TOC and consumed ozone as a function of the amount of ozone supplied. Up to 46 $mg \cdot L^{-1}$, TOC declined with ozone dosage up to a value for which it remained essentially constant. This initial zone (zone 1) corresponded with the reaction of the more readily oxidizable acids. In it, ozone was the limiting reagent and the reaction was mass-transfer controlled as revealed by the fact that no dissolved ozone (<0.01 $mg \cdot L^{-1}$) was detected in solution. In zone 2, ozone consumption slightly increased up to a value of 71 $mg \cdot L^{-1}$. In this intermediate zone, TOC depletion stabilized and the increased consumption of ozone indicated the presence of organic matter oxidized but not mineralized. This zone corresponded to chemical control and, accordingly, an increase in the concentration of dissolved ozone concentration was detected. At higher ozone dosages, above 71 $mg \cdot L^{-1}$ (zone 3), ozone consumption was almost constant and in parallel the concentration of ozone at the reactor outlet increased. This value was considered the upper operational limit. The maximum TOC depletion with non-catalytic ozonation was low, at about 35%, a figure that corresponds with the well-known behaviour of direct ozonation processes (von Gunten, 2003).

Fig. 7.1B represents the evolution of the concentration of individual carboxylic acids with ozone dosage. A good agreement was observed between the experimental TOC and the theoretical TOC calculated from the concentration of the acids detected with ion chromatography (>90%). Other organic reaction by-products were not detected. Maleic and formic acids were completely removed, acetic acid concentration

was slightly reduced and the amount of oxalic acid increased during treatment, the latter being the main component of the final mixture (around 60% TOC). Glyoxalic acid, an acid not present in the initial mixture, was detected as a reaction by-product. The glyoxalic acid concentration was detected for low ozone dosages to further reach a plateau and decrease thereafter with increased ozone input.

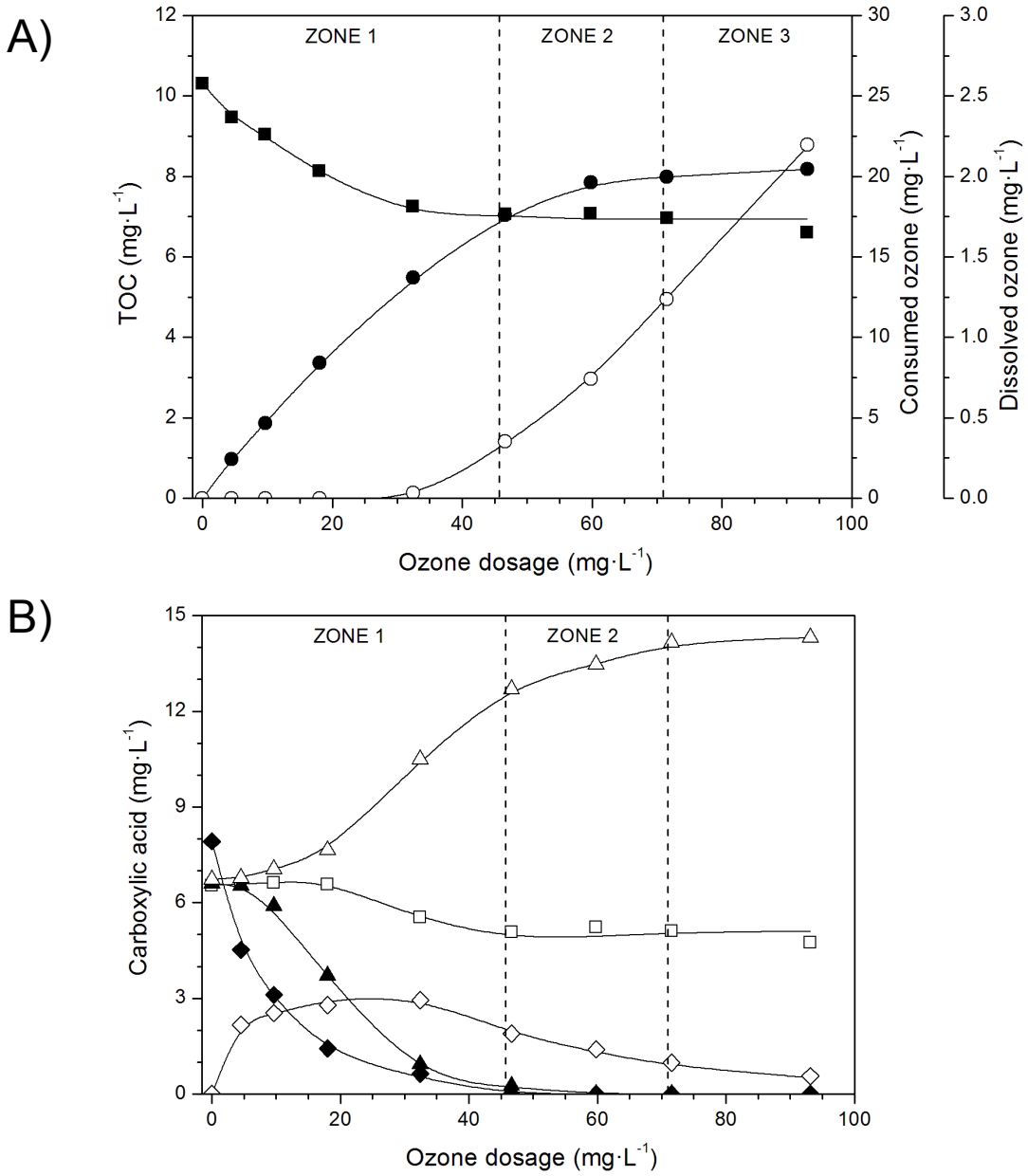
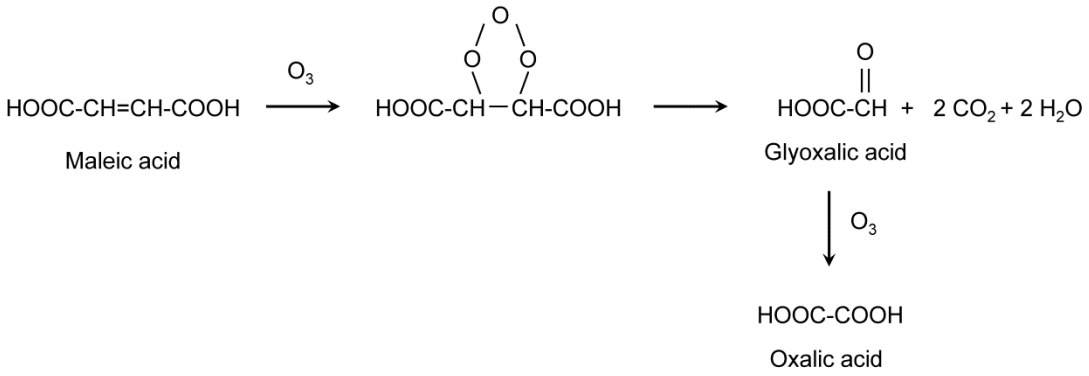


Fig. 7.1 Evolution of (A) TOC (■), consumed (●) and dissolved ozone (○) and (B) the concentration of acetic (□), glyoxalic (◊), formic (▲) maleic (♦) and oxalic acid (Δ) for different ozone dosages in the synthetic water matrix.

Maleic acid was the most reactive component and it was the only one oxidized for the lowest ozone dosages with the simultaneous evolution of glyoxalic acid. Under these conditions, maleic acid depletion (30.2 $\mu mol \cdot L^{-1}$) generated 30.3 $\mu mol \cdot L^{-1}$ of glyoxalic acid, displaying an almost stoichiometric conversion. This oxidation from maleic to glyoxalic acid has been previously reported together with the formation of formic acid (El-Raady and Nakajima, 2005, Sun *et al.*, 2006 and Leitzke and von Sonntag, 2009). In this study, a TOC reduction of 69.2 $\mu mol \cdot L^{-1}$ (almost two-fold maleic depletion) was observed. These facts suggest that roughly half of the maleic acid was converted to glyoxalic acid, with the rest being mineralized to CO_2. A tentative reaction pathway is presented in Scheme 7.2.



Scheme 7.2 Proposed maleic acid ozonation pathway.

At a higher ozone dosage, other reactions took place, such as the depletion of formic acid. Maleic and formic acids totally disappeared after dosing 46 $mg \cdot L^{-1}$ of ozone, after which no further TOC depletion took place (Fig. 7.1A). Final TOC removal, 3.41 $mg \cdot L^{-1}$, was in good agreement with the total mineralization of formic acid, 6.57 $mg \cdot L^{-1}$ (1.75 mg $TOC \cdot L^{-1}$), and the above-explained elimination of two CO_2 moles per mol of maleic acid depleted (1.67 mg $TOC \cdot L^{-1}$), suggesting that both acids essentially account for all TOC reduction. In spite of the reaction of glyoxalic acid, the concentration of which was reduced, it was not mineralized but rather oxidized to oxalic acid. This fact is well-documented and explains the fate of both acids (Rice and Browning, 1980, Caprio *et al.*, 1987 and El-Raady and Nakajima, 2005). Initially, glyoxalic concentration increased due to maleic acid oxidation, reducing at higher ozone dosages due to its oxidation to oxalic acid (Scheme 7.2). Acetic, glyoxalic and, particularly, oxalic acid were the main

contribution to final TOC in treated water (>90%), which is compatible with their well-known refractory character (Hoigné and Bader, 1983, Andreozzi *et al.*, 2000 and Rosal *et al.*, 2008).

3.1.2. Catalytic ozonation

Copper-catalysed continuous ozonation was carried out with increasing amounts of copper (from 1 to 250 $\mu g \cdot L^{-1}$) for a fixed ozone dosage of 71 mg L^{-1}, which represented the maximum conversion obtained with non-catalytic ozonation. Fig. 7.2 displays TOC reduction as copper concentration increased. A remarkable TOC depletion was observed even with the lowest concentration (1 $\mu g \cdot L^{-1}$), which increased with increasing copper concentration up to 20 $\mu g \cdot L^{-1}$, for which TOC removal reached around 75% (two-fold higher than that observed in non-catalysed reaction). Fig. 7.2 also represents the concentration of individual carboxylic acids. The strong influence of copper is apparent over oxalic and glyoxalic acids, the concentration of which decreased with the amount of added copper and became completely removed for 20 $\mu g \cdot L^{-1}$ of the catalyst. The depletion of both acids fitted well with the observed TOC reduction indicating the mineralization of both acids. After the removal of these acids, no more

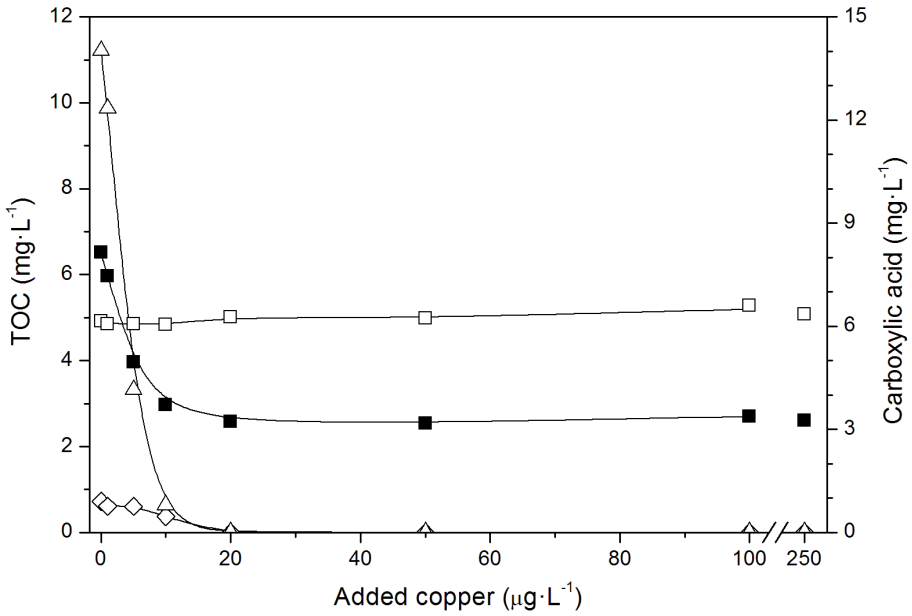


Fig. 7.2 Evolution of TOC (■) and the concentration of acetic (□), glyoxalic (◊) and oxalic (Δ) acid with added copper in the synthetic water matrix. Ozone dosage 71 $mg \cdot L^{-1}$

TOC depletion took place and the only acid detected was acetic acid, whose contribution to final TOC was essentially 100%. Oxalic acid accumulated in direct ozonation and got depleted in catalytic ozonation due the ability of copper in catalyzing its decomposition.

The two major mechanisms proposed in the literature for the homogeneous catalytic ozonation are the decomposition of ozone by metal ions leading to the generation of radicals and the formation of complexes between catalysts and the organic molecule followed by the oxidation of the former (Nawrocki and Kasprzyk-Horden, 2010). In order to elucidate the reaction pathway of copper oxalate, catalytic ozonation runs were carried out using *t*-butanol (30 mM) as a radical scavenger. The presence of *t*-butanol did not inhibit oxalate depletion, confirming that the catalysed reaction does not proceed by radical pathway but via complex formation (Nawrocki and Kasprzyk-Horden, 2010). Some authors claim that oxalic acid reacts relatively slow with hydroxyl radicals (Pines and Reckhow, 2002 and Petre *et al.*, 2013). Other works suggest that the catalytic ozonation of oxalate occurs via complex formation, which is in good agreement with our findings (Pines and Reckhow, 2002 and Beltrán *et al.*, 2005). MINTEQ chemical equilibrium model was used to calculate the chemical speciation of copper (Gustafsson, 2013). The modelling results of the synthetic water matrix showed that most copper concentration was present as oxalate complexes in the initial mixture (13 out of 20 $\mu g \cdot L^{-1}$, see Table 7.2), which displaced bicarbonate, the predominant complexing anion in the absence of oxalate. This fact is interesting because of the ubiquitous presence of radical scavengers in natural water and wastewater (mainly carbonates and bicarbonates), which could hamper the oxidation through hydroxyl radicals (Beltrán *et al.*, 2005 and Katsoyiannis *et al.*, 2011). Copper catalyst is highly active for the depletion of oxalic acid and, contrary to other transition metals, it is active at the natural pH of most surface waters and wastewaters (Beltrán *et al.*, 2005 and Nawrocki and Kasprzyk-Horden, 2010). It is also interesting to point out that the low concentration of homogeneous copper necessary for oxalic depletion should be taken into account while testing heterogeneous copper catalysts because a small leaching of the active could represent an important contribution.

Table 7.2 Predicted copper species concentration ($\mu g \cdot L^{-1}$) at different added copper concentration in synthetic water matrix as calculated by Visual MINTEQ 3.1.

Copper species	Added copper ($\mu g \cdot L^{-1}$)						
	1	5	10	20	50	100	250
Cu^{2+}	0.01	0.04	0.08	0.16	0.40	0.80	2.08
$CuOH^{+}$	0.01	0.06	0.13	0.26	0.65	1.31	3.40
$CuCO_3$(aq)	0.31	1.57	3.15	6.32	15.92	32.23	83.44
Cu-$(Oxalate)_2^{2-}$	0.47	2.33	4.65	9.29	23.08	45.72	110.96
Cu-Oxalate (aq)	0.19	0.93	1.85	3.71	9.27	18.56	46.54

3.2. Wastewater matrix

3.2.1. Non-catalytic ozonation

In this study we used real biologically treated wastewater as an alternative matrix for the carboxylic acids ozonation. The organic compounds present in the matrix before adding the organic acids were essentially refractory to ozonation under the working condition used in this study, achieving a mineralization value lower than 5% (Fig. 7.3). However, ozone was consumed up to 15 $mg \cdot L^{-1}$ as a result of partial oxidation reactions, which can be traced by the reduction (65%) of the specific ultraviolet absorption at 254 nm ($SUVA_{254}$); the parameter that provided an indirect measure of the

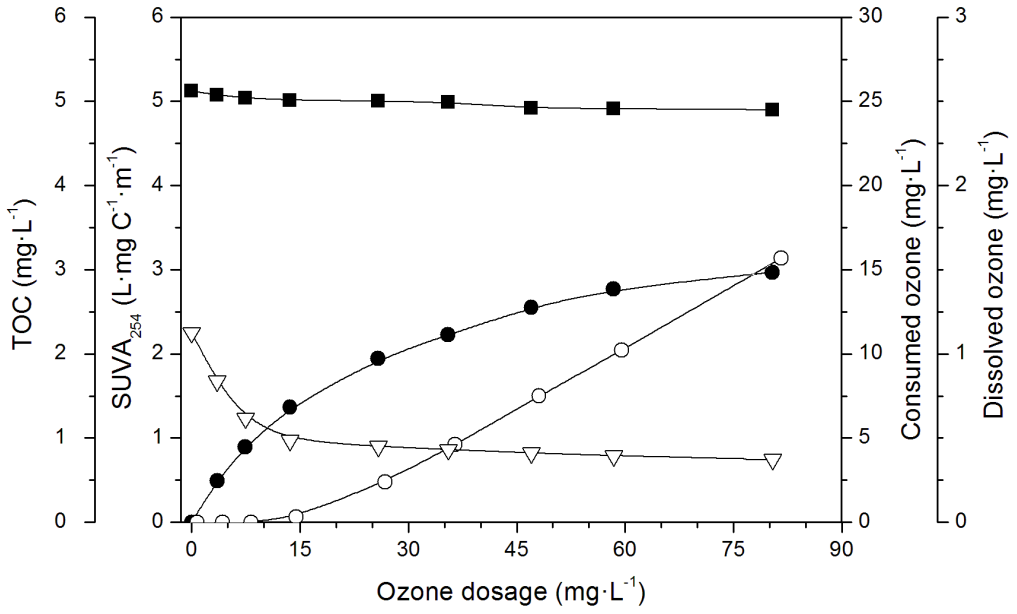


Fig. 7.3 Evolution of TOC (■), $SUVA_{254}$ (▽), consumed (●) and disolved (○) ozone for different ozone dosages in STP effluent.

aromaticity of the dissolved organic matter. The refractory character of wastewater TOC was previously reported (Rosal *et al.*, 2008).

The evolution of TOC and the ozone consumption during ozonation in the wastewater matrix spiked with organic acids are represented in Fig. 7.4A. A similar behaviour to the synthetic matrix (Fig. 7.1A) was observed. For lower ozone dosages (zone 1) TOC decreased with increasing ozone up to a value of 47 $mg \cdot L^{-1}$ for the latter

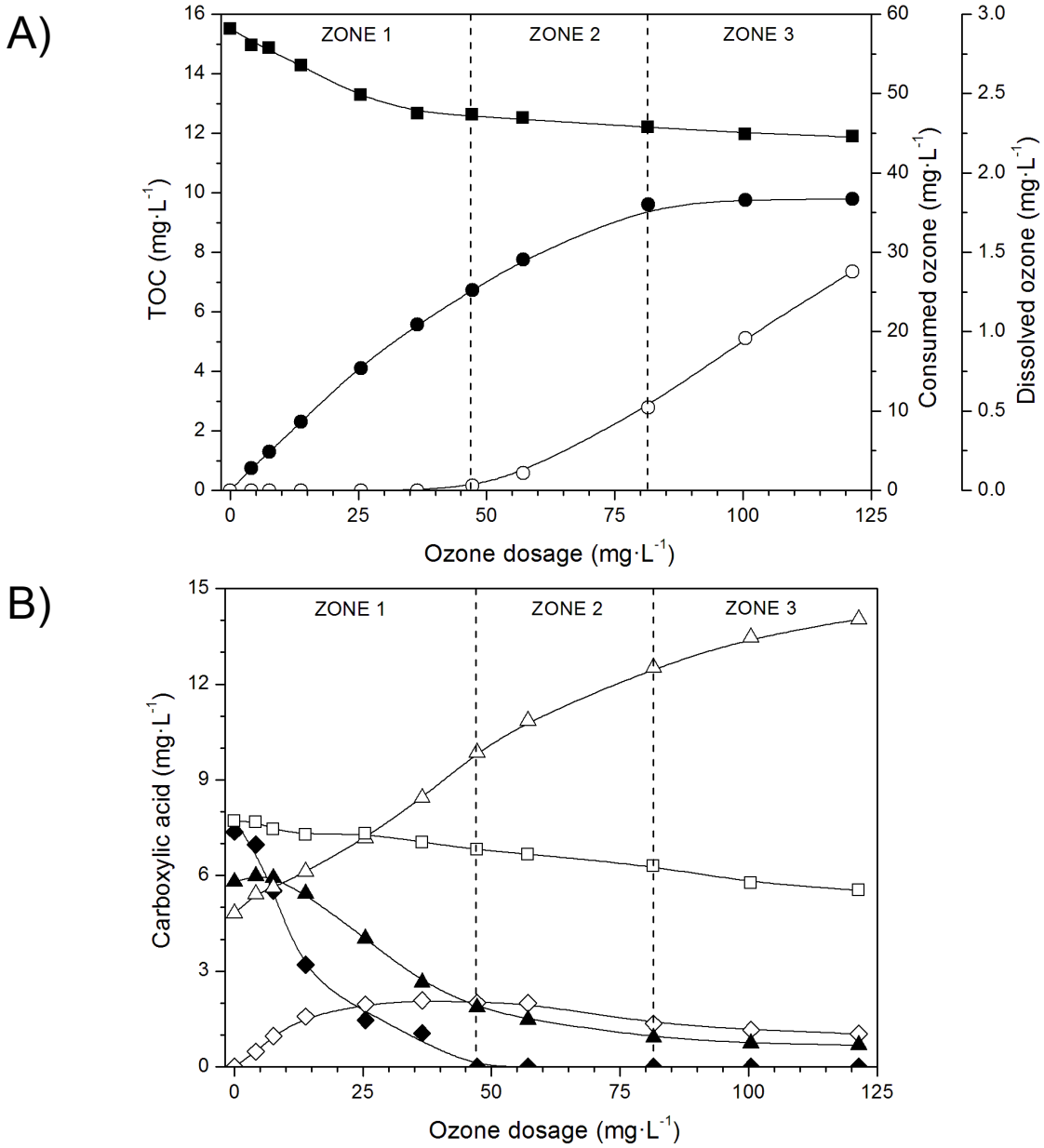


Fig. 7.4 Evolution of (A) TOC (■), consumed (●) and dissolved ozone (○) and (B) the concentration of acetic (□), glyoxalic (◊), formic (▲) maleic (♦) and oxalic acid (Δ) for different ozone dosages in STP effluent.

and remained constant afterwards. After the ozone dosage of 81 $mg \cdot L^{-1}$ mineralization did not further proceed. This value was taken as a reference for the treatments in the wastewater matrix described below. The maximum TOC removal was 22%, considerably lower than that observed in the synthetic matrix. Taking into account the refractory character of organic natural matter present in wastewater, it can be argued that TOC removal corresponded essentially to the depletion of the acids added to the matrix. Maximum ozone consumption was 37 $mg \cdot L^{-1}$, which was higher than the value obtained in the synthetic matrix and close to the sum of consumed ozone by the matrix, 15 $mg \cdot L^{-1}$ (Fig. 7.3), and by the depletion of carboxylic acids, 20 $mg \cdot L^{-1}$ (Fig. 7.1A). Fig. 7.4B represents the evolution of individual acids with increasing ozone dosage. The pattern was similar to that found in the synthetic water matrix with maleic acid being readily eliminated. Glyoxalic acid also appeared as an oxidation by-product and was further oxidized to oxalic acid, which accumulated steadily in treated wastewater. The final concentration of glyoxalic acid was noticeably higher in STP effluent and contrary to the synthetic matrix, formic acid was only partially removed.

3.2.2. Catalytic ozonation in wastewater

The catalytic ozonation in wastewater was carried out using copper concentrations ranging from 10 to 500 $\mu g \cdot L^{-1}$ and a fixed ozone dosage of 81 $mg \cdot L^{-1}$. Fig. 7.5 displays the evolution of TOC with the increasing copper concentration. Similarly to the synthetic matrix, a strong improvement of TOC depletion (around 45%) was achieved with the increasing catalyst concentration. The evolution of TOC can be explained as following that of individual acids also shown in Fig. 7.5. The concentration of glyoxalic acid increased initially to decrease when a higher amount of copper was added. The concentration of formic acid slightly increased with the copper concentration probably indicating that formic acid is a by-product of the oxidation of organic matter present in wastewater. Nevertheless, the main contribution to TOC depletion was due to the removal of oxalic acid. It is noteworthy that total oxalic acid depletion was not achieved even at the highest copper concentration (500 $\mu g \cdot L^{-1}$). No improvement was found in oxalic acid depletion for copper concentration above

100 µg·L^{-1}, which is five-fold the concentration required in the synthetic water matrix. The chemical copper speciation (MINTEQ model) in STP effluent using the available data (see Table 7.1) and common assumptions on the nature of organic matter in wastewater effluents (Pernet-Coudrier *et al.*, 2008), leads to a concentration of copper-oxalate complexes of 12 µg·L^{-1} at operational copper concentration of 100 µg·L^{-1} (Table 7.3). This value was near to the amount of copper-oxalate complexes (13 µg·L^{-1}) obtained for the synthetic matrix adding 20 µg·L^{-1}of copper.

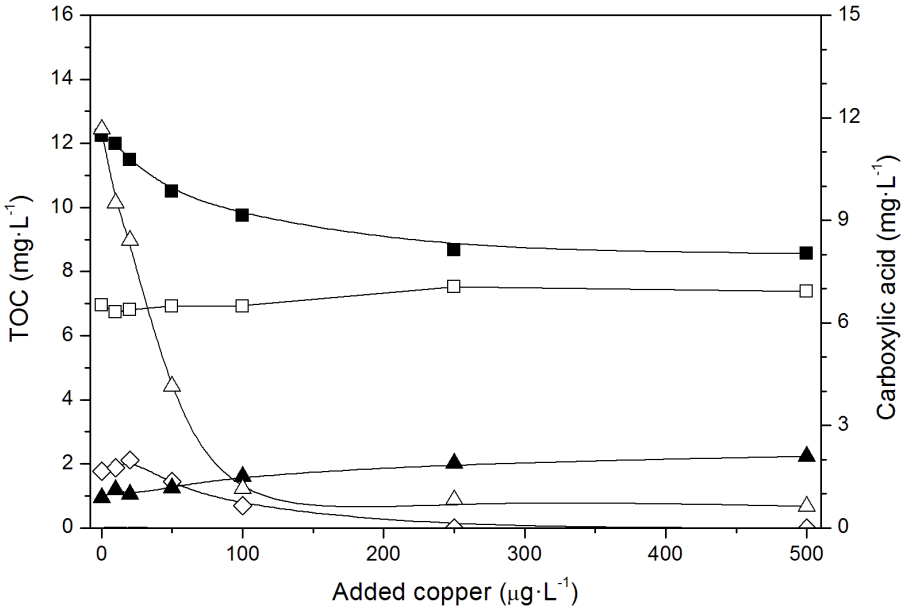


Fig. 7.5 Evolution of TOC (●) and the concentration of acetic (□), glyoxalic (◊), formic (▲) and oxalic (Δ) acid with added copper in STP effluent. Ozone dosage 81 mg·L^{-1}.

Table 7.3 Predicted copper species concentration (µg·L^{-1}) in STP effluent at different added copper concentration in STP effluent as calculated by Visual MINTEQ 3.1.

Copper species	Added copper (µg·L^{-1})					
	10	20	50	100	250	500
Cu^{2+}	0.01	0.07	0.57	2.14	9.08	23.2
$CuOH^{+}$	<0.01	0.03	0.22	0.81	3.43	8.76
$CuCO_3$(aq)	0.10	0.56	4.37	16.32	69.14	176.41
Cu-$(Oxalate)_2^{2-}$	0.03	0.15	1.14	4.23	17.58	43.15
Cu-Oxalate (aq)	0.04	0.25	1.95	7.26	30.46	76.27
Cu-EfOM[a]	9.81	18.91	41.50	68.32	116.41	162.18

[a] EfOM: STP effluent organic matter

3.3. Aquatic toxicity assessment

The minimum amount of copper used in this work which achieved the highest TOC depletion for synthetic matrix and STP effluent, 20 and 100 μg·L^{-1}, were well below the standard water quality regulated or recommended for different uses of reclaimed water. US EPA recommends a maximum of 200 (long-term) or 5000 μg·L^{-1} (short-term) of copper in water reused for irrigation (US EPA, 2004). Nonetheless, in spite of the good activity of copper catalysts in ozonation processes, concern about toxicity of treated water must be addressed in order to ensure the absence of negative impacts on receiving water bodies.

Aquatic toxicity data show that both water matrices, the mixture of organic acids and non-catalytically ozonated water did not present noticeable toxic effects on single-species tests (Table 7.4). On the contrary, the studied organisms were sensitive to copper presence as demonstrated by the low EC_{50} values in the three water matrices: Milli-Q water, synthetic matrix (*i.e.*, Milli-Q water with 276 mg·L^{-1} of $NaHCO_3$) and STP effluent. For ultrapure water, the reported aquatic toxicity values are in agreement with the data in the literature for *V. fischeri* (EC_{50} = 640 μg·L^{-1} in Heinlaan *et al.* (2008) and 740 μg·L^{-1} in Lappalainen *et al.* (2001)), *T. thermophila* (EC_{50} = 470 μg·L^{-1} in Gallego *et al.* (2007)), *P. subcapitata* (EC_{50} = 16.5 μg·L^{-1} in Heijerick *et al.* (2002) and 20 μg·L^{-1} in Aruoja *et al.* (2009)) and *D. magna* (EC_{50} = 18 μg·L^{-1} in Kim *et al.* (2006) and 24 μg·L^{-1} in Postma *et al.* (2009)).

The evolution of the effects of catalytically ozonated samples for different amounts of copper in synthetic water and STP effluent on the battery of biotests are presented in Fig. 7.6. Increased concentration of copper caused an increase in the toxic effects of studied organisms except for *V. fischeri*. These data suggest that, regardless of the possible combined effect of other compounds present or formed during ozonation, copper appeared to be the main source of toxicity. It is also interesting to note that very low amounts of copper led to a hormetic effect on both matrices with remarkable stimulation on *P. putida* and *P. subcapitata* growth, most probably due to the

assimilable organic matter (Thayanukul *et al.*, 2013), bicarbonate (Luzhøft *et al.*, 1999) and/or extra amounts of nitrate and phosphate (Selivanovskaya *et al.*, 2004).

Table 7.4 Effects of acid mixture addition and copper EC_{50} in three water matrices for the bioassay battery (mean ± 95% confidence interval).

	Bioassay				
Inhibition/ immobilization (%)	*V. fischeri*	*P. putida*	*T. thermophila*	*P. subcapitata*	*D. magna*
Synthetic matrix[a]	-2 ± 1	-10 ± 3	8 ± 1	-10 ± 1	6 ± 2
STP effluent	13 ± 4	-21 ± 1	15 ± 3	-40 ± 5	3 ± 1
Acid mixture in MQ water[b]	4 ± 1	-5 ± 1	5 ± 3	5 ± 2	12 ± 3
Acid mixture in synthetic matrix	-8 ± 1	-13 ± 4	10 ± 1	-12 ± 2	5 ± 1
Acid mixture in STP effluent	5 ± 2	-17 ± 3	9 ± 3	-45 ± 4	0 ± 1
Ozonated acid mixture in synthetic matrix[c]	-14 ± 5	9 ± 6	8 ± 3	-15 ± 7	10 ± 3
Ozonated acid mixture in STP effluent[d]	5 ± 3	-16 ± 4	6 ± 2	-42 ± 6	10 ± 2
Copper EC_{50} (µg·L^{-1})	*V. fischeri*	*P. putida*	*T. thermophila*	*P. subcapitata*	*D. magna*
In MQ water	820 ± 90	29.5 ± 3.5	400 ± 38	20.6 ± 2.6	20.5 ± 3.9
In synthetic matrix	1750 ± 180	21.9 ± 2.6	306 ± 41	29.8 ± 3.5	51.1 ± 8.8
In STP effluent	1730 ± 210	19.1 ± 3.6	284 ± 50	53.9 ± 9.5	293 ± 33

[a] Milli-Q water buffered with 276 mg·L^{-1} of $NaHCO_3$.
[b] Mixture of formic, acetic, oxalic and maleic acid with a concentration of 7 mg·L^{-1} each.
[c] Ozone dosage in non-catalytic process 71 mg·L^{-1}.
[d] Ozone dosage in non-catalytic process 81 mg·L^{-1}.

As can also be seen in Fig. 7.6, copper-catalysed samples caused a considerably lower toxicity in STP effluent than in the synthetic matrix, with the water matrix effect ratio (the ratio in terms of added copper in STP effluent and synthetic matrix in order to obtain a 50% of inhibition/immobilization) in the interval 1.6–4.4 except for *V. fischeri*. It is important to stress that the impact of copper on aquatic organisms does not only depend on its nominal concentration, but also on its bioavailability, which is influenced by water quality parameters such as pH, hardness, alkalinity and dissolved organic matter. Copper has been described as presenting high complexation capacity with both

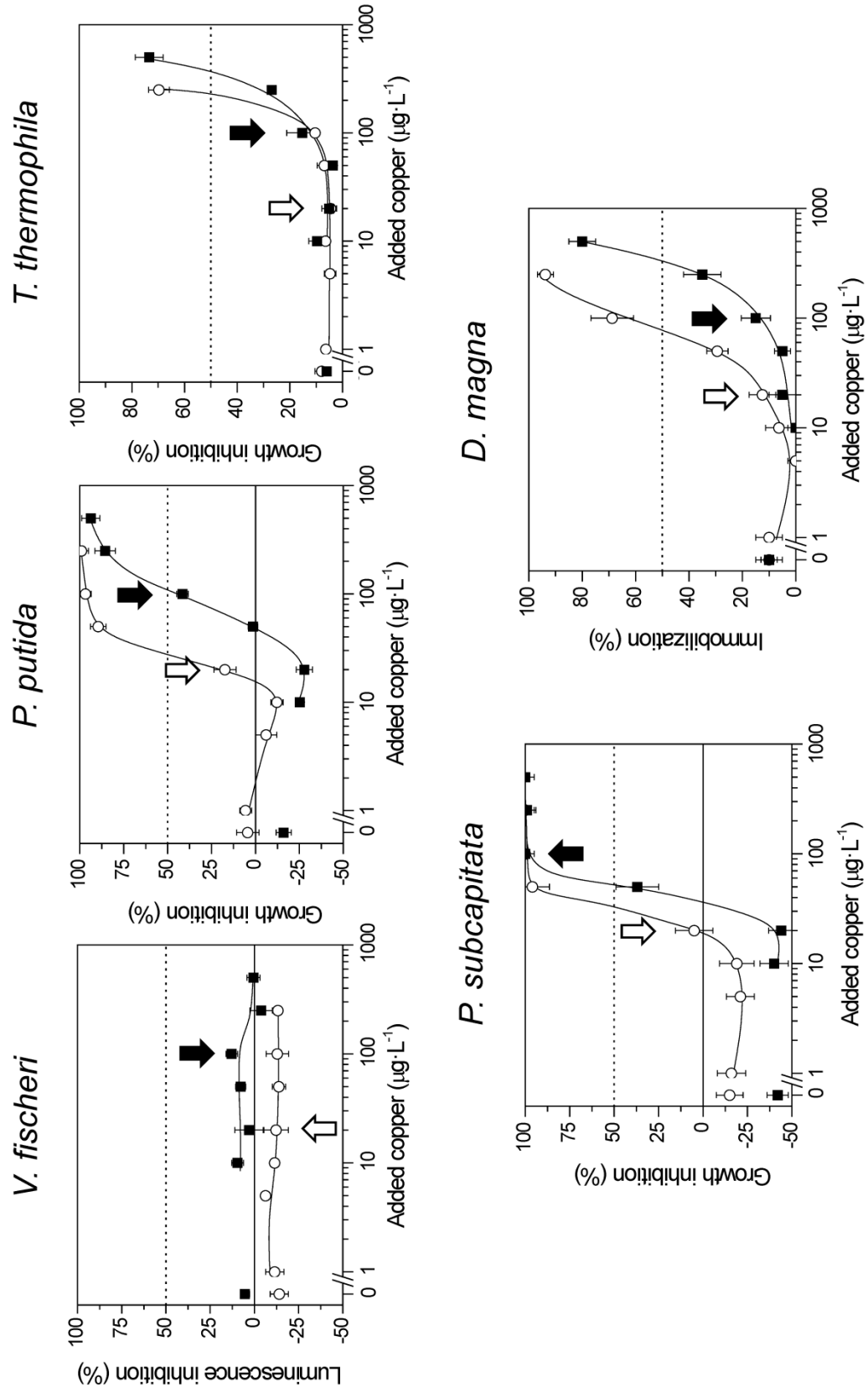


Fig. 7.6 Evolution of the effects of catalytic ozonated samples for different amount of added copper in the synthetic water matrix (○) and STP effluent (■) (mean ± 95% confidence interval). White and black arrows represent the operational concentration of added copper for synthetic and STP effluent, respectively.

inorganic (Stumm and Morgan, 1996) and organic ligands (Sarathy and Allen, 2005 and Karlsson *et al.*, 2006), which influences its effect on biological organisms. It has also been noted that the presence of natural organic matter considerably reduces copper toxicity to *V. fischeri* (Stauber, 2000 and Hsieh *et al.*, 2004). Heijerick *et al.* (2002) revealed that copper toxicity in natural waters to *P. subcapitata* (32–245 $\mu g \cdot L^{-1}$) is mainly determined by the concentration of dissolved organic carbon. Naddy *et al.* (2002) and De Schamphelaere and Jansen (2002) showed that copper EC_{50} values for *D. magna* in artificial media without organic matter vary between 4 and 57 $\mu g \cdot L^{-1}$. For natural water and wastewater, the presence of dissolved organic matter drastically decreases copper toxicity (34–1086 $\mu g \cdot L^{-1}$) as a consequence of copper-complexation (De Schamphelaere *et al.*, 2004 and Pernet-Coudrier *et al.*, 2008). Moreover, as the water matrix changes during the ozone treatment, the copper speciation also changes and consequently, so does the water toxicity. Thus, in the synthetic matrix, the sharp increase in the response curves (Fig. 7.6) started at a copper concentration of about 20 $\mu g \cdot L^{-1}$. For lower concentrations, oxalic and glyoxalic acid, whose copper complexation capacity is high (Petre *et al.*, 2013), were present in the mixture and probably contributed to a reduced copper bioavailability. For increased amounts of copper the main organic acid was acetic, whose complexation capacity is low (Bryan *et al.*, 2002), and a sharp toxicity increase was obtained accordingly. In STP effluent, the steep toxicity increase takes place at doses above 100 $\mu g \cdot L^{-1}$ except for *P. putida* and *P. subcapitata*, the organisms with higher sensitivity for copper in this water matrix.

Focus on the effects of the minimum amount of copper used to achieve the highest TOC depletion on the single species tests (see arrows in Fig. 7.6); catalytic ozonation in the synthetic matrix adding 20 $\mu g \cdot L^{-1}$ generated treated water with no significantly different inhibition/immobilization with respect to samples obtained from non-catalytic ozonation, causing an inhibition/immobilization below 15% for all single species tests. Otherwise, catalytically ozonated water from STP effluent using 100 $\mu g \cdot L^{-1}$ was notably toxic to *P. putida* (42% growth inhibition) and, particularly, to *P. subcapitata* (100% growth inhibition). For the rest of the species, the effect was below 15%. Despite adding an amount of catalyst five-fold higher in STP effluent than in synthetic water, the

toxicity was not affected in the same proportion as a consequence of the above-explained matrix effects.

4. Conclusions

Copper-catalysed continuous ozonation significantly improves organic acid mineralization, mainly due to its high performance in oxalic acid depletion at near neutral pH, with short reaction time and in water matrices with high scavenging rate.

The same copper concentration is less toxic in STP effluent than in the synthetic water matrix, an effect attributed to copper complexation with organic and inorganic compounds present in the wastewater that reduce its bioavailability.

Catalytic ozonation is also strongly influenced by the water matrix. The copper catalytic reaction proceeds through a selective complex reaction pathway so that complexation with STP effluent organic matter reduces the availability of metal for catalysis. Thus, in wastewater, a five-fold copper concentration is necessary to achieve similar oxalic depletion to that obtained in the synthetic water matrix.

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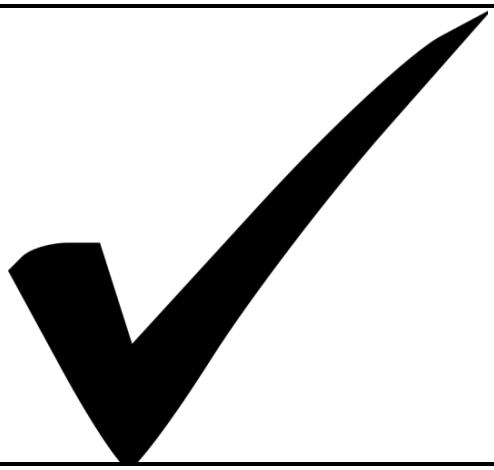
Chapter 8

Conclusions and outlooks



CONCLUSIONS AND OUTLOOKS

8.1. Conclusions

The current study set out to assess the potential environmental risks of emerging substances, such as personal care product preservatives and antibiotics, and to gain a deeper understanding of the continuous ozonation process for upgrading conventional STPs in order to minimize the discharge of these micropollutants to the receiving water bodies. Emerging pollutants enter into water bodies mainly through conventional STPs, where most of them are not efficiently removed. One way of minimizing the input of these micropollutants to the aquatic environment is to integrate additional treatment steps at STPs such as ozonation. The results highlight that:

1. Considerable aquatic toxicity of preservatives and antibiotics were observed on indigenous biological communities. The toxic effects of these mixtures need to be carefully evaluated on biological communities and any potential risk management options should be studied. Special attention may be placed on benzalkonium chloride and ofloxacin, the risk drivers of the mixture, on which should be performed mitigation measures such as source control by targeted restrictions and/or conventional STP upgrading for improving removal efficiencies of these micropollutants.

2. These risk driver emerging pollutants were effectively removed by continuous ozonation by means of the combined attack of ozone and hydroxyl radicals. Ozone treatment did not lead to a complete mineralization of the aforementioned micropollutants with the consequent accumulation of a mixture of intermediate transformation products and ozone-refractory compounds.

3. Liquid chromatography coupled to mass spectrometry (LC-ESI-MS(TOF), LC-ESI-MS(QTOF)) allows us to propose molecular structures for transformation products and degradation pathways for the removal of benzalkonium chloride and ofloxacin. The identified transformation products indicated that an

ozone/hydroxyl radical attack takes place on moieties directly responsible for the parent molecules' biological activities as well as leading to more polar molecules.

4. The further oxidation of intermediate transformation products gives rise to low molecular weight by-products such as carboxylic acids, which are accumulated in treated waters due to their refractory character towards ozonation. The copper-catalysed continuous ozonation process significantly improves the mineralization of organic acids, mainly because of its high performance levels in oxalic acid depletion.

5. Water matrix has a strong influence on both the ozone dose required for the removal of emerging pollutants and the optimum catalyst dose necessary to achieve a given degree of mineralization. Occurrence of dissolved organic carbon, suspended solids and reduced nitrogen species notably increases ozone dose as a consequence of competition reactions for ozone between water matrix components and target pollutants. Complexation of copper with STP effluent organic matter reduces the availability of metal for catalysis.

6. In the present study, the toxic effects of wastewaters were reduced proportionally with the depletion of the parent compounds. Toxicity values of ozonated waters were caused predominantly by the parent compounds, whereas the formation of transformation products did not significantly contribute to mixture toxicity. Nevertheless, ozone treated samples induced changes on the biodiversity of natural bacterial communities, mainly caused by easily assimilable compounds generated in the ozonation process. On the other hand, the degradation of pollutants that interact with engineered nanoparticles present in wastewaters, such as benzalkonium chloride, might cause an increase in the toxic-metal leaching from the nanomaterial and consequently, a toxicity enhancement in treated water.

As a general conclusion, it may be stated that continuous ozonation is clearly a suitable technology for upgrading conventional STPs, primarily due to its ability to minimise the release into receiving water bodies of emerging pollutants which pose a

risk to the freshwater ecosystem. For other emerging pollutants with potential environmental risks, the outline followed in the current study (risk assessment - continuous ozonation - chemical analysis - aquatic toxicity assessment) could be performed, with careful consideration given to any potential impacts on a case by case basis.

8.2. Outlooks

On the basis of the results from the present study several suggestions for the future research can be made, either to fill current knowledge gaps or to illustrate any emerging issues:

1. Engineered nanoparticles, in similar fashion to other emerging substances, enter into STPs or the aquatic environment as a part of a complex mixture. Co-occurrence with other compounds can influence their fate and toxicity. Research is needed to determine effective methods to detect and quantify engineered nanoparticles in environmental media and gain deeper understanding of their toxic mode of action. This valuable information can be used to study the joint effects of chronic exposures of nanomaterials and the co-existing emerging substances to obtain a comprehensive understanding of their potential hazards and any risks on the process performance of activated sludge and the aquatic ecosystem.

2. The overuse and/or misuse of antibiotics as well as their incomplete metabolization has led to the emergence and rapid spread of antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs). Recent concerns about the effects of ARB and ARGs released from STPs need to be addressed. Further research is required including the roll-out of proper monitoring programs and risk assessment in order to better protect public health and the natural ecosystem. The application of ozonation for the complete inactivation/elimination of ARB and ARGs in STP effluents should be intensely studied in order to establish the optimum conditions for its use.

3. Aquatic toxicity assessment of ozonated wastewater has been widely performed using single-species tests based on physiological or population-based parameters. Results of the present study suggest the need to use community assays based on structural endpoints for the continuous ozonation optimisation process in order to ensure adequate protection of the whole aquatic ecosystem. Further investigation on the chronic effect of ozone treated waters should be extended from single species testing to more complex experimental systems (*e.g.*, microcosms), or onto field evaluations in natural ecosystems.

ABBREVIATIONS

AF	Assessment factor
ARB	Antibiotic resistant bacteria
ARGs	Antibiotic resistance genes
AUC	Area under the curve
AOC	Assimilable organic carbon
AOX	Adsorbable organic halogens
AWC	Average well colour
BAC	Benzalkonium chloride
BNP	Bronopol
BOD	Biochemical oxygen demand
CA	Concentration addition
CAS	Chemical abstracts service
CI	Combination index
CMI/MI	Methylchloroisothiazolinone and methylisothiazolinone
COD	Chemical oxidation demand
CSTR	Continuous stirred tank reactor
D_m	Median dose
DBE	Double bond equivalent
DDD	Defined daily dose
DIU	Diazolidinyl urea
DOC	Dissolved organic carbon
DOM	Dissolved organic matter
DXY	Doxycycline
EC	European Community
EC_x	Effective concentration
ECHA	European Chemicals Agency
EMEA	European Medicines Agency
EQS	Environmental quality standards
ERY	Erythromycin
ESI	Electrospray ionization
EU	European Union
f_a	Fraction affected
f_u	Fraction unaffected
FNU	Formazin nephelometric unit
HPLC	High-performance liquid chromatography
IA	Independent action
IC	Ionic chromatography
IPBC	Iodopropynyl butylcarbamate
ICP-MS	Inductively coupled plasma mass spectrometry
ISO	International Organization for Standardization
IWW	Industrial wastewater
K_{OW}	Octanol water partition coefficient
LC-MS	Liquid chromatography coupled to mass spectrometry
LOEC	Lowest observed effect concentration

LOQ	Limit of quantification
MEC	Measured environmental concentration
MLSS	Mixed liquor suspended solids
MNZ	Metronidazole
nMDS	Non-metric multidimensional scaling
NOEC	No observed effect concentration
ND	Not detected
NTU	Nephelometric turbidity units
NPs	Nanoparticles
OD	Optical density
OECD	Organisation for Economic Co-operation and Development
OFX	Ofloxacin
PAC	Activated carbon adsorption
PEC	Predicted environmental concentration
pK_a	Acid dissociation constant
PNEC	Predicted no effect concentration
POP	Persistent organic pollutant
PPB	Propylparaben
PPCP	Pharmaceuticals and personal care product
QAC	Quaternary ammonium compound
QTOF/MS	Quadrupole time-of-flight mass spectrometry
REACH	Registration, Evaluation and Authorization of Chemicals
RQ	Risk quotient
SDA	Sequential deletion analysis
SEPA	Swedish Environmental Agency
SMX	Sulfamethoxazole
SPE	Solid phase extraction
SRM	Selected reaction monitoring
STP	Sewage treatment plant
STU	Sum of toxic unit
SUVA	Specific ultraviolet absorption
TCS	Triclosan
TIS	Toxicant-induced succession
TMP	Trimethoprim
TOC	Total organic carbon
TOF/MS	Time-of-flight mass spectrometry
TP	Transformation product
TSQ/MS	Triple-stage quadrupole mass spectrometry
TSS	Total suspended solids
TU	Toxic unit
UNEP	United Nations Environmental Programme
US EPA	United States Environmental Policy Agency
VSS	Volatile suspended solids
WFD	Water framework directive
ZPT	Zn pyrithione