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CONTRIBUTION A L'EVALUATION DES RISQUES ECOTOXICOLOGIQUES DES EFFLUENTS HOSPITALIERS : BIOCONCENTRATION, BIOACCUMULATION ET BIOAMPLIFICATION DES RESIDUS PHARMACEUTIQUES

le 26/06/2015 devant le jury composé de :

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Articles publiés ou à soumettre dans un journal national ou international avec comité de lecture

Orias F., Rambla M., Rodriguez J., Brosselin V., Guillou C., Perrodin Y., *à soumettre*. Comparative acute ecotoxicity of tamoxifen citrate and amiodarone hydrochloride and their respective active ingredients.

Perrodin Y., Bazin C., Orias F., Bastide T., Wigh A., Berlioz-Barbier A., Vuillet E., Wiest L. *in press*. A posteriori assessment of ecotoxicological risks linked to building a hospital, *Chemosphere*

Orias F., Simon L., Perrodin Y., 2015. Respective contributions of diet and medium to the bioaccumulation of pharmaceutical compounds in the first levels of an aquatic trophic web. *Environmental Science and pollution Research Disponible en ligne depuis le 28 août 2015*

Orias F., Simon L., Mialdea G., Clair A., Brosselin V., Perrodin Y., 2015. Bioconcentration of ^{15}Ntamoxifen at environmental concentration in liver, gonad and muscle of *Danio rerio*. *Ecotoxicology and Environmental Safety* **120** : 457–462

Perrodin Y., Orias F., Boillot C., Brackers de Hugo A., Jean J., Panouilleres M., Emmanuel E. 2015. Évaluation des risques écotoxicologiques liés aux effluents hospitaliers et recommandation de gestion. *Revue des Sciences de l'Eau* **28** : 211–280

Orias F., Bony S., Devaux A., Aubrat M., Durrieu C., Hombert T., Wigh A. Perrodin Y., 2015. Tamoxifen ecotoxicity and resulting risks for aquatic ecosystems. *Chemosphere* **128** : 79–84.

Orias F., Simon L., Perrodin Y., 2015. Experimental assessment of the bioconcentration of ^{15}Ntamoxifen in Pseudokirchneriella subcapitata. *Chemosphere* **122** : 251–256.

Perullini M., Orias F., Durrieu C., Jobbágy M., Bilmes S. A., 2014. Co-encapsulation of *Daphnia magna* and Microalgae in silica matrices, a stepping stone toward a portable Microcosm. *Biotechnology Reports* **4** : 147–150.

Orias F. & Perrodin Y., 2014. Pharmaceuticals in hospital wastewater : their ecotoxicity and contribution to the environmental hazard of the effluent. *Chemosphere* **115** : 31–39.

Orias F. & Perrodin Y., 2014. Les résidus pharmaceutiques des effluents hospitaliers : Quels dangers pour les écosystèmes ? *L'Actualité Chimique* **381** : 17–23.

Orias, F. & Perrodin Y., 2013. Characterisation of the ecotoxicity of hospital effluents : A review. *Science of The Total Environment* **454 - 455** : 250–276.

Communications orales dans un congrès national ou international avec comité de lecture

Orias F. « Bioconcentration des résidus pharmaceutiques des effluents hospitaliers » *In* : Orias F., Simon L. Perrodin Y., *Conférence Eau & Santé : Les médicaments dans le cycle urbain de l'eau*, 26-27 mars 2015, Genève-Annemasse.

Perrodin Y., « Ecotoxicological Risk Assessment of Hospital Waste Water and Management Recommendations » *In* : Perrodin Y., Orias F., Boillot C., Jean J., Brackers de Hugo A., Angerville R., Panoullières M., Emmanuel E., *10th International congress of the GRUTTEE*, Octobre 2014, Limoges.

Orias F. « Suivi de la bioconcentration d'un résidu pharmaceutique grâce aux isotopes stables. » *In* : Orias F., Simon L. Perrodin Y., *Colloque de la SEFA*, 1-2 juillet 2014, Besançon.

Orias F. « Ecotoxicity of the pharmaceuticals compounds of hospital effluent » *In* : Orias F. & Perrodin Y., *Pharmaceuticals in the Environment : Is there a problem ?*, 3-4 juin 2013, Nîmes.

Communication par affiche dans un congrès national ou international

Orias F., Simon L., Perrodin Y., « Comparison of ^{15}N tamoxifen bioconcentration in aquatic organisms (microalgae, daphnids and fishes) », *SETAC Europe 25th Annual Meeting - Environmental protection in a multi-stressed world : challenges for science, industry and regulators.* 3-7 mai 2015, Barcelone, Espagne.

Orias F., Simon L., Perrodin Y. « Étude de la bioconcentration, de la bioaccumulation et de la bioamplification par marquage isotopique. », *Colloque d'Ecophysiologie Animale*, 7-9 nov. 2013, Lyon.

Clément B., Herbach U., Lamonica D., Orias F., Lopes C., Nézondet F., Charles S., « Towards ecotoxicological modelling in a laboratory microcosm - a first step focused on daphnid-algae interactions. » *SETAC Europe 23rd Annual Meeting - Building a Better Future : Responsible Innovation and Environmental Protection.* 12-16 mai 2013, Glasgow, UK.

Résumé-Abstract

Contribution à l'évaluation des risques écotoxicologiques des effluents hospitaliers : bioconcentration, bioaccumulation et bioamplification des résidus pharmaceutiques

Les hôpitaux génèrent des effluents riches en résidus pharmaceutiques (RP), fonctions de leurs activités de soins et de diagnostic. Certains de ces RP sont aujourd'hui retrouvés de manière ubiquitaire dans les écosystèmes aquatiques, en raison de leurs propriétés persistantes et/ou de leur émission continue. La variété de ces RP est telle qu'il est nécessaire de les hiérarchiser, en fonction des risques qu'ils représentent pour l'Environnement, à des fins d'étude et de gestion. Un de ces risques est le transfert des RP bioaccumulables (i.e. K_{ow} élevé et faible biodégradabilité) dans les chaînes alimentaires, via les processus de bioconcentration, de bioaccumulation et de bioamplification. L'objectif principal de cette thèse est de caractériser expérimentalement la bioconcentration et la bioaccumulation de molécules identifiées comme prioritaires dans des travaux précédents. Le composé modèle que nous avons choisi est le tamoxifen, molécule utilisée contre le cancer du sein et déjà retrouvé dans l'Environnement. Les organismes étudiés, issus des trois niveaux trophiques de la chaine alimentaire modèle, sont *Pseudokirchneriella subcapitata*, *Daphnia magna* et *Danio rerio*. Pour mesurer la teneur de cette molécule dans les organismes, nous avons développé une méthode d'analyse reposant sur l'utilisation d'une molécule marquée par un isotope stable, le ^{15}N tamoxifen. Nous avons mesuré des facteurs de bioconcentration (BCF) allant de 12800 chez *D. magna* à 85600 dans le foie de *D. rerio* en passant par 21500 chez *P. subcapitata*. Chez ces derniers, nous avons également évalué la part du régime alimentaire dans la bioaccumulation du tamoxifen. Nous avons observé que plus la concentration dans le milieu d'exposition est faible, plus le régime alimentaire contribue à la bioaccumulation. Ces travaux de thèse présentent de nombreuses perspectives que l'on peut regrouper autour de deux axes : connaissance de l'écotoxicité des RP et de l'écotoxicologie isotopique.

Contribution to ecotoxicological risk assessment of hospital effluents : bioconcentration, bioaccumulation & biomagnification of pharmaceutical compounds

Hospitals generates effluents rich in pharmaceuticals compounds (PC), notably because of care and diagnostics activities. Some of these PCs are ubiquitous in aquatic ecosystems owing to its persistent properties and/or because of continuous releasing in environment. The diversity of these PCs is so strong that it is necessary to prioritize them, considering risks that PCs represents for the Environment, in order to manage and study these compounds. One of these risks is the transfer of bioaccumulatives PCs (i.e. PCs with high K_{ow} and low biodegradability) along trophic webs, via bioconcentration, bioaccumulation and biomagnification processes. The main objective of this thesis is to characterize bioconcentration and bioaccumulation of molecules identified as priority in previous studies. The model compound choose in our work is the tamoxifen, a molecule used in the treatment of breast cancer and already found in Environment. Organisms studied, typical from three trophic levels of the model trophic chain, are *Pseudokirchneriella subcapitata*, *Daphnia magna* and *Danio rerio*. In order to measure content of tamoxifen in organisms, we developed an innovative analytic method based on the use of stable isotopes labelled tamoxifen : ^{15}N tamoxifen. We succeeded to measure bioconcentration factors (BCF) from 12800 in *D. magna* to 85600 in liver of *D. rerio* including BCF of 21500 in *P. subcapitata*. In this latter, we also assessed the contribution of dietary route to the total contamination of *D. magna* by tamoxifen. We observed that the more the medium concentration was weak, the more the dietary route contribute to the contamination. These works shows numerous perspectives that we can gather inside two axes : ecotoxicity knowledge of PCs an isotopic ecotoxicology.

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Liste des abréviations

AFNOR	:	Association Française de Normalisation
BAF	:	Facteur de bioaccumulation
BCF	:	Facteur de bioconcentration
Cr	:	*Chlamydomonas reinhardtii*
Cv	:	*Chlorella vulgaris*
Dm	:	*Daphnia magna*
EH	:	Effluent hospitalier
HPLC	:	Chromatographie en phase liquide à haute performance
HQ	:	Quotient de danger
ISO	:	Organisation Internationale de Normalisation
K_{ow}	:	Coefficient de partage octanol/eau
MEC	:	Concentration mesurée dans l'Environnement
PCB	:	Polychlorobyphényle
PNEC	:	Concentration prédite comme sans effet
Ps	:	*Pseudokirchneriella subcapitata*
QSAR	:	Relation quantitative structure / activité
RP	:	Résidu pharmaceutique
STEP	:	Station d'épuration
TMX	:	Tamoxifen
Vf	:	*Vibrio fisheri*

INTRODUCTION GENERALE

De nos jours, les résidus pharmaceutiques (RP) sont omniprésents dans les écosystèmes aquatiques :

- **Le compartiment acqueux**. Au sein de ce compartiment central, nous pouvons distinguer deux sous-compartiments contaminés : (i) Les eaux de surface c'est-à-dire les lacs (Blair et al., 2013; Fick et al., 2009), les rivières (Kolpin et al., 2002; Loos et al., 2009) et les eaux estuariennes et côtières (Claessens et al., 2013; Thomas and Hilton, 2004) ; (ii) Les eaux profondes, c'est-à-dire les nappes phréatiques (Barnes et al., 2008; Loos et al., 2010).
- **Le compartiment sédimentaire**. De plus en plus d'études montrent que les sédiments sont contaminés par les RP, tant en milieu continental (Schultz et al., 2010; Silva et al., 2011) qu'en milieu estuarien (Vazquez-Roig et al., 2012; Yang et al., 2011).

Santos et al. (2010) ont publié une revue bibliographique qui recense notamment les concentrations auxquelles on retrouve les résidus pharmaceutiques dans différents environnements aquatiques (e.g. rivières, affluents et effluents de stations d'épurations, eaux profondes, sédiments, ...).

- **Le compartiment biologique**. En 2014, Zenker et al. (2014), ont publié une revue bibliographique qui regroupe l'ensemble des mesures des résidus pharmaceutiques dans les organismes vivants dans les rivières.

De nombreuses sources sont responsables de cette pollution aux RP (Figure 1). Il en existe 5 majeures :

- **Les effluents domestiques**. Les effluents domestiques correspondent à tous les effluents issus des habitations. En termes de flux, ces effluents représentent la plus grande source de résidus pharmaceutiques (Langford and Thomas, 2009). Quand les médicaments sont consommés, ils sont ensuite excrétés via l'urine et les fèces sous forme plus ou moins métabolisées : on parle alors de résidus pharmaceutiques ou de résidus médicamenteux. Une fois excrétés, ces RP se retrouvent dans les effluents domestiques qui sont eux même envoyés vers la station d'épuration. Une des sources de pollutions aux RP pour les écosystèmes peut intervenir à ce moment du processus d'acheminement des effluents domestiques. En effet, lorsque le réseau d'acheminement est unitaire, la station d'épuration est parfois « court-circuitée »lors des orages et une quantité non négligeable (jusqu'à 15%) d'effluent est directement émise vers le milieu récepteur (le plus souvent une rivière) sans aucun traitement (Chèvre et al., 2013). Lorsque le réseau d'acheminement est séparatif, les effluents domestiques sont traités au sein de la station d'épuration. Mais les stations d'épuration (STEP) ne sont pas conçues pour traiter des molécules aussi complexes et variées que celles présentes dans la pharmacopée mondiale. Ainsi, si les STEP peuvent être efficaces pour abattre à près de 100% certains RP (e.g. paracetamol), elles ne parviennent pas à traiter aussi efficacement tous les RP.

– **Les effluents d'industries pharmaceutiques**. Plusieurs études ont montré que les effluents de l'industrie de conditionnement et/ou de formulation des médicaments sont à l'origine d'une pollution des rivières non négligeable (Collado et al., 2014; Sanchez et al., 2011). En effet, malgré une pression réglementaire qui impose de traiter ces effluents, cela n'est pas toujours suffisant. Cardoso et al. (2014) illustrent qu'en fonction du type de médicament conditionné ou formulé sur un site donné, on peut observer des effets liés à l'activité de tel ou tel médicament sur les organismes vivants en aval du rejet de l'effluent (e.g. anabolisant qui augmente la masse des poissons touchés).

– **Les élevages et l'aquaculture** : Pour traiter les animaux d'élevages contre les pathogènes, les éleveurs ont recours à des médicaments vétérinaires qui, comme chez l'Homme, sont plus ou moins métabolisés et se retrouvent dans les lisiers une fois utilisés. De la même manière que dans les stations d'épurations à « usage humain », les stations de traitement de lisiers animaux ne peuvent pas éliminer 100% des résidus pharmaceutiques. De plus, certains lisiers sont simplement stockés dans des bassins et infiltrés dans le sol (Bartelt-Hunt et al., 2011). Ainsi, on pourra retrouver une quantité non négligeable de RP émise vers l'environnement (Sarmah et al., 2006). En ce qui concerne l'aquaculture, que ce soit en milieu marin ou continental, le problème est plus complexe puisque les fèces ne sont pas récupérées. Ainsi, que ce soit les RP ou les médicaments dispensés en excès, tous se retrouve directement dans l'environnement (Burridge et al., 2010; Kolodziej et al., 2004).

– **Les effluents hospitaliers**. En termes de concentrations, les effluents hospitaliers sont les plus concentrés en RP parmi les sources précitées (Verlicchi et al., 2010). Les établissements hospitaliers sont le siège d'activités très variées qui vont être responsables de l'extrême diversité de contaminants présents dans les effluents hospitaliers. Les hôpitaux sont des établissements accueillant du public et hébergeant des patients. La première activité va être à l'origine d'une pollution organique « classique »(i.e. lingerie, cuisine, eaux usées) assez proche de celles des effluents domestiques. Il existe au sein des hôpitaux des activités qui lui sont beaucoup plus spécifiques, telles que le nettoyage intensif des surfaces pour limiter l'apparition de maladies nosocomiales, ou encore telles que la désinfections de sondes médicales. On retrouvera alors dans les effluents des quantités extrêmement importantes de surfactants et de désinfectants, dont des produits chlorés. Mais les deux activités très spécifiques du milieu hospitalier qui concernent plus directement ces travaux de thèse sont les activités de soins et de diagnostic de pathologie. Ces activités vont être à la source d'émission de RP dans les effluents hospitaliers. Ensuite, ces effluents vont, dans la quasi-totalité des cas, directement, sans presqu'aucun traitement préalable, rejoindre les STEP via le même réseau de canalisation que les effluents domestiques. On retrouve ainsi les mêmes risques de pollution que ceux liés au

« court-circuit »de la STEP ou à l'inefficacité du traitement de la STEP. Notons qu'une partie des effluents hospitaliers subit tout de même un prétraitement : il s'agit des eaux usées issues de la médecine nucléaire. Les effluents issus de ce service sont simplement stockés plusieurs jours le temps que la radioactivité de l'iode utilisé soit redescendue à son seuil réglementaire. Après ce stockage, ces effluents rejoignent également la STEP par le réseau de canalisation municipal.

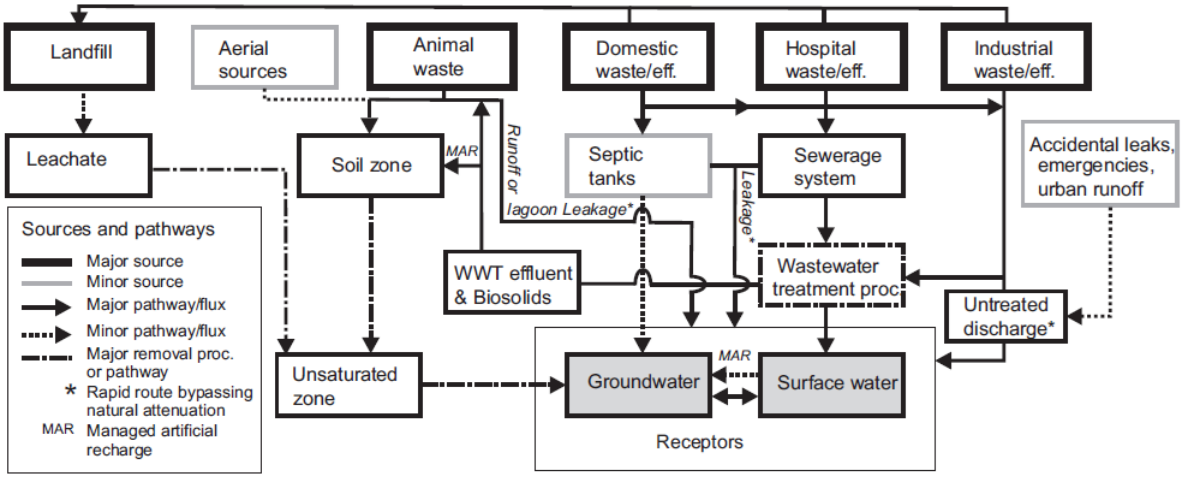

Figure 1 *Diagramme représentant les sources et les flux de RP dans les différents compartiments écosystémique (Lapworth et al., 2012)*

Compte-tenu du fort potentiel polluant de cette dernière source de RP, de plus en plus d'étude s'intéressent aux risques écotoxicologiques que peuvent représenter ces effluents (Brackers de Hugo et al., 2013; Emmanuel et al., 2005; Kümmerer, 2001; Verlicchi et al., 2012). Cependant, que ces risques soient envisagés selon une approche globale (i.e. matrice complète) ou spécifique (i.e. substance par substance), ce n'est que très récemment que les chercheurs se sont intéressés à la bioaccumulabilité des substances qui peuvent être présentes dans les effluents hospitaliers. Grâce à une approche théorique (i.e. « Quantitative Structure/Activity Relation »), Jean et al. (2012) ont établi une liste de 14 molécules bioaccumulables prioritaires à étudier (Tableau 1).

Table 1 *Liste des 14 molécules prioritaires (Jean et al., 2012). En gras les molécules les plus bioaccumulables de chaque catégorie.*

Forte consommation	Perturbation endocrinienne	Ecotoxicité potentielle
	Tamoxifen	
Amiodarone	Ethynilestradiol	**Ritonavir**
Dextropropoxyphene	Mitotane	Hexetidine
Desloratadine	Norgestimate	Itraconazole
Nicardipine	Amitryptiline	Telethromycine
	Mifepristone	

Dans un premier temps, ils ont sélectionné 70 molécules prioritaires sur la base de leur bioaccumulabilité évaluée à partir de leur lipophilie. Cette liste a été ensuite ramenée à 14 molécules prioritaires sur la base d'une analyse multicritère. Les molécules finales sélectionnées ont été classées dans 3 groupes en fonction du risque additionnel (en plus de la bioaccumulabilité) qu'elles pouvaient représenter pour les écosystèmes :
(i) molécules consommées en très forte quantité au sein des hôpitaux étudiés ; (ii) molécules présentant un risque de perturbation endocrinienne ; (iii) molécules présentant un potentiel écotoxicologique. Pour nos travaux, nous avons choisi les composés les plus bioaccumulables de ces 3 groupes de risque, respectivement, l'amiodarone, le tamoxifen et le ritonavir.

Pour mieux comprendre le fil conducteur qui a guidé mes travaux de thèse, le diagramme suivant (Figure 2) présente les objectifs fixés au début de la thèse avec les questions que nous nous sommes posées pour y répondre.

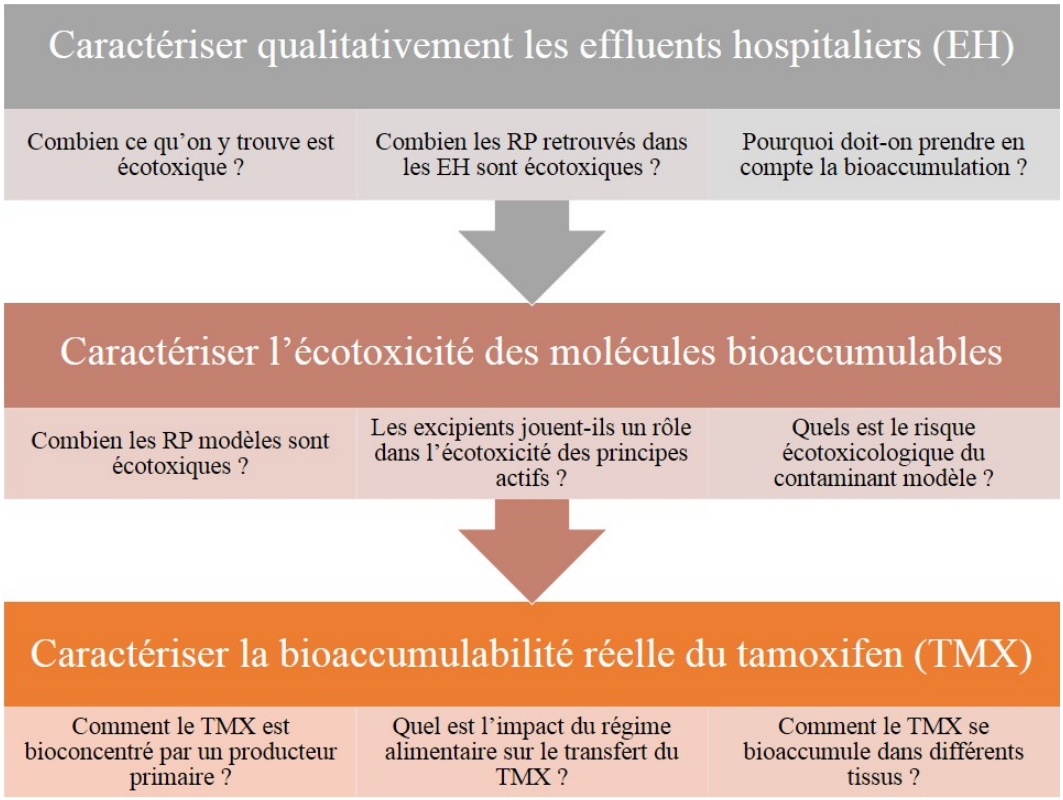

Figure 2 *Diagramme illustrant les objectifs de la thèse et les questions posées pour y répondre*

Dans la **première partie** de ce document, nous présentons un état de l'art en trois chapitres. Le **premier chapitre** correspond à une revue bibliographique sur la caractérisation des effluents hospitaliers ainsi que sur l'écotoxicité de tous les contaminants déjà mesurés dans les effluents hospitaliers. Le **deuxième chapitre** se focalise sur l'impact écotoxicologique des RP au sein de ces effluents particuliers. Enfin, le **troisième chapitre** correspond une revue historique de la Découverte et la Recherche autour des problématiques de bioconcentration, bioaccumulation et de la bioamplification des contaminants dans les organismes aquatiques.

Ensuite, dans une **deuxième partie**, nous présentons les résultats de l'étude de l'écotoxicité des trois contaminants modèles. Dans le **premier chapitre**, nous effectuons une batterie de bioessais afin de caractériser l'écotoxicité aiguë et chronique de chacun des contaminants choisis. Dans un **second chapitre**, nous vérifions la pertinence de l'utilisation des principes actifs plutôt que des médicaments (i.e. principe actif et excipient) dans les tests d'écotoxicité en comparant leurs écotoxicités respectives. Finallement, dans un **troisième chapitre**, nous avons évalué le risque écotoxicologique du contaminant modèle le plus toxique : le tamoxifen.

Enfin, dans une **troisième partie**, nous présentons les différentes expérimentations relatives à la bioconcentration et à la bioaccumulation de notre contaminant modèle. Cette dernière partie est divisée en **3 chapitres** correspondant chacun à l'étude du transfert dans un niveau trophique : **producteur primaire**, **consommateur primaire** et **consommateur secondaire**.

Chacune de ces trois parties est constituée de la compilation d'articles scientifiques publiés ou soumis dans des revues internationales à comité de lecture. Ces publications sont précédées d'un paragraphe introductif sur les enjeux et la démarche de chaque article présenté. A la suite de chaque article présenté, un paragraphe de synthèse rassemble les principaux résultats.

Enfin, dans la **conclusion générale**, nous effectuons un bilan des travaux effectués au cours de ces trois années de thèse ainsi qu'un bilan sur les **perspectives générales** et les suites éventuelles à donner aux résultats obtenus.

Première partie

APPROCHE BIBLIOGRAPHIQUE DE L'ECOTOXICITE DES EFFLUENTS HOSPITALIERS

L'étude bibliographique présentée ci-après correspond à un état des lieux des recherches scientifiques menées dans le domaine des effluents hospitaliers d'une part, et autour de la problématique « transfert des contaminants dans la biosphère », d'autre part. Dans le **premier chapitre**, nous avons fait un inventaire des contaminants déjà mesurés dans les effluents hospitaliers avec leur écotoxicité respective. Dans le **deuxième chapitre** nous avons effectué un focus sur l'écotoxicité liée aux résidus pharmaceutiques présents dans les effluents hospitaliers et au danger environnemental potentiel lié à leur présence. Enfin, dans un **troisième chapitre**, nous avons pris du recul sur les recherches menées autour de la problématique de bioconcentration, bioaccumulation et bioamplification des contaminants en général, et des résidus pharmaceutiques en particulier.

Chapitre 1

Ecotoxicité des effluents hospitaliers

1.1 Enjeux et démarche

La première démarche adoptée dans ces travaux a été de caractériser les effluents hospitaliers (EH). Or, plusieurs auteurs s'étaient déjà attachés à caractériser la composition des effluents hospitaliers. Ces travaux de caractérisation répondaient cependant à des problématiques très éloignées les unes des autres : contribution des EH à la présence de molécules illicites (Lin et al., 2010), contribution des EH à la charge polluante dans les effluents urbains (Langford and Thomas, 2009; Ort et al., 2010; Verlicchi et al., 2010), développement de méthode analytique multi-résidus (Gros et al., 2013) ou encore la caractérisation des contaminants dans les effluents hospitaliers (Boillot et al., 2008; Kümmerer et al., 2000; Tauxe-Wuersch et al., 2006; Weissbrodt et al., 2009; Yuan et al., 2013). D'autres auteurs se sont intéressés au risque écotoxicologique potentiel des effluents hospitaliers selon deux approches différentes mais néanmoins complémentaires : une approche « substance » (Perrodin et al., 2012; Verlicchi et al., 2012) ou une approche « matrice » (Emmanuel et al., 2005; Zgorska et al., 2011). Dans cette revue bibliographique, nous avons réuni au sein d'un même article toutes les données obtenues ces 20 dernières années sur la composition des effluents hospitaliers. Cela représente 173 prélèvements sur 115 hôpitaux à travers le monde. Pour aller plus loin, nous avons également réunis toutes les données d'écotoxicité disponibles pour les composés recherchés dans ces effluents ainsi que les données d'écotoxicité lié à l'effluent complet. Ainsi, nous avons caractérisé la toxicité des effluents hospitaliers selon les deux approches précitées : (i) une approche « substance » qui s'intéresse à l'écotoxicité de tous les contaminants recherchés dans les effluents ; (ii) une approche « matrice » qui rassemble tous les tests d'écotoxicité réalisés sur l'effluent entier.

1.2 Article 1 : *Characterisation of the ecotoxicity of hospital effluents : a review*

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Review

Characterisation of the ecotoxicity of hospital effluents: A review

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HIGHLIGHTS

- All studies on the hospital wastewater chemistry or ecotoxicity were examined.
- 155 PNEC (Predictive Non Effect Concentration) were calculated.
- For this assessment, priority was given to the experimental values.
- Very ecotoxic compounds have been identified.
- Bioaccumulation is rarely considered in the prioritisation methods.

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ABSTRACT

The multiple activities that take place in hospitals (surgery, drug treatments, radiology, cleaning of premises and linen, chemical and biological analysis laboratories, etc.), are a major source of pollutant emissions into the environment (disinfectants, detergents, drug residues, etc.). Most of these pollutants can be found in hospital effluents (HWW), then in urban sewer networks and WWTP (weakly adapted for their treatment) and finally in aquatic environments. In view to evaluating the impact of these pollutants on aquatic ecosystems, it is necessary to characterise their ecotoxicity. Several reviews have focused on the quantitative and qualitative characterisation of pollutants present in HWW. However, none have focused specifically on the characterisation of their experimental ecotoxicity. We have evaluated this according to two complementary approaches: (i) a "substance" approach based on the identification of the experimental data in the literature for different substances found in hospital effluents, and on the calculation of their PNEC (Predicted Non Effect Concentration), (ii) a "matrix" approach for which we have synthesised ecotoxicity data obtained from the hospital effluents directly. This work first highlights the diversity of the substances present within hospital effluents, and the very high ecotoxicity of some of them (minimum PNEC observed close to 0,01 pg/L). We also observed that the consumption of drugs in hospitals was a predominant factor chosen by authors to prioritise the compounds to be sought. Other criteria such as biodegradability, excretion rate and the bioaccumulability of pollutants are considered, though more rarely. Studies of the ecotoxicity of the particulate phase of effluents must also be taken into account. It is also necessary to monitor the effluents of each of the specialised departments of the hospital studied. These steps is necessary to define realistic environmental management policies for hospitals (replacement of toxic products by less pollutant ones, etc.).

Contents

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1. Introduction

The very wide range of activities performed by hospitals (care, diagnostics, hygiene, maintenance, etc.) lead them to use a great variety of potentially ecotoxic substances, such as surfactants, disinfectants, drugs and radionuclides (Kummerer et al., 1998; Kümmerer, 2001; Boillot et al., 2008). Once used or excreted by a patient, these substances (except radionuclides which are generally stocked in hospital before releasing) combine with the hospital wastewater (HWW) (Kümmerer, 2001; Langford and Thomas, 2009) in metabolised and non-metabolised forms, either stable or unstable, and then flow directly into the municipal wastewater network, generally without any prior treatment (Emmanuel et al., 2004). However, Waste Water Treatment Plants (WWTP) are not usually adapted to treat the pollutants present in these specific effluents (Ternes, 1998; Heberer, 2002; Joss et al., 2005). Moreover, HWW can be over 150 times more concentrated in micropollutants than urban effluents (Verlicchi et al., 2010). This could contribute towards explaining the presence of hospital pollutants not only in WWTPs and their effluents (Brown et al., 2006; Langford and Thomas, 2009), but also in different compartments of the environment (surface water, groundwater, sediments, etc.) (Santos et al., 2010; Vazquez-Roig et al., 2012).

Given the large number of substances used in hospitals, it is necessary to rank the risks they represent for host aquatic ecosystems. Concerning the specific case of pharmaceutical compounds (PhCp), and in the framework of increasingly generalised monitoring of their diffusion into the environment in recent years, several classifications of drugs to be monitored with priority have been proposed (Halling-Sørensen et al., 1998; Jjemba, 2006; Besse and Garric, 2008; Kumar and Xagoraraki, 2010; Sui et al., 2012). To do this, different criteria have been taken into account, such as consumption and excretion rate or, more rarely, biodegradability, bioaccumulability and the ecotoxicity of PhCp.

In view to setting up an HWW monitoring procedure, it is necessary to draw up a list of priority compounds to be dosed in these very specific effluents. To do this, we make use of existing characterisation studies, including several reviews that have focused on the quantitative and qualitative characterisation of HWW (Kümmerer, 2001; Verlicchi et al., 2012a, 2012b). These works merit completion by characterising the ecotoxicity of these effluents, and the compounds they transport, taking into account the potential effects at very low concentrations (Kidd et al., 2007) of certain compounds present in HWW. Escher et al. (2011) performed an initial study to this end, based on a theoretical approach based on QSAR (Quantitative Structure Activity Relation).

In this review, we specifically focus on the experimental characterisation of the ecotoxicity of HWW, a subject that has not been examined in-depth up to now. This review was carried out according to two complementary approaches: (i) the "substance" approach for which we first identified all the compounds (disinfectants, detergents, drugs, metabolites and others) sought in HWW. For each compound, we then sought to establish its PNEC (Predicted Non Effect Concentration) in relation to the available experimental ecotoxicity data. When not enough experimental ecotoxicity data were available in the literature, we used ECOSAR data (Sanderson et al., 2004) which, in addition to QSAR data, take into account experimental ecotoxicity data on structurally similar molecules, (ii) the "matrix" approach for which we grouped the experimental ecotoxicity values measured directly on the HWW.

2. Characterisation of ecotoxicity according to the "substance" approach

2.1. Methodological approach

Characterising the ecotoxicity of effluents using the "substance" approach consists in comparing the concentration of each substance (pharmaceutical compounds, detergents, disinfectants, etc.) sought in the hospital effluents with its PNEC. As the latter is rarely available in the literature, notably for pharmaceutical compounds, it was often established specifically for this review. To achieve this, we first carried out bibliographical research essentially, but not only, relying on the ECOTOX EPA and Wikipharma (Molander et al., 2009) databases. This research included the identification of the trophic level of the species tested, according to the classification presented in Table 1. The chronicity of the tests was also recorded. The tests considered as chronic here are those that measure the effect of contaminants on the reproduction of organisms (transmission from one generation to another) and on the sex of individuals (e.g. population sex ratio, induced intersex).

Once the synthesis of existing ecotoxicity data had been established, an "extrapolation factor" (EF) was applied to the lowest toxicity values obtained in view to establishing the PNEC of the substance concerned. The approach to assigning EFs is explained in Table 2. It uses the method proposed by the EU (European Union) in the Technical Guidance Document (TGD, 2003) issued in relation to launching chemical substances on the market. However, several adaptations and improvements have been achieved. In particular, when ecotoxicological data on fungi (e.g. *Glomus intraradices*) were available, we considered them as decomposers. Regarding these organisms, we considered these tests as chronic when the tests lasted several weeks and the number of spores was used as the measurement parameter. Likewise, tests on periphyton were classified as chronic tests on decomposers. In the case where the only data available corresponded to an EC (Effective Concentration) higher than those tested, we applied the EF to this EC. For certain compounds without chronic tests, acute data were not available for three different trophic levels. In this case, the ECOSAR data were considered as acute

Table 1
Identified trophic levels and definitions (TGD, 2003).

Trophic level	TGD definition	Example
Primary producers	Primary producers photo-/chemo-autotrophically synthesise organic compounds using inorganic precursors.	Algae, cyanobacteria, macrophytes,...
Primary consumers	They live mainly on living or dead autotrophic organisms or on microorganisms.	Crustacea, molluscs, protozoa (non-carnivorous), ...
Secondary consumers	They live mainly on primary consumers.	Rotatoria, fishes, predatory copepods,...

data and the EF was applied to the lowest value. Nevertheless, for the PNEC calculation of a compound, we need at least acute ecotoxicity data (theoric (ECOSAR) or experimental) for species from three different trophic levels. Without this minimum information, the PNEC could not be calculated.

It should be noted that although the "algae" and "macrophyte" tests (those measuring growth rates and not photosynthetic activity) were classed among the chronic tests, they did not permit lowering the EF when they were only ecotoxicological data available.

Lastly, when rotifers were the only organisms subjected to chronic tests at trophic level 2C (denoted "Bc" in the different tables), the EF was not reduced as is indicated in the classification of the TGD.

Regarding metals, ecotoxicological data are much more numerous (except for gadolinium (Gd) and platinum (Pt)). Thus we report the PNEC calculated by the INERIS, when available, using statistical methods (INERIS, 2005). To give an idea of the robustness of these PNEC, we also report the HC5 (Hazardous Concentration for 5% of species tested) and IC90 (Confidence Index of 90%). Since these data were not available for Gd and Pt, we calculated the PNEC using the same method as for the other molecules (PhCp, disinfectants, detergents, other molecules).

2.2. Results

The concentrations of 297 pollutants measured in the HWW, and the PNEC established (143 PNECs based on experimental data and 61 PNECs based on theoric data) for each substance are given in Tables 3a to 3u, then analysed "therapeutic family by therapeutic family" (Classified according Anatomical, Therapeutics and Chemical classification system called ATC) for the PhCp and "type of use" by "type of use" for the other substances. Table 3u contains all references found in Tables 3a to 3t.

2.2.1. Pharmaceutical compounds

2.2.1.1. Alimentary tract and metabolism (Table 3a). Within this therapeutic class, 7 compounds were detected in hospital effluents. One compound (sulfaguanidine) was never detected. Two compounds attracted particular attention: nifuroxazide and ranitidine. The

Table 2
Extrapolation factor to derive a $PNEC_{water}$.
(Inspired from TGD, 2003).

Available data	EF
At least one short-term L(E)C50 from each of three trophic levels of the base-set (fish, daphnia and algae)	1000
One long term chronic data (either fish or invertebrate)	100
Two long term from species representing two trophic levels (fish and/or daphnia and/or algae)	50
Long-term NOECs from at least three species (fish and/or daphnia and/or algae and/or decomposers) representing three trophic levels	10
Statistics method (e.g. species sensitivity distribution)	5–1 (justify case by case)
Field data or model ecosystem	Reviewed case by case

Table 3a
Inventory of compounds sought in hospital wastewater with their corresponding concentration, ecotoxicity data and PNEC calculation — alimentary tract and metabolism.

N° CAS	Compound	HWW conc. (µg/L)		Ref.	ECOSAR (µg/L) (Sanderson et al., 2004)			Experimental ecotoxicity												EF	PNEC (µg/L)	Ref.
								Tested Species	Trophic level			Chronic test				The most sensitive species						
		Min	Max		Algae	Daphnia	Fish		D	P	1C	2C	D	P	1C	2C	Endpoint	Parameter	Species	Value (µg/L)			
51481-61-9	Cimetidine	<0.003	0.033	32	6495	35000	572000	3	1	0	1	1	0	0	0	0	EC50	Immobilization	*D. magna*	271300	1000	271	36
76824-35-6	Famotidine	0.035	0.29	32	22000	242000	4615000	2	0	0	1	1	0	0	0	0	EC50	Immobilization	*D. magna*	398000	1000	22	37
		0.094		17																			
10238-21-8	Glibenclamide (Glyburide)	0.05	0.11	32	418	197	176																
965-52-6	Nifuroxazide	0.1	2.56	32	328	581	555																
58-74-2	Papaverine	0.001	0.002	15	1430	830	1000																
66357-35-5	Ranitidine	0.4	1.7	10	10000	64000	1076000	3	0	0	2	1	0	0	1	1	NOEC	Growth	*C. dubia*	2	50	0.040	36
		1.565		62																			
		0.24	4.1	32																			
57-67-0	Sulfaguanidine	<0.001		17	354052	395	30455	9	2	6	1	0	0	5	1	0	EC50	Growth	*L. minor*	220	50	4.40	38; 39

HWW conc.: concentration measured in hospital wastewater; D: decomposers; P: primary producer; 1C: primary consumer; 2C: secondary consumer; Ref.: all references are grouped in Table 3u.

Table 3b
Inventory of compounds sought in hospital wastewater with their corresponding concentration, ecotoxicity data and PNEC calculation — cardio-vascular system.

N° CAS	Compound	HWW conc. (µg/L)		Ref.	ECOSAR (µg/L) (Sanderson et al., 2004)			Experimental ecotoxicity												EF	PNEC (µg/L)	Ref.
								Tested species	Trophic level				Chronic test				The most sensitive species						
		Min	Max		Algae	Daphnia	Fish		D	P	1C	2C	D	P	1C	2C	Endpoint	Parameter	Species	Value (µg/L)			
37517-30-9	Acebutolol (HCl)	0.185		17	5376	20000	306000	0													1000	5.376	
29122-68-7	Atenolol	0.045	0.053	15	11000	83000	1461000	5	1	1	1	2	0	1	0	1	NOEC	Index condition	*P. promelas*	1000	50	20	36
		0.1	122	10																			
		2.2	6.6	32																			
		1.6	6.5	25																			
		0.796	2.134	28																			
		2.315		62																			
		1.607		17																			
134523-00-5	Atorvastatin	0.003	0.006	15	384	165	86	6	1	2	2	1	1	0	2	0	LOEC	Fresh weight	*L. gibba*	19	100	0.19	36
		0.062	0.31	32																			
63659-18-7	Betaxolol	<0.002	0.02	32	1240	1673	20000	1	0	0	1	0	0	0	0	0	EC50	Immobilization	*D. magna*	>300000	1000	1.24	36
41859-67-0	Bezafibrate	<0.001	2.9	32	6730	3536	3811	6	0	0	4	2	0	0	1	1	NOEC	Population growth inhitbition	*C. dubia*	23	50	0.46	36
		0.063		62																			
		0.001		17																			
75847-73-3	Enalapril	0.091	0.4	32	21000	26000	93000	1	0	0	1	0	0	0	0	0	LC50	Mortality	*T. platyurius*	184000	1000	21	36
49562-28-9	Fenofibrate	0.6		11	80	358	48	4	0	1	2	1	0	0	1	1	NOEC	Population growth inhitbition	*C. dubia*	39	50	0.78	36
		<0.001	0.026	32																			
54-31-9	Furosemide	0.2	0.45	15	103000	79000	166000	4 + C	1	0	3	1	1	0	1	1	LOEC	Structure and metabolism of community	Biofilm	10	10	1	36
		2.037		62																			
		5.3	18	32																			
25812-30-0	Gemfibrozil	1.1	7.3	11	2179	1033	930	9	1	1	4	3	0	0	1	1	LOEC	Decrease of plasmatic testosterone	*C. auratus*	15	50	0.3	36
		<0.003	0.064	32																			
		<0.001	1.11	18																			
		<0.134		17																			
58-93-5	Hydrochlorothiazide	0.54	2.4	32	317000	394000	1909000	0													1000	317	
		1.995		62																			
83915-83-7	Lisinopril	<0.012	0.61	32	333000	4293000	85033000	0													1000	333	
51384-51-1	Metoprolol	0.51	1.2	32	2908	8244	116000	9	1	2	3	3	0	1	1	1	LOEC	Liver structure alteration	*O. mykiss*	1	10	0.1	36
		0.419	25.097	31																			
		1.325		62																			
		0.145		17																			
73573-88-3	Mevastatin	0.068	2	32	239	2401	273	0													1000	0.239	
		<0.01		17																			
42200-33-9	Nadolol	<0.001	0.0034	32	5021	19000	290000	4	0	0	3	1	0	0	0	0	LC50	Mortality	*C. dubia*	>100000	1000	5.021	40
6493-05-6	Pentoxifylline	0.302		17				0															
13523-86-9	Pindolol	<0.001	0.26	32	3175	10000	147000	0													1000	3.175	
81093-37-0	Pravastatin	0.064	1.1	32	100000	104000000	497	0													1000	0.497	
		<0.001		17																			
525-66-6	Propanolol	0.2	6.5	10				22	2	5	10	5	0	4	2	2	LOEC	Reproduction success	*O. latipes*	0,5	10	0.05	36
		0.116		62																			
		<0.002	0.094	32																			
		<0.0005	0.225	18																			
		0.03	0.603	32																			
		0.042		17																			
79902-63-9	Simvastatin	0.002		15	92	416	56	4	0	1	2	1	0	1	0	0	LOEC	Growth rate	*N. spinipes*	0,16	1000	0.0002	41; 42
3930-20-9	Sotalol	0.35	6.7	32	13000	42000	243000	3	1	1	1	0	0	0	0	0	EC50	Immobilization	*D. magna*	>300000	1000	13	36
		0.7		62																			
		<0.01		17																			
137862-53-4	Valsartan	3.032		62				0															
52-53-9	Verapamil	0.03		62			36	12	1	1	7	3	0	1	0	0	LOEC	Length	*P. promelas*	600	1000	0.6	36; 42

HWW conc.: concentration measured in hospital wastewater; D: decomposers; P: primary producer; 1C: primary consumer; 2C: secondary consumer; Ref.: all references are grouped in Table 3u; +C: ecotoxicity data available for tests on community of organisms (several species exposed together).

Table 3c
Inventory of compounds sought in hospital wastewater with their corresponding concentration, ecotoxicity data and PNEC calculation – dermatologicals.

N° CAS	Compound	HWW conc. (µg/L)		Ref.	ECOSAR (µg/L) (Sanderson et al., 2004)			Experimental ecotoxicity										EF	PNEC (µg/L)	Ref.
								Tested Species	Trophic level				Chronic test				The most sensitive species				
		Min	Max		Algae	Daphnia	Fish		D	P	1C	2C	D	P	1C	2C	Endpoint	Parameter	Species	Value (µg/L)			
23593-75-1	Clotrimazole	0.004	0.011	15	0.112	0.023	0.09	2 + C	1	0	1	1	1	0	0	0	LOEC	Sterol synthesis	Peryphyton	0.000005	100	0.00000005	36; 42
27220-47-9	Econazole	<0.0012	0.028	28				0															
86386-73-4	Fluconazole	3.445		62				2	0	1	0	1	0	0	0	0	LC50	Mortality	*O. latipes*	> 100000			42
2013-58-3	Meclocycline	<0.007		31				0															
83-43-2	Methylprednisolone	1.42		62	34	26	29	0													1000	0.026	42
69-72-7	Salicylic acid	23.4	70.1	11	48	16	1.288	11	1	5	4	1	0	4	3	0	LOEC	Maturity	*D. longispina*	1800	50	36	36
		0.9	2.4	32																			
		0.034	0.04	28																			
3380-34-5	Triclosan	<0.044		11	0.296	0.107	0.022	31 + C	3	11	6	12	1	4	4	7	LOEC	Biomass	Biofilm	0.12	5	0.024	44

HWW conc.: concentration measured in hospital wastewater; D: decomposers; P: primary producer; 1C: primary consumer; 2C: secondary consumer; Ref.: all references are grouped in Table 3u; +C: ecotoxicity data available for tests on community of organisms (several species exposed together).

concentrations of these molecules in the HWW were 11-fold and 100-fold higher, respectively, than the PNEC established. Although the PNEC of the nifuroxazide is based on ECOSAR data (thus less robust), that of ranitidine is based on experimental data. It is also noteworthy that within this group, 4 molecules detected in the hospital effluents did not present any experimental ecotoxicity.

2.2.1.2. Cardio-vascular system (Table 3b). Of the 22 compounds sought in this class, 9 were detected at values 6 (bezafibrate) to 250 (metoprolol) fold higher than the PNEC. All the compounds sought were detected in the HWW. Furthermore, 8 compounds had never been subject to an experimental ecotoxicological study. Pentoxifylline had not been subject to data modelling either.

2.2.1.3. Dermatologicals (Table 3c). One of the seven compounds of this class was remarkable, namely clotrimazole, which was measured at concentrations 200,000 fold higher than its PNECs! The high toxicity of compounds from this group is of great interest given the utilisation of these products, which are all antimicrobial. Although the utilisation of these products is important locally, the impact of these compounds on WWTP could be considered as significant, as they function on the principle of biological degradation of pollutants by micro-organisms. Moreover, regarding triclosan, the experimental ecotoxicity data (acute and chronic and on all the trophic levels) are very complete, leading to an EF of 5. However, the PNEC remains twice as high as the limit of detection (LOD) (Kosma et al., 2010), making it difficult to ascertain the real ecotoxicity of triclosan in this hospital effluent. Not enough (for PNEC calculation) or not at all ecotoxicological data was available for methylprednisolone, fluconazole and econazole, the latter detected in the effluent and identified as highly bioaccumulable and slightly biodegradable (Jean et al., 2012). Therefore the PNEC of these compounds could not be calculated.

2.2.1.4. Genito-urinary system and sex hormones (Table 3d). Only one (clomiphen) of the nine compounds of this group was never detected. Four of them had a MCW_{max} (Highest Measured Concentration in Wastewater)/PNEC ratio between 620 (estriol) and 28,750 (estradiol). It is noteworthy that the LODs of certain compounds were up to 1000 times higher than their PNECs (e.g. estradiol), exhibiting the strong ecotoxicity of these compounds. It should be noted that no ecotoxicological data were available for three compounds detected in the effluents analysed: raloxifen, cilastatin and mifepristone, the latter identified as particularly bioaccumulable and slightly biodegradable (Jean et al., 2012).

2.2.1.5. Anti-infectives for systemic use (Tables 3e to 3j). It is in this class that we found the largest number of compounds measured in HWWs. Although the anti-infectives not only include antibiotics (e.g. antivirals), research are almost limited to the latter. This emphasizes the omnipresence of consumption in the choice of compounds studied. Indeed, antibiotics are among the drugs most frequently consumed. A number of ecotoxicological data were available as these compounds have already been subject to numerous studies. However, no ecotoxicological data were available for 6 of the 60 compounds measured. Furthermore, 9 compounds were measured in the effluents at concentrations more than 1000-fold higher than their PNEC, while the maximum concentration of ampicillin was more than 500,000-fold higher. In spite of the large number of experimental ecotoxicological data, few chronic tests have been carried out for these compounds.

2.2.1.6. Antineoplastics and immunomodulant agents (Table 3k). Generally, little attention has been devoted to antineoplastics (eight without experimental ecotoxicological data, including two without any ecotoxicological data) in spite of sometimes high MCW_{max}/PNEC ratios (244 000 for 5-fluorouracil). Among these compounds, ifosfamide is an interesting example. Despite having detected in 6 different

Table 3d
Inventory of compounds sought in hospital wastewater with their corresponding concentration, ecotoxicity data and PNEC calculation — genito-urinary system and sex hormones.

N° CAS	Compound	HWW conc. (µg/L)		Ref.	ECOSAR (µg/L) (Sanderson et al., 2004)			Experimental ecotoxicity												EF	PNEC (µg/L)	Ref.
								Tested species	Trophic level				Chronic test				The most sensitive species						
		Min	Max		Algae	Daphnia	Fish		D	P	1C	2C	D	P	1C	2C	Endpoint	Parameter	Species	Value (µg/L)			
57-63-6	17α-ethinylestradiol	<0.0003		31	677	234	40	45 + C	1	2	14	28	1	1	6	9	NOEC	Sprawn egg per couple	*P. promelas*	0,0001	2	0.00004	36; 42; 47
		<0.01		17																			
		<0.025	0.432	18																			
		<0.0004		28																			
50-28-2	17β-estradiol	<0.003	0.072	31	800	277	44	35	0	2	9	24	0	1	3	8	LOEC	Induce intersex	*O. latipes*	0,0004	50	0.000008	36; 42
		<0.01		17																			
		<0.025	0.23	18																			
82009-34-5	Cilastatin	1.037		62				0															
911-45-5	Clomiphen	<0.0005		21				0															
50-27-1	Estriol	0.18	0.785	31	4390	1451	15	3	0	0	1	2	0	0	0	2	LOEC	Induce intersex	*O. latipes*	0,75	100	0.0075	36; 42
		4.651		17																			
53-16-7	Estrone	0.007	0.047	31	1660	56	74	12	0	0	4	8	0	0	1	2	LOEC	Induce intersex	*O. latipes*	0,008	50	0.00016	36; 42
		<0.01		17																			
		<0.025	0.415	18																			
98319-26-7	Finasteride	0.00032	0.00436	21				3	0	1	1	1	0	0	0	0	LEC50	Mortality	*O. mykiss*	20000	1000	20	55
84371-65-3	Mifepristone	0.0064	0.195	21				0															
84449-90-1	Raloxifen	0.00674	0.022	21				0															
57-83-0	Progesterone	0.009		17	3300	1000	733	3	0	0	2	1	0	0	1	1	LOEC	Population sex ratio	*D. magna*	100	50	2	42; 46

HWW conc.: concentration measured in hospital wastewater; D: decomposers; P: primary producer; 1C: primary consumer; 2C: secondary consumer; Ref.: all references are grouped in Table 3u; +C: ecotoxicity data available for tests on community of organisms (several species exposed together).

Table 3e
Inventory of compounds sought in hospital wastewater with their corresponding concentration, ecotoxicity data and PNEC calculation — anti-infective for systemic use — β-lactams.

N° CAS	Compound	HWW conc. (µg/L)		Ref.	ECOSAR (µg/L) (Sanderson et al., 2004)			Experimental ecotoxicity												EF	PNEC (µg/L)	Ref.
								Tested species	Trophic level			Chronic test				The most sensitive species						
		Min	Max		Algae	Daphnia	Fish		D	P	1C	D	P	1C	2C	Endpoint	Parameter	Species	Value (µg/L)			
26787-78-0	Amoxicillin	<0.032	0.218	64	69.15	247.9	12.6	10	1	5	3	0	5	0	0	NOEC	Growth	*S. leopolensis*	0.78	1000	0.00078	36, 42
69-53-4	Ampicillin	<0.010	5.08	18	45712	148776	797502	15	1	9	3	2	0	9	0	1	LOEC	Normal expression of p53	*D. rerio*	0.0005	50	0.00001	36; 42
		<0.004		64																		
		0.053		17																		
61-72-3	Cloxacillin	<0.005		17	36466	23495	36139	0												1000	23.495	
66-79-5	Oxacillin	<0.0025		17				0														
		<0.048		64																		
61-33-6	Penicillin G (Benzylpenicillin)	0.85	5.2	2	206708	174685	431277	2	0	2	0	0	0	2	0	0	EC50	Growth	*M. aeruginosa*	6	1000	0.006	36, 42
		<0.002		18																		
		<0.002		64																		
		ND		29																		
		<0.005		17																		
87-08-1	Penicillin V (Phénoxyméthylpenicilline)	<0.011		64	28121010	61968500	814000000	0												1000	28121	
		<0.01		2																		

HWW conc.: concentration measured in hospital wastewater; D: decomposers; P: primary producer; 1C: primary consumer; 2C: secondary consumer; Ref.: all references are grouped in Table 3u; ND: compound not detected.

studies between 1997 and 2012 (Table 3k), there is still any experimental data concerning its ecotoxicity. Otherwise, it should be noted that three compounds present LODs much higher than the PNECs.

2.2.1.7. Musculoskeletal system (Table 3l). Diclofenac and ibuprofen are the two compounds that attract attention in this group. In spite of a relatively low EF, their MCW_{max}/PNEC ratios remain very high (746 and 302 respectively).

2.2.1.8. Nervous system (Table 3m). With 47 compounds identified, nervous system category is the investigated in HWW after anti-infectives for systemic use. Caffeine has the highest MCW_{max}/PNEC ratio in this class (3.64×10^6). However, the EF is high since there were no chronic ecotoxicity data. Paracetamol and propofol are also of note with a ratio close to 200. Although the PNEC of propofol is based on ECOSAR data, that of paracetamol is more robust with an EF of 50. Otherwise, pollutants of this category are almost always higher than LOD (mepirizole only compound of this category not detected). It should also be noted that only a few compound have experimentally studied on more than three species.

2.2.1.9. Compounds for veterinary use (Table 3n). Only five compounds of this class were detected in the HWW, at concentrations always lower than the PNEC. Nevertheless, 4 of the 5 PNECs calculated are based on theoric data.

2.2.1.10. Respiratory system (Table 3o). No experimental ecotoxicity data are available for the compounds grouped in this class except for dexamethasone and ECOSAR data was available for four out of six compounds in this category. Globally, the MCW_{max} remained higher than the PNEC obtained with the ECOSAR and experimental data (except for clenbuterol).

2.2.1.11. Iodine compound medium (Table 3p). These compounds are very rarely measured in HWW in spite of the presence in relatively high concentrations in the environment (Santos et al., 2010) and in HWW. Furthermore, very few ecotoxicity data are available. Those for which ECOSAR data are available have an MCW_{max} that remains lower than PNEC.

2.2.1.12. Metabolites of PhCp (Table 3q). Very little information exists on the ecotoxicity of the metabolites of PhCp. Only two compounds had experimental ecotoxicity data available: 2′2′-difluorodeoxyuridine by Zounkova et al. (2010) and clofibric acid (clofibrate metabolite but also a pesticide). The available MCW_{max} is far below the PNEC, but very few organisms have been tested.

2.2.2. Non-pharmaceutical compounds

2.2.2.1. Disinfectants (Table 3r). The reaction of bleach, very often used in hospitals to disinfect apparatus and premises, with the organic compounds present in HWW, leads to the production of numerous organohalogen compounds (Emmanuel et al., 2004).

Generally, the ecotoxicity of the compounds of this group has been quite well identified, despite there being an imbalance once again between the available chronic tests and acute tests. Glutaraldehyde, the disinfectant used classically for disinfecting instruments and medical probes, was detected in certain effluents at very high concentrations with respect to its ecotoxicity. The MCW_{max}/PNEC ratio observed can reach up to 4960.

2.2.2.2. Alcohols (Table 3r). Ten alcohols, stemming from different uses or from the metabolism of initial compounds (Boillot et al., 2008), were detected in HWW at sometimes very high concentrations with respect to their ecotoxicity. In the case of 2-propanol, the MCW_{max}/PNEC ratio reached 321,930.

Table 3f
Inventory of compounds sought in hospital wastewater with their corresponding concentration, ecotoxicity data and PNEC calculation — anti-infective for systemic use — macrolides.

N° CAS	Compound	HWW conc. (μg/L)		Ref.	ECOSAR (μg/L) (Sanderson et al., 2004)			Experimental ecotoxicity												EF	PNEC (μg/L)	Ref.
								Tested species	Trophic level				Chronic test				The most sensitive species						
		Min	Max		Algae	Daphnia	Fish		D	P	1C	2C	D	P	1C	2C	Endpoint	Parameter	Species	Value (μg/L)			
83905-01-5	Azithromycin	<0.003	1.04	32	1630	2873	4100	2	0	1	1	0	0	1	0	0	EC50	Growth	*P. subcapitata*	19	1000	0.019	36; 37
		0.139		62																		
		0.085	0.113	64																		
		0.227		17																		
81103-11-9	Clarithromycin	0.02	14	32	1746	3110	4590	6	0	1	3	2	0	1	1	1 (Bc)	EC50	Growth	*P. subcapitata*	2	50	0.04	36; 42
		2.555		62																		
		0.113	0.973	64																		
		<0.5	2	26																		
		0.721		17																		
18323-44-9	Clindamycin	0.983		62				1	1	0	0	0	0	0	0	0	EC50	Protein synthesis	*P. falciparum*	2.2			42
		0.184	1.465	64																		
		0.341		17																		
114-07-8	Erythromycin	0.06	0.32	32	3898	7822	17109	19	0	11	4	4	0	11	1	1 (Bc)	EC50	Growth inhibition	*P. subcapitata*	20	50	0.4	36; 42
		<0.5	27	26																		
		0.01	0.03	10																		
		<0.01	0.261	4																		
16846-24-5	Josamycin	<0.003	0.015	32	1979	3537	910	0												1000	0.91	
		<0.0025		17																		
154-21-2	Lincomycin	0.3	2	2	12979	82356	139157	8	1	2	3	2	0	0	1	1 (Bc)	EC50	Growth	*P. subcapitata*	70	50	1.4	42; 52
		<0.005	0.174	4																		
		<0.009	0.119	64																		
		ND		29																		
3922-90-5	Oleandomycin	<0.01		17	6719	18000	51000	0												1000	6.719	
80214-83-1	Roxithromycin	<0.004	0.14	32	3238	6205	11662	6	1	2	2	1	0	2	0	0	NOEC	Growth	*P. subcapitata*	10	1000	0.01	36
		<0.5	1	26																		
		0.023		62																		
		<0.009		64																		
		<0.01	2.189	4																		
		<0.0025		17																		
8025-81-8	Spiramycin	0.001		15				2	0	2	0	0	0	0	0	0	EC50	Growth	*M. aeruginosa*	5			42
		<0.002	0.11	32																		
		<0.012		64																		
		<0.01		17																		

HWW conc.: concentration measured in hospital wastewater; D: decomposers; P: primary producer; 1C: primary consumer; 2C: secondary consumer; Ref.: all references are grouped in Table 3u.

Table 3g
Inventory of compounds sought in hospital wastewater with their corresponding concentration, ecotoxicity data and PNEC calculation — anti-infective for systemic use — Quinolones.

N° CAS	Compound	HWW conc. (μg/L)		Ref.	ECOSAR (μg/L) (Sanderson et al., 2004)			Experimental ecotoxicity												EF	PNEC (μg/L)	Ref.
								Tested species	Trophic level				Chronic test				The most sensitive species						
		Min	Max		Algae	Daphnia	Fish		D	P	1C	2C	D	P	1C	2C	Endpoint	Parameter	Species	Value (μg/L)			
28657-80-9	Cinoxacin	<0.001		64	23.69	21.08	56.93	1	1	0	0	0	0	0	0	0	NOEC	Bioluminescence	*V. fisheri*	26.22	1000	0.021	36
85721-33-1	Ciprofloxacin	0.85	2	2	132217	991485	8096293	10 + C	3	4	1	3	1	4	1	0	EC50	Growth	*M. aeruginosa*	5	10	0.5	36; 42
		<0.038	54.049	31																			
		2	83	14																			
		3.6	101	20																			
		1.1	25.8	5																			
		<0.005	0.217	4																			
		0.7	124.5	8																			
		5.329	7.494	64																			
		<0.003	0.051	26																			
		0.8	4.4	25																			
		31.98		62																			
		0.46	5.03	29																			
		1.4	26	32																			
		9.449	76.167	28																			
		0.751		17																			
74011-58-8	Enoxacin	0.058	0.48	32	150221	1271253	11652579	1	1	0	0	0	0	0	0	0	NOEC	Bioluminescence	*V. fisheri*	2.88	1000	0.00288	36
42835-25-6	Flumequine	0.003		17	61925	40763	38279	9	1	5	2	1	0	5	0	0	EC50	Bioluminescence	*V. fisheri*	2.64	1000	0.00264	36; 42
		<0.002		64																			
100986-85-4	Levofloxacin	<0.07		5				8	1	5	1	1	1	5	0	0	EC50	Growth	*M. aeruginosa*	7.9	1000	0.0079	36; 42
98079-51-7	Lomefloxacin	<0.08		5	110000	693000	4771000	5	1	4	0	0	0	3	0	0	NOEC	Bioluminescence	*V. fisheri*	2	1000	0.002	36
		<0.005	1.162	4																			
389-08-2	Nalidixic acid	0.186		17	272960	217359	253640	2	1	0	1	0	0	0	0	0	NOEC	Bioluminescence	*V. fisheri*	72	1000	0.072	36; 42
		<0.002	<0.005	64																			
70458-96-7	Norfloxacin	<0.07	15.2	5	161730	1232512	14040449	11	1	10	0	0	0	9	0	0	NOEC	Growth population	*V. fisheri*	1.6	1000	0.0016	36; 42
		<0.005	1.62	4																			
		<0.01		2																			
		5.933		62																			
		0.022	0.51	32																			
		<8	44	26																			
		<0.028	0.327	64																			
		0.131		17																			
82419-36-1	Ofloxacin	0.2	7.6	20	132217	991485	8096293	13	2	6	3	2	0	6	1	1 (Bc)	NOEC	Bioluminescence	*V. fisheri*	1.1	50	0.022	36
		4.9	35.5	2																			
		<0.07		5																			
		1.6	4.24	4																			
		3.3	37	32																			
		2.978	10.368	64																			
		<8	44	26																			
		1.088		17																			
14698-29-4	Oxolinic acid	0.005		17	280435	220919	254644	6	1	3	1	1	0	3	1	0	NOEC	Bioluminescence	*V. fisheri*	0.731	100	0.00731	36; 42
		<0.002		64																			
70458-92-3	Pefloxacin	0.062		17				0															
51940-44-4	Pipemidic acid	<0.005		64	447410	8926999	195000000	0													1000	447	
		0.178		17																			

HWW conc.: concentration measured in hospital wastewater; D: decomposers; P: primary producer; 1C: primary consumer; 2C: secondary consumer; Ref.: all references are grouped in Table 3u; +C: ecotoxicity data available for tests on community of organisms (several species exposed together).

Table 3h
Inventory of compounds sought in hospital wastewater with their corresponding concentration, ecotoxicity data and PNEC calculation – anti-infective for systemic use – Sulfonamides.

N° CAS	Compound	HWW conc. (µg/L)		Ref.	ECOSAR (µg/L) (Sanderson et al.. 2004)			Experimental ecotoxicity												EF	PNEC (µg/L)	Ref.
								Tested species	Trophic level				Chronic test				The most sensitive species						
		Min	Max		Algae	Daphnia	Fish		D	P	1C	2C	D	P	1C	2C	Endpoint	Parameter	Species	Value (µg/L)			
127-71-9	Sulfabenzamide	<0.003		64	18.45	0.071	1.297	0													1000	0.000071	
68-35-9	Sulfadiazine	0.029	0.38	32	153936	252	12443	8	2	5	1	0	0	5	1	0	EC50	Growth	*L. minor*	70	100	0.7	36; 38
		<0.006		64																		
		<0.005	0.253	4																		
		1.896		62																		
		0.05		17																		
122-11-2	Sulfadimethoxine	<0.001	0.0086	18	24709	89	1756	9	1	4	3	1	0	3	0	0	EC50	Growth	*L. gibba*	248	1000	0.248	53; 54
		<0.005		4																		
		<0.016		64																		
		<0.001		17																		
127-79-7	Sulfamerazine	0.001		17	77201	168	5955	10	2	2	0	6	0	1	0	0	EC50	Growth	*L minor*	680	1000	0.68	38; 42; 55
		<0.019		64																		
57-68-1	Sulfamethazine (Sulfadimidine)	<0.001		17	38608	112	2842	7	1	2	2	2	0	1	1	0	EC50	Growth	*L. gibba*	1277	100	12.77	42; 53; 54
		<0.5		26																		
		<0.002	0.03	32																		
		<0.0005	0.0028	18																		
		<0.005		4																		
		<0.01		2																		
		ND		29																		
144-82-1	Sulfamethiazole (Sulfamethizole)	<0.001		17	60229	146	4567	4	2	2	0	0	0	1	0	0	EC50	Growth	*L. minor*	2540	1000	2.54	38
		<0.012		64																		
723-46-6	Sulfamethoxazole	0.4	1.2	2	51328	129	3869	19	2	5	5	7	1	5	1	2(Bc)	LOEC	Growth	*S. leopolensis*	5;9	10	0.59	36; 42; 48
		<0.004	4.107	31																		
		0.4	12.8	20																		
		<0.005	1.06	4																		
		0.8	4.4	25																		
		<0.001	7.35	18																		
		0.065	0.2	64																		
		0.9	6.5	32																		
		<0.5	6	26																		
		3.476		62																		
		25.3		29																		
		0.642	1.87	28																		
		0.647		17																		
63-74-1	Sulfanilamide	0.019		17	140747	206	11582	0													1000	0.206	
144-83-2	Sulfapyridine	<0.003	<0.01	64	47.213	0.122	3.544	1	0	0	1	0	0	0	0	0	EC50	Development	*H. attenuata*	21610	1000	0.000122	42
		0.251		62																		
72-14-0	Sulfathiazole	ND		29	37386	107	2761	12	1	2	2	7	0	2	0	0	LOEC	Growth	*L. gibba*	100	1000	0.1	53; 54
		<0.009		64																		
		<0.001		17																		
515-64-0	Sulfisomidine	<0.012		64	38608	112	2842	0													1000	0.112	
		<0.001		17																		
127-69-5	Sulfisoxazole (sulfafurazol)	<0.001		17	25726	86	1850	10	2	2	0	6	0	1	0	0	EC50	Growth	*L. minor*	620	1000	0.62	38; 55
		<0.004		64																		

(continued on next page)

Table 3h (*continued*)

N° CAS	Compound	HWW conc. (μg/L)		Ref.	ECOSAR (μg/L) (Sanderson et al., 2004)			Experimental ecotoxicity												EF	PNEC (μg/L)	Ref.
								Tested species	Trophic level				Chronic test				The most sensitive species						
		Min	Max		Algae	Daphnia	Fish		D	P	1C	2C	D	P	1C	2C	Endpoint	Parameter	Species	Value (μg/L)			
738-70-5	Trimethoprim	0.028	7.26	29	2629	90	795579	20	1	10	4	5	0	10	1	0	LOEC	Decrease metabolic activity	*D. polymorpha*	0;29	50	0.0058	36; 42
		0.068	1.6	32																		
		<0.5	6	26																		
		0.01	0.03	10																		
		2.9	5	2																		
		0.93		62																		
		0.6	7.6	20																		
		<0.002	14.993	31																		
		0.05	0.216	64																		
		<0.005	0.174	4																		
		1.04		17																		

HWW conc.: concentration measured in hospital wastewater; D: decomposers; P: primary producer; 1C: primary consumer; 2C: secondary consumer; Ref.: all references are grouped in Table 3u; ND: not detected.

Table 3i
Inventory of compounds sought in hospital wastewater with their corresponding concentration, ecotoxicity data and PNEC calculation – anti-infective for systemic use – Tetracyclines.

N° CAS	Compound	HWW conc. (µg/L)		Ref.	ECOSAR (µg/L) (Sanderson et al.. 2004)			Experimental ecotoxicity												EF	PNEC (µg/L)	Ref.
								Tested species	Trophic level				Chronic test				The most sensitive species						
		Min	Max		Algae	Daphnia	Fish		D	P	1C	2C	D	P	1C	2C	Endpoint	Parameter	Species	Value (µg/L)			
57-62-5	Chlortetracycline	<0.006	0.069	31	32237	167585	520	11	2	4	3	2	1	4	0	0	LOEC	Growth inhibition	*L. gibba*	100	1000	0.1	36; 42
		<0.005	0.011	18																			
		<0.018		64																			
		ND		29																			
		<0.008	0.094	32																			
		<0.005		17																			
127-33-3	Demeclocycline	<0.003	0.052	31	44574	379599	749	0													1000	0.749	
564-25-0	Doxycycline	<0.005	0.403	31	50473	566315	865	3	1	2	0	0	1	1	0	0	EC50	Spore production	*G. intraradices*	37	100	0.37	36
		0.6	6.7	20																			
		<0.034		64																			
		<5		27																			
		<0.015	0.97	32																			
10118-90-8	Minocycline	<15	531.7	27	25000	221000	397	2	1	1	0	0	0	1	0	0	EC50	Protein synthesis	*P. falciparum*	178	1000	0.178	42; 56
79-57-2	Oxytetracycline	<0.012	3.743	31	167016	6535171	3279	38	2	15	11	10	1	13	2	1 (Bc)	EC50	Growth inhibiion	*A. flosaquae*	25	10	2.5	36; 42
		<0.01		4																			
		<0.01		2																			
		<0.007	1.3	32																			
		<0.003		64																			
		<0.002	0.014	18																			
		ND		29																			
		<0.005		17																			
60-54-8	Tetracycline	<0.015	4.178	31	49322	515013	843	12	2	6	3	1	1	5	1	0	LOEC	Growth inhibition	Synecchocysti sp.	10	10	1	36; 42
		<0.024		64																			
		<0.01		4																			
		<0.002	0.455	18																			
		<0.5	158	27																			
		<0.007	0.033	32																			
		0.059		17																			

HWW conc.: concentration measured in hospital wastewater; D: decomposers; P: primary producer; 1C: primary consumer; 2C: secondary consumer; Ref.: All references are grouped in Table 3u; ND: not detected.

Table 3j
Inventory of compounds sought in hospital wastewater with their corresponding concentration, ecotoxicity data and PNEC calculation – anti-infective for systemic use – Others.

N° CAS	Compound	HWW conc. (µg/L)		Ref.	ECOSAR (µg/L) (Sanderson et al., 2004)			Experimental ecotoxicity												EF	PNEC (µg/L)	Ref.
								Tested species	Trophic level				Chronic test				The most sensitive species						
		Min	Max		Algae	Daphnia	Fish		D	P	1C	2C	D	P	1C	2C	Endpoint	Parameter	Species	Value (µg/L)			
50370-12-2	Cefadroxil	ND		29	154203	1087662	36909	0													1000	36.9	
25953-19-9	Cefazolin	6.221		17				1	0	1	0	0	0	1	0	0	EC50	Growth	*P. subcapitata*	>1000000			36
		<0.049		64																			
63527-52-6	Cefotaxim	0.413		17				0															
		<0.023	0.089	64																			
38821-53-3	Cefradine	0.113		17	101717	602410	10021483	0													1000	101	
		0.203	0.854	29																			
55268-75-2	Cefuroxime	<0.125		31	135489	859810	5279742	3	0	1	1	1	0	1	0	0	NOEC	Growth	*P. subcapitata*	91000	1000	91	48
		<0.024		64																			
15686-71-2	Cephalexin (Cefalexine)	2.457		17				1	0	1	0	0	0	1	0	0	EC50	Growth	*L., gibba*	>1000000			42
		<0.004		64																			
21593-23-7	Cephapirin (Cefapirin)	0.005		17				0															
		<0.018		64																			
56-75-7	Chloramphenicol	<0.004	0.036	32	29	27	547	17	2	2	9	4	0	1	0	0	EC50	Bioluminescence	*V. fisheri*	60	1000	0.06	36; 42
		<0.5		26																			
		0.001		17																			
1403-66-3	Gentamycin	0.2	7.6	22	71000	1569000	35070000	3	0	1	1	1	0	1	0	0	EC50	Growth	*cyanobacteria*	69	1000	0.069	49
		1	5	26																			
443-48-1	Metronidazole	1.8	9.4	10	6830	51000	898000	13	1	3	4	5	0	3	1	0	LOEC	Histopathological lesion	*T. zillii*	10000	100	100	36; 42
		0.1	90.2	20																			
		3.388		62																			
		0.067	0.643	64																			
		0.26	1.64	32																			
		1.591		17																			
196618-13-0	Oseltamivir	0.025		62				0															
155213-67-5	Ritonavir	0.108		62				0															
15318-45-3	Thiamphenicol	0.004		17	48	57	1074	5	0	5	0	0	0	5	0	0	EC50	Growth	*P. subcapitata*	8860	1000	8.86	50; 51
1404-90-6	Vancomycin	1.311		29	1103000	6810000	322000														1000	322	

HWW conc.: concentration measured in hospital wastewater; D: decomposers; P: primary producer; 1C: primary consumer; 2C: secondary consumer; Ref.: all references are grouped in Table 3u; ND: not detected.

Table 3k
Inventory of compounds sought in hospital wastewater with their corresponding concentration, ecotoxicity data and PNEC calculation – antineoplastics and immunomodulant agents.

N° CAS	Compound	HWW conc. (µg/L)		Ref.	ECOSAR (µg/L) (Sanderson et al., 2004)			Experimental ecotoxicity												EF	PNEC (µg/L)	Ref.
								Tested species	Trophic level				Chronic test				The most sensitive species						
		Min	Max		Algae	Daphnia	Fish		D	P	1C	2C	D	P	1C	2C	Endpoint	Parameter	Species	Value (µg/L)			
51-21-8	5-fluorouracil	<0.005	27	12			9678000	7	2	2	1	2	0	2	1	0	LOEC	Embryo development	*X. laevis*	0,05	50	0.001	36; 42
		20	122	23																		
		<0.005	0.027	33																		
		0.4	0.9	25																		
		<0.015		35																		
		ND	0.03	3																		
120511-73-1	Anastrozole	0.003	0.0037	21				0														
446-86-6	Azathioprine	<0.005	0.032	34	12000	94000	1661000	0												1000	12	
50-18-0	Cyclophosphamide	<0.002	0.021	31	8168	1795000	70000	5	1	1	2	1	0	1	0	0	LO EC	Increase of righting time	*A. rubens*	18000	1000	18	36
		<0.002	2	34																		
		0.161		62																		
		0.5	0.8	25																		
		0.3	0.9	3																		
23214-92-8	Doxorubicin	0.1	0.5	24	735	358	355	3	1	1	1	0	0	1	0	0	NOEC	Immobilization	*D. magna*	10	1000	0.01	36
		<0.01		34																		
56420-45-2	Epirubicin	0.1	1.4	24	735	358	355	0													1000	0.355	
33419-42-0	Etoposide	<0.005	0.38	34	55000	149000	5283	3	1	1	1	0	0	1	0	0	LOEC	Growth	*P. subcapitata*	10000	1000	10	36
		0.11	0.6	3																		
107868-30-4	Exemestane	<0.002		21				0														
95058-81-4	Gemcitabine	<0.0009	0.038	12				3	1	1	1	0	0	1	1	0	LOEC	Growth	*D. subcapitatus*	10000	50	200	36
3778-73-2	Ifosfamide	<0.002	0.338	31	8168	1795000	70000	0												1000	8.2	
		<0.1	1	25																		
		0.895		62																		
		<0.006	1.914	13																		
		<0.002	10.647	34																		
		0.2	0.9	3																		
112809-51-5	Letrozole	0.002	0.00238	21				1	0	0	0	1	0	0	0	1	LOEC	Fecundity and fertility	*O. latipes*	5	100	0.05	57
59-05-2	Methotrexate	<0.002	4.689	34	192000	5659	3,83E+08	5	1	1	2	1	0	1	0	0	EC50	Growth inhibition	*T. pyriformis*	45000	1000	45	36
		ND	0.12	3																		
671-16-9	Procarbazine	<0.005		34	2797000	4765000	39951000	0													1000	2797	
10540-29-1	Tamoxifen	0.0002	0.0082	21	482	20	16000	6	0	0	4	2	0	0	1	2(Bc)	LOEC	Development	*S. purpuratus*	0,019	50	0.00038	42; 57; 58
		0.003	0.022	15																		
		>0.001	<0.004	35																		
		<0.001	<0.002	32																		
89778-26-7	Toremifene	<0.0001		21				0														
57-22-7	Vincristine	<0.02		34	1.881	3.339	4.874	0													1000	0.00181	

HWW conc.: concentration measured in hospital wastewater; D: decomposers; P: primary producer; 1C: primary consumer; 2C: secondary consumer; Ref.: all references are grouped in Table 3u; ND: not detected.

Table 3l
Inventory of compounds sought in hospital wastewater with their corresponding concentration, ecotoxicity data and PNEC calculation — musculoskeletal system.

N° CAS	Compound	HWW conc. (µg/L)		Ref.	ECOSAR (µg/L) (Sanderson et al., 2004)			Experimental ecotoxicity												EF	PNEC (µg/L)	Ref.
								Tested species	Trophic level				Chronic test				The most sensitive species						
		Min	Max		Algae	Daphnia	Fish		D	P	1C	2C	D	P	1C	2C	Endpoint	Parameter	Species	Value (µg/L)			
78-44-4	Carisoprodol	0,005	0,01	15	1592	97000	7,629	0													1000	0.007629	
64-86-8	Colchicines	0,009		17	34000	10000	29000	1	0	1	0	0	0	0	0	0	EC50	Physiology	*P. malhamensis*	1500000	1000	1500	42
15307-86-5	Diclofenac	0,06	1,9	10	7706	4238	4944	17	1	7	4	5	0	7	3	1 (Bc)	LOEC	Liver, kidney and gills cellular alteration	*O. mykiss*	1	50	0.02	36
		<0,126	6,3	11																			
		0,238	14,934	31																			
		0,17	0,53	32																			
		0,833		62																			
		<0,01	70	18																			
		0,286		17																			
		0,028	6,88	29																			
		<0,001	0,011	28																			
36330-85-5	Fenbufen	0,015		17	22553	14646	22842	0													1000	14.646	
29679-58-1	Fenoprofen	<0,0075		17				0															
15687-27-1	Ibuprofen	7	8,93	11	7510	4322	5460	16 + C	2	6	4	5	1	6	2	2 (Bc)	LOEC	Reproduction	*O. latipes*	1	5	0.2	36
		0,069	8,957	31																			
		1,5	151	10																			
		0,38	3,2	32																			
		<0,025	0,3	18																			
		0,282		17																			
		ND		29																			
		1,614	1,729	28																			
53-86-1	Indometacin	0,002	0,008	15	6852	3614	3922	2	0	0	1	1	0	0	0	0	LC50	Mortality	*T. platyurus*	16140	1000	16.14	42
		0,069		62																			
		<0,005		17																			
		0,31	4,1	32																			
22071-15-4	Ketoprofen	0,2	0,35	15	29331	19739	32759	3	1	1	1	0	0	1	0	0	EC50	Growth	*P. subcapitata*	2000	1000	2	36
		17,4	0,17	25																			
		<0,01		17																			
		<0,01	0,231	18																			
		1,1	9,8	32																			
		0,143	0,401	28																			
66635-83-4	Ketorolac	0,2	59,5	10				1	0	0	0	1	0	0	0	0	LC50	Mortality	*L. macrochirus*	1480000			55
61-68-7	Mefenamic acid	0,001	0,014	15	998	428	323	4	1	1	1	1	0	1	0	0	EC50	Immobilization	*T. platyurus*	3900	1000	3.9	36; 42
		6,14		62																			
		0,1	0,75	32																			
		ND		29																			
22204-53-1	Naproxen	<0,0437	21,3	11	22952	15144	24279	15	1	4	7	3	0	4	1	1 (Bc)	EC50	Growth population	*C. dubia*	330	50	6.6	36
		0,34	11	32																			
		<0,01	1,01	18																			
		<5,6		62																			
		ND		29																			
		0,47		17																			
50-33-9	Phenylbutazone	0,01	0,17	32	231	197	26	1	0	1	0	0	0	1	0	0	NOEC	Growth population	*P. subcapitata*	> 250000	1000	0.026	42
36322-90-4	Piroxicam	<0,001		17			220	0															
57-96-5	Sulfinpyrazone	<0,001		17				0															

HWW conc.: concentration measured in hospital wastewater; D: decomposers; P: primary producer; 1C: primary consumer; 2C: secondary consumer; Ref.: all references are grouped in Table 3u; +C: ecotoxicity data available for tests on community of organisms (several species exposed together), ND: not detected.

Table 3m
Inventory of compounds sought in hospital wastewater with their corresponding concentration, ecotoxicity data and PNEC calculation — nervous system.

N° CAS	Compound	HWW conc. (µg/L)		Ref.	ECOSAR (µg/L) (Sanderson et al., 2004)			Experimental ecotoxicity												EF	PNEC (µg/L)	Ref.
								Tested species	Trophic level				Chronic test				The most sensitive species						
		Min	Max		Algae	Daphnia	Fish		D	P	1C	2C	D	P	1C	2C	Endpoint	Parameter	Species	Value (µg/L)			
50-78-2	Acetylsalicylic acid(aspirin)	1.54	384	29	4.6705	885.8537	55.0836	13	1	2	7	3	0	2	2	0	NOEC	Number of viable offspring	*D. longispina*	320	50	6.4	36; 42
28981-97-7	Alprazolam	ND	0.032	63	0.628	0.508	5.406	0													1000	0.000508	
129722-12-9	Aripiprazole	ND	0.033	63				0															
77-26-9	Butalbital	0.011	0.48	32			15.3000	0															
58-08-2	Caffeine	12.3	42	11	6852	4.7000	80.5000	8	0	0	3	5	0	0	0	0	LOEC	Development	*X. laevis*	0.05	1000	0.00005	42
		15.6		17																			
		12.1	182	29																			
298-46-4	Carbamazepine	0.03	0.07	10	8146	6359	1.4000	33 + C	4	9	13	8	2	8	4	2(Bc)	LOEC	Decrease of cyanobacteria biomass	Periphytic community	10	5	2	36; 42
		<0.0167	1.7	11																			
		ND	0.24	63																			
		0.7	2.7	15																			
		0.64	1.2	32																			
		0.222		62																			
		0.018	6.08	29																			
		0.003	0.036	28																			
		0.163		17																			
303-49-1	Chlorimipramine	ND	0.089	63				0															
50-53-3	Chlorpromazine	ND	0.364	63	0.233	0.088	0.786	2	0	1	1	0	0	1	0	0	LC50	Mortality	*T. platyurus*	380	1000	0.000088	42
59729-33-8	Citalopram	0.019	0.322	63	0.729	0.635	6.878	1	0	0	1	0	0	0	1	0	LC50	Mortality	*C. dubia*	3900	100	0.00635	42
5786-21-0	Clozapine	0.295	13.2	63	1,468	2.147	26	1	0	0	0	1	0	0	0	0	LOEC	Mortality	*P. promelas*	30.8	1000	0.0014	42
50-36-2	Cocaine	0.05		19	2281	4905	1.3000	0													1000	2.281	
76-57-3	Codeine	0.378		19	4464	1.6021	60	0													1000	0.06	
		0.01	5.7	10																			
		0.26	3.2	32																			
439-14-5	Diazepam	<0.001	0.038	32	1424	2258	2.8000	12	1	1	6	4	0	1	0	0	LOEC	Regeneration of digestive region	*H. vulgaris*	10	1000	0.01	36; 42
		0.069		62																			
437-38-7	Fentanyl	0.019		19	674	539	5720	0													1000	0.539	
1622-62-4	Flunitrazepam	0.0063		19	2877		9.9000	0															
54910-89-3	Fluoxetine	0.001	0.002	15	345	178	1716	27	0	6	12	9	0	6	6	2	LOEC	Delayed development	*R. pipiens*	0.029	10	0.0029	36; 42
		<0.03		62																			
		<0.002	0.069	32																			
		ND	0.021	63																			
		<0.005		17																			
54739-18-3	Fluvoxamine	ND	0.435	63				7	0	4	3	0	0	1	1	0	EC50	Growth inhibition	*P. subcapitata*	62	50	1.24	36
60142-96-3	Gabapentin	19.4		62	196	3234	6.7672	0													1000	0.196	
6740-88-1	Ketamine	0.206		19	861	1072	1.3000	0													1000	0.861	
102767-28-2	Levetiracetam	11.02		62				0															
137-58-6	Lidocaine (lignocaine)	9.133		62	2.609	7.523	107	0													1000	0.00261	
846-49-1	Lorazepam	0.17	0.79	32	2008	3761	4.8000	0													1000	2	
		ND	0.353	63																			
18694-40-1	Mepirizole (Epirizole)	<0.005		17	3.0489	2.8503	8.3926	0													1000	28.5	
537-46-2	Methamphetamine	0.26		19				0															
57-27-2	Morphine	1.24		19	6545	2.7561	93	1	0	0	1	0	0	0	0	0	LOEC	Immunotoxic effect	*E. complanata*	8840	1000	0.09	36
		3.679		62																			
72-69-5	Nortriptyline	0.002	0.007	15	2.5555	2.2558	6.0090	1	0	0	1	0	0	0	0	0	EC50	Spherical déformation	*S. ambiguum*	370	1000	0.37	36
132539-06-1	Olanzapine	0.3	0.127	63				0															
604-75-1	Oxazepam	1.123		62	1.921	3.789	49	0													1000	0.0019	
		0.186	0.942	63																			

(continued on next page)

Table 3m (*continued*)

N° CAS	Compound	HWW conc. (µg/L)		Ref.	ECOSAR (µg/L) (Sanderson et al., 2004)			Experimental ecotoxicity												EF	PNEC (µg/L)	Ref.
								Tested species	Trophic level				Chronic test				The most sensitive species						
		Min	Ma x		Algae	Daphnia	Fish		D	P	1C	2C	D	P	1C	2C	Endpoint	Parameter	Species	Value (µg/L)			
103-90-2	Paracetamol (Acetaminophen)	0.5	29	10	9.3816	2.7585	1071	24	2	5	11	6	0	4	1	0	EC50	Mortality	*S.proboscideus*	346	50	6.92	36; 42
		1.4	5.9	32																			
		<0.002	186.5	18																			
		107		62																			
		0.271	63.1	29																			
		5.421	1369	31																			
		56.11	98.85	28																			
		3.1	21.2	11																			
		36.95		17																			
61869-08-7	Paroxetine	0.013	0.019	15	1.5699	1.2627	2.8595	5	0	1	3	1	0	1	1	0	LOEC	Reproduction	*C. dubia*	440	50	8.8	36
		ND		63																			
		<0.002	0.076	32																			
		<0.0025		17																			
76-74-4	Pentobarbital	0.011	0.15	32			123000	1	0	0	0	1	0	0	0	0	EC50	Mortality	*P. promelas*	4.9500			36
58-39-9	Perphenazine	ND	0.012	63	0.854	0.71	7.613	0													1000	0.00071	
60-80-0	Phenazone (Antipyrine)	0.05	0.2	15	276	675	306	0													1000	0.276	
		0.162		62																			
		<0.0434	2.5	11																			
		<0.003	<0.008	32																			
50-06-6	Phenobarbital	<0.001	0.36	32				10	2	0	6	2	0	0	0	0	EC50	Immobilization	*D. magna*	878	800	1.1	36; 42
125-33-7	Primidone	0.383		62	69	73	265	0													1000	0.069	
2078-54-8	Propofol	1.1	10.1	25	895	306	42	0													1000	0.042	
479-92-5	Propyphenazone	<0.003	0.1	32	0.242	0.349	0.086	0													1000	0.000086	
111974-69-7	Quetiapine	ND	4.967	63				0															
106266-06-2	Risperidone	0.003	0.069	63	1.119	1.125	13	0													1000	0.001	
79617-96-2	Sertraline	0.009	0.01	15	1.3086	1.0359	2.2820	12	1	4	3	4	0	4	2	0	EC50	Growth	*P. subcapitata*	12.1	50	0.242	36; 42
		0.009	0.106	63																			
15676-16-1	Sulpiride	0. 432	10.83	63	8.264	43	688	0													1000	0.008	
76-75-5	Thiopental	0.763		63				0															
27203-92-5	Tramadol	0.958		63				0															
144-11-6	Trihexyphenidyl	0.007	0.153	63				0															
93413-69-5	Venlafaxine	0.811		63				0															
151319-34-5	Zaleplon	ND	0.033	63				0															
146939-27-7	Ziprasidone	ND	0.004	63				2	0	0	2	0	0	0	2	0	NOEC	Reproduction	*D. magna*	11	100	0.11	36

HWW conc.: concentration measured in hospital wastewater; D: Decomposers; P: Primary producer; 1C: Primary Consumer; 2C: Secondary consumer; Ref.: all references are grouped in Table 3u; +C: Ecotoxicity data available for tests on community of organisms (several species exposed together), ND: Not detected.

Table 3n
Inventory of compounds sought in hospital wastewater with their corresponding concentration, ecotoxicity data and PNEC calculation — compounds for veterinary use.

N° CAS	Compound	HWW conc. (µg/L)		Ref.	ECOSAR (µg/L) (Sanderson et al., 2004)			Experimental ecotoxicity												EF	PNEC (µg/L)	Ref.
								Tested species	Trophic level				Chronic test				The most sensitive species						
		Min	Max		Algae	Daphnia	Fish		D	P	1C	2C	D	P	1C	2C	Endpoint	Parameter	Species	Value (µg/L)			
41937-02-4	Brombuterol	<0.0025		17																			
57775-29-8	Carazolol	<0.001	0.0023	32	1539	2497	31000	0													1000	1.539	
6804-07-5	Carbadox	<0.005		17																			
80370-57-6	Ceftiofur	<0.003		64																			
112398-08-0	Danofloxacin	<0.005	<0.008	32																			
		<0.012		64																			
98106-17-3	Difloxacin	<0.005		17	60000	214000	863000	0													1000	60	
551-92-8	Dimetridazole	0.019		17																			
93106-60-6	Enrofloxacin	<0.004	<0.005	32				8	1	3	3	1	0	3	1	0	EC50	Growth	*M. aeruginosa*	49	50	0.98	36; 42
		ND		29																			
		<0.008		64																			
		0.04		17																			
43210-67-9	Fenbendazole	<0.001		17	318	506	512	1	0	0	0	1	0	0	0	0	EC50	Devlopment	*D. rerio*	24	100	0.24	59
73231-34-2	Florfenicol	ND		29				6	0	4	2	0	0	4	0	0	EC50	Population growth	*T. chui*	1300	1000	1.3	42; 50; 60
		<0.0005		17																			
115550-35-1	Marbofloxacin	0.003		17																			
		<0.004		64																			
20574-50-9	Morantel	<0.0025		17	1344	145	156	0													1000	0.145	
113617-63-3	Orbifloxacin	<0.001		64																			
53716-50-0	Oxfendazole	<0.001		17	3592	10541	37166	1	0	0	0	1	0	0	0	0	EC50	Development	*D. rerio*	68000	1000	3.596	59
97825-25-7	Ractopamine	<0.005		17																			
98105-99-8	Sarafloxacin	<0.001		17				2	0	2	0	0	0	2	0	0	EC50	Growth	*P. subcapitata*	16000			41
000080-35-3	Sulfamethoxypyridazine	<0.001		17	82990	180	6407	4	2	2	0	0	0	2	0	0	EC50	Growth	*L. minor*	1510	1000	1.51	38
		<0.005		64																			
1220-83-3	Sulfamonomethoxine	<0.001		17	82990	180	6407	0													1000	0.18	
		<0.001		18																			
122-16-7	Sulfanitran	ND		64				0															
59-40-5	Sulfaquinoxaline	<0.001		17	37384	114	2733	5	0	4	1	0	0	4	1	0	LOEC	Growth	*S. dimorphus*	20	50	0.4	39
108050-54-0	Tilmicosin	0.014	0.35	32	665	1007	872	0													1000	0.665	
		<0.008		64																			
1401-69-0	Tylosin	<0.01		4	16302	66797	27413	6	1	4	1	0	1	4	1	0	LOEC	Growth	*P. subcapitata*	64	50	1.28	36
		<0.01		2																			
		<0.012		64																			
		<0.005		18																			
		ND		29																			
		<0.001	<0.002	32																			

HWW conc.: concentration measured in hospital wastewater; D: decomposers; P: primary producer; 1C: primary consumer; 2C: secondary consumer; Ref.: all references are grouped in Table 3u.

Table 3o
Inventory of compounds sought in hospital wastewater with their corresponding concentration, ecotoxicity data and PNEC calculation — respiratory system.

N° CAS	Compound	HWW conc. (µg/L)		Ref.	ECOSAR (µg/L) (Sanderson et al., 2004)			Experimental ecot oxicity												EF	PNEC (µg/L)	Ref.
								Tested species	Trophic level				Chronic test				The most sensitive species						
		Min	Max		Algae	Daphnia	Fish		D	P	1C	2C	D	P	1C	2C	Endpoint	Parameter	Species	Value (µg/L)			
37148-27-9	Clenbuterol	<0.002	1.119	32	2376	40	475	0													1000	0.04	
		<0.0025		17																			
50-02-2	Dexamethasone	0.147		62	41	32	37	9	0	0	3	6	0	0	1	0	LOEC	Weight	*A. mexicanus*	100	100	1	36; 42
79794-75-5	Loratadine	<0.001	0.026	32	49	142	21	0													1000	0.021	
18559-94-9	Salbutamol	0.026	0.14	32	5837	5730	1158	0													1000	1.158	
		0.022		17																			
23031-25-6	Terbutaline	0.038		17				0															
41570-61-0	Tulobuterol	<0.001		17				0															

HWW conc.: concentration measured in hospital wastewater; D: decomposers; P: primary producer; 1C: primary consumer; 2C: secondary consumer; Ref.: all references are grouped in Table 3u.

Table 3p
Inventory of compounds sought in hospital wastewater with their corresponding concentration, ecotoxicity data and PNEC calculation — iodine compounds medium.

N° CAS	Compound	HWW conc. (µg/L)		Ref.	ECOSAR (µg/L) (Sanderson et al., 2004)			Experimental ecotoxicity												EF	PNEC (µg/L)	Ref.
								Tested species	Trophic level				Chronic test				The most sensitive species						
		Min	Max		Algae	Daphnia	Fish		D	P	1C	2C	D	P	1C	2C	Endpoint	Parameter	Species	Value (µg/L)			
737-31-5	Diatrizoate	348.7		62				0															
136949-58-1	Iobitridol	<0,1	3213	25				0															
66108-95-0	Iohexol	<12		62				0															
		<20	1700	33	45705000	97190000	12000000000	0													1000	45705	
78649-41-9	Iomeprol	0,35	1390	25				0															
		439		62																			
		28	2400	33																			
62883-00-5	Iopamidol	<10	1120	33	5363000	8592000	64725000	0													1000	5363	
		2599		62																			
73334-07-3	Iopromide	<5	1390	33	25600	55086	609000000	4	0	1	1	2	0	1	1	0	EC50	Reproduction	*D. magna*	> 1000000	100	256	42
		170.6		62																			
28179-44-4	Ioxitalamic acid	342		62	2857	3154	12412000	0													1000	2.857	
		15	550	33																			

HWW conc.: concentration measured in hospital wastewater; D: decomposers; P: primary producer; 1C: primary consumer; 2C: secondary consumer; Ref.: all references are grouped in Table 3u.

Table 3q
Inventory of compounds sought in hospital wastewater with their corresponding concentration, ecotoxicity data and PNEC calculation — metabolites.

N° CAS	Parent compound	Metabolite	HWW Conc. (µg/L)		Ref.	Experimental ecotoxicity												EF	PNEC (µg/L)	Ref.
						Tested species	Trophic level				Chronic test				The most sensitive species					
			Min	Max			D	P	1C	2C	D	P	1C	2C	Endpoint	Parameter	Species	Value (µg/L)		
114248-23-6	Gemcitabine	2'2'-difluorodeoxyuridine	<0.009	0.84	12	3	1	1	1	0	0	1	1	0	LOEC	Immobilization	*D. magna*	100000	50	2000	61
83-15-8	Dipyrone, Metamizole, Phenazone	4-acetyl aminoantipyrin (Ampyrone)	57.1	77.4	7	0															
			225		62																
83-07-8		4-aminoantipyrine	101		62	0															
58-15-1		4-dimethylaminoantipyrine	<0.14		62	0															
1672-58-8		4-formyl-aminoantipyrin	17.81	25.99	7	0															
			47.88		62	0															
519-98-2		4-methylaminoantipyrine	218		62	0															
?	?	4/5-methylbenzotriazole	223		62	0															
6703-27-1	Codeine	6-acetylcodeine	<0.002		19	0															
2784-73-8	Morphine	6-acetylmorphine	<0.0005	0.039	19	0															
56392-14-4	Atenolol	Atenolol acid	9.84		62	0															
519-09-5	Cocaine	Benzoylecgonine	0.029		19	0															
882-09-7	Clofibrate	Clofibric acid	0.009		17	12	0	5	3	4	0	5	1	0	LOEC	Mortality	*B. calyciflorus*	740	50	14.8	42
			<0.07		62																
34245-14-2	Verapamil	D617	0.155		62	0															
63950-05-0	Doxorubicin	Doxorubicinol	<0.01		35	0															
?	Tetracycline	Epitetracycline	<0.5	18.9	27	0															
59319-72-1	Erythromycine	Erythromycin-H2O	<0.5	83	26	0															
			0.676		17																
			<0.001	6.11	18																
			< 0.005	0.827	4																
			ND		29																
514-53-4	Chlortetracycline	Iso-chlorotetracycline	<0.01	0.02	4	0															
4812-40-2	Metronidazole	Metronidazole-OH	0.15	0.887	64	0															
127-74-2	Sulfadiazine	N-acetylsulfadiazine	<0.025		64	1	0	1	0	0	0	1	0	0	EC50	Growth	Cyanobacteria	101000			51
127-73-1	Sulfamerazine	N-acetylsulfamerazine	<0.033		64	0															
100-90-3	Sulfamethazine	N-acetylsulfamethazine	<0.007		64	0															
31750-48-8	Tamoxifen	N-desmethyltamoxifen	<0.001		21	0															
187227-45-8	Oseltamivir	Oseltamivir carboxylate	0.151		62	0															
19395-41-6	Methylphenidate	Ritalinic acid	0.295		62	0															

HWW conc.: concentration measured in hospital wastewater; D: decomposers; P: primary producer; 1C: primary consumer; 2C: secondary consumer; Ref.: all references are grouped in Table 3u.

Table 3r
Inventory of compounds sought in hospital wastewater with their corresponding concentration, ecotoxicity data and PNEC calculation — non-pharmaceuticals compounds.

N° CAS	Group	Parent compound	Conc. HWW (µg/L)		Ref.	Experimental ecotoxicity												EF	PNE C (µg/L)	Ref.
						Tested species	Trophic level				Chronic test				The most sensitive species					
			Min	Max			D	P	1C	2C	D	P	1C	2C	Endpoint	Paramètre	Species	Value (µg/L)			
67-56-1	Alcohol	Methanol	760		1	65 + C	1	16	29	19	1	16	0	1	LC50	Mortality	*A. imbecillis*	37020	50	740,4	42
64-17-5	Alcohol	Ethyl alcohol (Ethanol)	1700		1	55	0	13	25	17	0	3	4	0	EC50	Reproduction	*D. magna*	14000	10	1400	42
67-63-0	Alcohol	2-propanol	3670		1	20	0	4	8	8	0	4	0	0	EC50	Growth	*C. vacuolata*	11.4	1000	0.00114	42
75-65-0	Alcohol	Tert-butanol (Tertiobutanol)	<100		1	8	0	0	4	4	0	0	0	0	NOEC	Mortality	*C. riparius*	1600000			42
71-23-8	Alcohol	1-Propanol	<100		1	32	0	5	19	8	0	5	0	0	EC50	Mortality	*O. latipes*	600000	1000	600	42
78-92-2	Alcohol	2-Butanol	<100		1	8	0	1	3	4	0	1	0	0	LC50	Mortality	*X. laevis*	1530000	1000	1530	42
78-83-1	Alcohol	2-Méthyl-1-propanol	<100		1	21	0	4	7	10	0	4	1	0	NOEC	Reproduction	*D. magna*	4000	50	80	42
565-67-3	Alcohol	2-(3)-Pentanol	<100		1	0															
17015-11-1	Alcohol	3-Hexanol	<100		1	0															
111-27-3	Alcohol	1-Hexanol	<100		1	12 + C	1	5	3	4	1	5	0	0	LOEC	Population growth rate	*M. aeruginosa*	12000	50	240	42
589-55-9	Alcohol	4-Heptanol	<100		1	0															
111-87-5	Alcohol	1-Octanol	<100		1	22 + C	1	5	10	7	1	5	1	0	NOEC	Reproduction	*D. magna*	1000	10	100	42
71-55-6	HVOC	1,1,1-Trichloroethane	<0.5		1	17	0	6	3	8	0	6	1	0	EC50	Population biomass	*C. reinhardtii*	536	50	10.7	42
79-00-5	HVOC	1,1,2-Trichloroethane	<0.5		1	23	0	7	11	5	0	7	0	0	LC50	Mortality	*P. platessa*	5500	1000	5.5	42
75-34-3	HVOC	1,1-Dichloroethane	<0.5		1	2	0	0	1	1	0	0	0	0	LOEC	Mortality	*P. promelas*	500000			42
75-35-4	HVOC	1,1-Dichloroethene	<0.5		1	10	0	4	2	4	0	4	0	0	EC50	Population biomass	*C. reinhardtii*	9120	1000	9.12	42
96-18-4	HVOC	1,2,3-Trichloropropane	<0.5		1	7	0	3	3	1	0	3	0	0	LC50	Mortality	*C. marinus*	20	1000	0.02	42
106-93-4	HVOC	1,2-Dibromethane	<0.5		1	0															
107-06-2	HVOC	1,2-Dichloroethane	<0.5		1	22	0	7	8	7	0	7	0	0	NOEC	Food comsumption	*D. rerio*	1820	1000	1.82	42
74-97-5	HVOC	Bromochloromethane	<0.5		1	1	0	0	0	1	0	0	0	0	LC50	Mortality	*C. carpio*	67000			42
75-27-4	HVOC	Bromodichloromethane	<0.5		1	1	0	0	1	0	0	0	0	0	EC50	Population growth rate	*T. pyriformis*	240000			42
156-59-2	HVOC	cis-Dichloroethene	<0.5		1	1	0	0	0	1	0	0	0	0	LOEC	Mortality	*P. promelas*	500000			42
594-18-3	HVOC	Dibromodichloromethane	0.7		1	0															
75-09-2	HVOC	Dichloromethane	<0.5		1	25	0	12	4	9	0	12	0	0	EC50	Behavioral changes (general)	*D. rerio*	65500	1000	65.5	42
75-69-4	HVOC	Freon 11	<0.5		1	0															
76-13-1	HVOC	Freon 113	2.6		1	0															
127-18-4	HVOC	Tetrachloroethene	<0.5		1	30	0	8	13	9	0	8	2	0	NOEC	Reproduction	*D. magna*	400	50	8	42
56-23-5	HVOC	Tetrachloromethane	<0.5		1	20	0	4	8	8	0	4	0	0	EC50	Population biomass	*C. reinhardtii*	246	1000	0.246	42
156-60-5	HVOC	trans-Dichloroethene	<0.5		1	0															
75-25-2	HVOC	Tribromethane	0.6		1	0															
79-01-6	HVOC	Trichloroethene	<0.5		1	35	0	6	18	11	0	6	1	0	EC50	Mortality	*D. japonica*	1700	50	34	42
67-66-3	HVOC	Trichloromethane	2.4		1	33	0	9	10	14	0	9	2	0	EC50	Mortality	*O. mykiss*	1240	50	24.8	42
75-01-4	HVOC	Vinyl chlorides	<0.5		1	1	0	0	1	0	0	0	0	0	EC50	Mortality	*T. pyriformis*	405000			42
67-64-1	Other	Acétone	7300		1	66	0	11	40	15	0	11	4	0	LC50	Mortality	*D. magna*	10000	50	200	42
8001-54-5	Other	Benzalkonium (HCl)	6000		14	30	2	13	10	5	0	3	0	0	LOEC	Chlorophyll fluorescence	*E. canadensis*	5	1000	0.005	43; 42
95-14-7	Other	Benzotriazole	23.57		62	1	0	0	1	0	0	0	0	0	EC50	Population growth rate	*T. pyriformis*	29270			42
631-64-1	Other	Dibromoacetic acid	5.42	58.29	30	1	0	1	0	0	0	1	0	0	EC50	Mortality	*P. subcapitata*	69000			42
79-43-6	Other	Dichloroacetc acid	5.19	257.84	30	4	0	1	2	1	0	1	0	0	EC50	Mortality	*N. spinipes*	23000	1000	23	42
93-72-1	Other	Fenoprop	<0.0025		17	22	0	6	4	12	0	6	0	0	EC50	Mortality	*P. californica*	340	1000	0.34	42
111-30-8	Other	Glutaraldéhyde	<500	3720	9	8	0	1	3	4	0	1	0	0	EC50	Immobilization	*D. magna*	750	1000	0.75	42
50-00-0	Other	Formaldehyde	70		1	55	0	9	21	25	0	9	0	0	EC50	Immobilization	*C. virginica*	300	1000	0.3	42
79-11-8	Other	Monochloroacetic acid	<0.55	22.14	30	5	0	1	2	2	0	1	1	1 (Bc)	EC50	Growth	*P. subcapitata*	28	50	0.56	42
76-03-9	Other	Trichloroacetic acid	<0.82	19.14	30	18	0	5	5	8	0	5	0	0	EC50	Mortality	*S. proboscideus*	1200	1000	1.2	42

HWW conc.: concentration measured in hospital wastewater; D: decomposers; P: primary producer; 1C: primary consumer; 2C: secondary consumer; Ref.: all references are grouped in Table 3u; + C: ecotoxicity data available for tests on community of organisms (several species exposed together); HVOC: halogenated volatile organic compounds.

Table 3s
Inventory of compounds sought in hospital wastewater with their corresponding concentration, ecotoxicity data and PNEC calculation — gadolinium and platinum.

Element	HWW conc. (µg/L)		Ref.	Experimental ecotoxicity												EF	PNEC (µg/L)	Ref.
				Tested species	Trophic level				Chronic test				The most sensitive species					
	Min	Max			D	P	1C	2C	D	P	1C	2C	Endpoint	Parameter	Species	Value (µg/L)		
Gadolinium (Gd)		100	14	3	0	1	1	1	0	1	0	0	LC50	Mortality	*H. azteca*	150	1000	0.15	42
		32	33															
	<0.5	10.3	28															
Platinum (Pt)	<0.01	3.58	14	7	0	1	5	1	0	1	1	0	EC50	Immobilization	*T. tubifex*	61	50	1.22	42
	4.7	145	16															
	<0.003	0.125	13															
		13	33															

HWW conc.: concentration measured in hospital wastewater; D: decomposers; P: primary producer; 1C: primary consumer; 2C: secondary consumer; ref.: all references are grouped in Table 3u.

2.2.2.3. Detergents (Table 3r). Detergents, which are sometimes sought in HWW, were always subject to global measurements (anionic detergents, cationic detergents, non-ionic detergents) in the works listed, which did not allow calculating the PEC/PNEC ratio or the MCW_{max}/PNEC ratio for each substance concerned. This was unfortunate given their level of concentration and the toxicity of some detergent compounds (Boillot et al., 2008).

2.2.2.4. Heavy metals (Table 3s and 3t). The PNECs of nine of the eleven metals sought were obtained by a statistical method (SSD), giving them considerable robustness. There was insufficient ecotoxicological data on gadolinium and platinum to estimate an SSD (less than ten species). It was observed that the LOD of five metals was higher than the PNECs. Mercury had the lowest PNEC (0.01 µg/L) of the elements measured and detected in the HWW, while the highest MCW_{max}/PNEC ratios were obtained with copper and zinc (101 and 62 respectively).

3. Characterisation of ecotoxicity through a "matrix" approach

3.1. Methodology

Contrary to the "substance" approach, the "matrix" approach considers the impact of the whole matrix on organisms and the community of organisms. This approach comprises several levels of study: (i) monospecific bioassays: species are exposed one by one to a range of concentrations of the matrix; (ii) multispecific bioassays (cosmos): communities of species are exposed together to a range of concentrations of the matrix.

This approach has a major advantage when characterising the ecotoxicity of a mixture since the response of the organisms exposed to the matrix can be observed directly. Furthermore, it permits understanding the combined effect of substances (antagonism, synergy, or additivity). However, it is impossible to determine which compounds are responsible for the mixture's ecotoxicity. "Matrix" and "substance"

Table 3t
Inventory of compounds sought in hospital wastewater with their corresponding concentration, ecotoxicity data and PNEC calculation (From INERIS, 2005) — Metals.

N° CAS	Element	Conc. HWW (µg/L)		Ref.	Statistical data from INERIS, 2005				
					HC5	IC90	EF	PNEC (µg/L)	
		Min	Max		µg/L				
7440-22-4	Silver (Ag)	<2		1	0.203		10	0.0203	
7440-38-2	Arsenic (As)	10		1	22.3	2.9 - 80.7	5	4.4	
		<1	2.4	28					
		11		6					
7440-47-3	Chromium (Cr)	<8		1	7.8	3.4 - 15.2		Cr6	Cr3
		<5		28				4.1	4.7
		<4		6					
7440-50-8	Copper (Cu)	162		1	3.15	1.0 - 2.4	2	1.6	
		8.9	73	28					
		112		6					
7439-92-1	Lead (Pb)	12		1	15.1	6.6 - 27.6	3	5	
		<3.5		6					
		<1	2.6	28					
7439-97-6	Mercury (hg)	0.04	2.6	14				Organic form	Inorganic form
		<2		1					
		<5		28				0.01	0.27
		1.65		33					
		<0.5		6					
7440-02-0	Nickel (Ni)	<2		1			10	0.5	
		<5		28					
		<0.7		6					
7440-66-6	Zinc (Zn)	147		1	17.2	8.1 - 29.2	2	8.6	
		0.03	250	28					
		536		6					
7440-43-9	Cadmium (Cd)	<5		1				< 50 mg/L CaCO3	> 50 mg/L CaCO3
		<0.5		28					
		<7		6				0.21	0.75

HWW conc.: concentration measured in hospital wastewater; HC5: hazardous concentration for 5% of species tested; IC90: confidence index of 90%; Ref.: references for HWW values are grouped in Table 3u and ecotoxicity data are from INERIS, 2005.

approaches therefore complement each other when attempting to ascertain the ecotoxicity of a matrix.

3.2. Results (Table 4)

Data on the ecotoxicity of effluents are much less numerous than data on the substances that compose them. Only ten studies performed on different hospitals are currently available. The ecotoxicity values measured concern only ten organisms, all studies confounded. Only two species were exposed "chronically" to HWW: *Ceriodaphnia dubia* 7 days (Boillot et al., 2008) and *Brachionus calyciflorus*72 h (Perrodin et al., 2012).

These works nonetheless provide a wealth of information on the specific characteristics of these effluents: (i) there is very considerable variability in the ecotoxicity of effluents between hospitals (EC50 of a few % or less for the most ecotoxic effluents, and nearly 100% for the least ecotoxic effluents); (ii) as with many other effluents, toxicity varies greatly as a function of the organism tested (toxicity up to 20 times higher for an organism in comparison to another with the same effluent); (iii) when bioassays permit, the ecotoxicity of non-filtered effluents is greater than that of filtered effluents, therefore demonstrating toxicity linked to the particular fraction (Boillot et al., 2008); (iv) when samples were taken throughout a day of hospital activity, a marked variation of ecotoxicity was recorded between diurnal and nocturnal periods. During the diurnal period, considerable variations of ecotoxicity were observed as a function of the different hospital activities in progress (cleaning premises, operating theatre blocks, chemistry laboratory, etc.) (Boillot et al, 2008).

Lastly, globally, it has been observed that the toxicity of HWW is generally higher than that measured in urban effluents (Santiago et al., 2002).

4. Discussion

4.1. Results obtained by the "substance" approach

4.1.1. Consumption… but not alone!

Before carrying any measurement campaign, whether for HWW or other effluents, it is necessary to choose a list of priority substances to be measured. In existing studies on HWW, the consumption of drugs and other substances is the predominant criteria for choosing these compounds and it is the only criterion in certain cases. This criterion is certainly logical and pertinent (it is pointless to measure compounds that will never be found in the effluents), but other criteria may also be important. Thus, on reading the results of the studies identified in this review, three types of criteria appear important to take into account: (i) those that influence the presence (or detectability) of the compound in the effluent (e.g., consumption, excretion rate, excretion site (ambulatory care or not), analytical constraints). These criteria permit increasing the probability of detecting the compound sought in the effluent, (ii) those that take into account the compound's toxicity (e.g. ecotoxicity, cytotoxicity). The advantage of these criteria is especially to evaluate the impact that toxic compounds can have on the operation of a WWTP (and thus indirectly generate greater pollution) and on organisms of the environment receiving the effluents, (iii) those that take into account the fate and persistence of the compound in the environment (e.g. biodegradability, solubility, photodegradation (DellaGreca et al., 2007)). This type of criterion could be helpful for understanding why some compounds cross the "barrier" of the WWTP without any removal (Verlicchi et al., 2012b).

However, measurements previously available of a compound in the environment should not influence the compounds to be measured. Indeed, if measurements are performed on what has already been investigated, compounds that are potentially present and which may present a high ecotoxicological risk may be overlooked.

Table 3u
Matching between references and publications in Tables 3a to 3t.

1	Boillot et al. (2008)
2	Brown et al. (2006)
3	Catastini et al. (2009)
4	Chang et al. (2010)
5	Duong et al. (2008)
6	Emmanuel et al. (2005)
7	Feldmann et al. (2008)
8	Hartmann et al. (1999)
9	Jolibois et al. (2002)
10	Gómez et al. (2006)
11	Kosma et al. (2010)
12	Kovalova et al. (2009)
13	Kümmerer and Helmers (1997)
14	Kümmerer (2001)
15	Langford and Thomas (2009)
16	Lenz et al. (2005)
17	Lin et al. (2008)
18	Lin and Tsai (2009)
19	Lin et al. (2010)
20	Lindberg et al. (2004)
21	Liu et al. (2010)
22	Löffler and Ternes (2003)
23	Mahnik et al. (2004)
24	Mahnik et al. (2007)
25	Mullot et al. (2010)
26	Ohlsen et al. (2003)
27	Pena et al. (2010)
28	Perrodin et al. (2013)
29	Sim et al. (2011)
30	Sun and Gu (2007)
31	Thomas et al. (2007)
32	Verlicchi et al. (2012a)
33	Weissbrodt et al. (2009)
34	Yin et al. (2010)
35	Tauxe-Wuersh et al. (2006)
36	Wikipharma (Molander et al. 2009)
37	Cunningham et al. (2006)
38	Białk-Bielińska et al. (2011)
39	De Liguoro et al. (2010)
40	Huggett et al. (2002)
41	Santos et al. (2010)
42	EPA ECOTOX database
43	Beveridge et al. (1998)
44	Brausch and Rand (2011)
45	Sanderson and Thomsen (2009)
46	Snell and DesRosiers (2008)
47	Kidd et al. (2007)
48	Grung et al. (2008)
49	Straub et al. (2012)
50	Lai et al. (2009)
51	Eguchi et al. (2004)
52	Isidori et al. (2005)
53	García-Galán et al. (2012)
54	Park and Choi (2008)
55	Park (2005)
56	Stoichev et al. (2011)
57	Besse et al. (2012)
58	DellaGreca et al. (2007)
59	Carlsson et al. (2013)
60	Liu et al. (2012)
61	Zounkova et al. (2010)
62	Kovalova et al. (2012)
63	Yuan et al. (2013)
64	Gros et al. (in press)

4.1.2. The numerous "orphan" compounds of ecotoxicity data

No experimental ecotoxicity data existed for 48% of the 297 compounds sought in the different studies. Of these compounds, 21% were the subject of ECOSAR data (Sanderson et al., 2004). This method is certainly controversial (Madden et al., 2009), but it is the only information available to us at present for these compounds without experimental ecotoxicity data. For example, no experimental data is available for ifosfamide, detected in the HWW and also in the environment (Besse et al., 2012). For the remaining compounds (17%) any ecotoxicological

Table 4
Ecotoxicity of raw hospital wastewater.

Reference	Species	Exposition duration	Parameter	Endpoint	Hospital	Toxicity (% of HWW)
SOCOTEC Environment (1994)	*D. magna*	24 h	Mortality	EC50	A	48.7–>90
					B	2.1–8.1
					C	46.3–>90
	V. fisheri	15 min	Bioluminescence	EC50	A	7.9–18.6
					B	2.1–5.7
					C	25–53.5
Villegas-Navarro et al. (1997)	*D. magna*	24 h	Mortality	EC50	D	0.7 ± 0.3
					E	50.3 ± 8
	D. magna	48 h	Mortality		D	0.4 ± 0.2
					E	33.2 ± 6.6
Emmanuel et al. (2004)	*V. fisheri*	5 min	Bioluminescence	EC50	F	1.42 ± 0.25
		15 min				3.4 ± 0.7
	D.magna	24 h	Mortality	EC50		44 ± 34
Emmanuel et al. (2005)	*V. fisheri*	5 min	Bioluminescence	EC50	G	40
		15 min				23.8
		30 min				21.7
	P. subcapitata	72 h	Growth			1.8
	D. magna	24 h	Mortality			1.6
		48 h				1.4
Tsakona et al. (2007)	*V. fisheri*	5 min	Bioluminescence	EC50	H	15.6
		15 min				6.8
Boillot et al. (2008)	*D. magna*	24 h	Mortality	EC20	I	19
		48 h				17
	V. fisheri	15 min	Bioluminescence			15
		30 min				14
	P. subcapitata	72 h	Growth			7
	C. dubia	24 h	Mortality			18
		48 h	Mortality			18
		7 d	Growth			23
		7 d	Reproduction			5
	L. minor	7 d	Growth			100
Berto et al. (2009)	*D. magna*	48 h	Mortality	LOEC	J	4
	P. subcapitata	72h	Fluorescence			16
Zgorska et al. (2011)	*V. fisheri*	?	Bioluminescence	EC50	K	46.3
	A. salina	24 h	Mortality			59.9
	D. magna	48 h	Mortality			20.8
	T. platyurus	24 h	Mortality			22.6
	P. subcapitata	72 h	Growth			18.8
Perrodin et al. (2012)	*P. subcapitata*	72 h	Growth	EC20	L	100
						100
	H. incongruens	72 h	Mortality			100
						100
	B. calyciflorus	72 h	Mortality			14
						11.5

data, either modelled or experimental, is available. This is especially interesting since many compounds such as econazole and mifepristone are detected in effluents, and have already been identified as having priority in the literature due to their bioaccumulability and their low biodegradability (Jean et al., 2012). Globally, 105 others compound are detected in HWW without experimental ecotoxicity.

4.1.3. Over-high limits of detection (LOD)

For some compounds, it is not possible to evaluate their ecotoxicity in hospital effluents as their limits of detection are higher than the PNEC established. Therefore care must be taken before concluding on the toxicity of these compounds: a compound not detected does not mean that it is necessarily absent or inoffensive. Thus, before measuring a compound in view to assessing its contribution to the ecotoxicity of a given effluent, it is important to consider the LOD. Furthermore, it is also necessary to ask the question of what one wishes to measure, especially regarding metals. Indeed, the ecotoxicity of a metal can differ according to its chemical formula (e.g. $CdCl_2$ or $Cd(OH)_2$), and to whether it is inorganic or organic (e.g. Hg or $Hg–CH_3$) (INERIS, 2005). The hardness of the medium can also influence ecotoxicity, thus it is interesting to obtain this parameter during measurements.

4.1.4. Limits of the European method of calculating PNECs

The recommendations of the TGD (2003) for calculating PNEC were very useful for this synthesis on the ecotoxicity of the substances present in HWW, but present certain limits. The latter include in particular the use of an available number of data about species belonging to different "trophic levels" to determine the EF. The organisms are grouped into 4 trophic levels (primary (C1) and secondary consumers (C2), primary producers (P) and decomposers (D)). Organisms with very different metabolisms are present at each level (e.g. C1: insects/protozoa, C2: rotifers/fishes) with equally important roles in the functioning of the ecosystem (e.g. epibenthic, endobenthic and pelagic). This grouping by trophic level would be interesting if we had sufficient data for the compounds of interest relating to the phenomena of bioamplification and bioaccumulation. However, the effects generated by the contaminants are linked more closely to the metabolism of the organism considered than to the trophic level to which it belongs. Grouping organisms by major metabolic class could indicate progress. In this way, the organisms exposed would be more susceptible, within the same class, to undergo the same impacts. Thus the weight accorded to the individuals of one class or another would be the same for all the organisms of this class (not like the rotifer or the fish which although belonging to the same trophic level do not allow applying the same EF when chronic data exist for both organisms).

Furthermore, with the current classification, some organisms are very difficult to classify (omnivorous, change of trophic level on becoming adults, etc.) or cannot be classified (*Eumycota*) according to current classification.

Moreover, this classification does not take into account the species diversity of the organisms tested. Thus a compound with a long term EC50 for two species of different trophic levels (fishes and daphnia) can have an EF of 50 (e.g. ranitidine) while acute tests of a compound on 30 species will have an EF of 1000 (e.g. chloramphenicol).

4.1.5. There is nothing automatic about antibiotics!

Among the PhCp measured, antibiotics are over-represented (20% of the compounds measured). On the contrary, PhCp such as anitivirals are almost absent from the measures performed (only 2 antivirals (ritonavir and oseltamivir) among 60 compounds). Nonetheless, Jean et al. (2012) identified 7 antivirals as prevalently bioaccumulable compounds as ritonavir found in HWW (Kovalova et al., 2012). This observation is a good illustration of the preference given to the quantity consumed in comparison to other criteria, such as "bioaccumulabity", for choosing the compounds to be measured.

4.1.6. "Drug focused" measurements

Curing patients is not the only activity performed in hospitals, and compounds other than drugs are used. However, very few studies have focused on the other products present in HWW. Among these other "compounds", mention can be made of: (i) disinfectants including bleach, glutaraldehyde and detergents. These compounds may be present in large quantities and contribute to a great extent to the toxicity of HWW (Boillot et al., 2008); (ii) metabolites produced by metabolism (e.g. erythromycine H_2O) and reactions between organic compounds and the chlorine present in HWW (e.g. dichloracetic acid). These compounds must be considered in the same way as "parent" compounds, since their toxicity can be far greater than the original compounds (Daniel et al., 1981; Wu et al., 2009); (iii) products used for diagnostics such as ICMs, present in large quantities in both hospital effluents and the environment (Santos et al., 2010). What is more, there is practically no ecotoxicological data relating to these compounds; (iv) metals that can stem from the ageing of hospital piping, leaching of buildings by rainwater (sometimes mixed with wastewater (e.g. Cu, Pb)), and drugs (e.g. Gd, Pt). Regarding the latter, the speciation or chemical form is never specified. However, this information is necessary to evaluate the toxicity linked to their presence. Indeed, most of the time, neutral or inorganic forms are often far less toxic and/or bioavailable than ionised or organic forms (e.g. for mercury and cadmium).

4.1.7. Limits of ATC classification

This ATC classification system was chosen for practical reasons. It permits easy comparison with pharmacological and metabolic data. Moreover, it allows communication between specialists of disparate disciplines such as chemists, ecologists and biologists. However, it is based on a human metabolic classification, not necessarily consistent with the other living organisms used in ecotoxicology.

Another artefact linked to this classification is the presence of compounds for veterinary use in HWW. Some compounds are not used exclusively for medical purposes; they can also be used for veterinary purposes. What is more, they can be formed after "parent" compounds have reacted with other compounds. Certain of the compounds found can be considered as corresponding to metabolites of compounds for human use since some molecules are structurally very similar. In addition, some compounds could belong to several categories and so being hard to classify.

4.2. Results obtained by the "matrix" approach

4.2.1. The variability of ecotoxicity values

Theoretically, the data available relating to the ecotoxicity measured in the effluents make it possible to perform a more realistic evaluation of their ecotoxicity, but they are difficult to interpret given their variability. This variability is linked to the diversity of the activities practised in the hospitals considered. The period of the year, the geographic position or the number of patients is one of the parameters that can influence the toxicity of HWW. The moment of sampling during a 24 h period can also influence the concentration of pollutants in the effluent. For example, Boillot et al., demonstrated that the period of the day in which the effluent was most ecotoxic was between 9 a.m. and 1 p.m., a period of intense activity in the hospital.

4.2.2. Ecotoxicity linked to the particulate phase of the effluent

Few authors have focused on measuring the particulate phase of HWW effluents (Löffler and Ternes, 2003; Boillot et al., 2008; Langford and Thomas, 2009). However, the concentration in contaminant can be up to five times greater when measuring a non-filtered sample (Löffler and Ternes, 2003). This adsorption of pollutants in the particulate phase of the effluent and the consequences it implies on the ecotoxicity of the effluent as a whole have already been observed with other types of effluents (Corapcioglu and Jiang, 1993; Grolimund and Borkovec, 2001; Han et al., 2006; Massoudieh and Ginn, 2008; Mc Gechan and Lewis, 2002). It is essential to take this into account to perform an accurate assessment of the ecotoxicity of HWW.

4.2.3. Variation of ecotoxicity linked to the desorption of VOCs in the gaseous phase

The presence of highly volatile substances in HWW (e.g. HVOC, residual active chlorine, etc.), and the subsequent potential transfer in gaseous phase can lead to a considerable variation of the ecotoxicity of HWW through time. This phenomenon can occur in particular when HWW flows into urban sewer systems, or during the transport and conservation of samples in the laboratory under inappropriate conditions.

4.3. Comparison between "substance" and "matrix" approaches

The comparison of the composition of HWW with the ecotoxicity of the corresponding effluents could provide a clearer explanation of the source of their ecotoxicity and, on this basis, lead to recommendations on the compounds to be treated with priority, or to be eliminated from consumption in hospitals. This task, although tempting at first glance, is always difficult to perform due to the considerable number of phenomena to be taken into account regarding the ecotoxicity of an effluent. Mention can be made here of the phenomena of ecotoxicological synergy and/or antagonism liable to exist between the compounds of the same effluent, as well as the effect of the effluent's pH and hardness on ecotoxicity.

In the present case, the exercise is even more difficult, given the scarcity of studies available that link "in-depth chemical analysis" with "ecotoxicity" for the same effluent.

5. Conclusion and perspectives

This review first highlighted the very great diversity of compounds present in HWW. Given the numerous activities practised in hospitals, with the latter sometimes sharing sewer systems carrying rainwater, a very large number of different substances are discharged into the effluents (disinfectants, detergents, drugs, heavy metals, etc.).

Two complementary approaches have been used to assess the ecotoxicity of HWW. The "substance" approach allows identifying the presence of very toxic compounds (minimum PNEC observed close to 10^{-8} µg/L). This ecotoxicity was observed for both the short term (acute toxicity) and the long term (chronic toxicity). The conclusions of the "matrix" approach are similar, emphasising strong ecotoxicity of the global effluent in some cases. These two approaches also underline the great complexity of HWW, with considerable qualitative variability (type of compounds (PhCp, detergents, metabolites, etc.)) and quantitative variability (concentrations of the different compounds

detected), hence potential ecotoxicity that varies greatly as a function of the case considered.

The work also brings to the fore the predominance, in the studies and practices of environmental agencies, of consumption as a criterion for selecting the compounds to be dosed. Excretion rate and biodegradability are sometimes taken into account as supplementary criteria. As for the "bioaccumulability" and "ecotoxicity" of substances, they are rarely taken into account for choosing the compounds to be dosed, whereas they have a major impact on the risk of these compounds for ecosystems.

To obtain further knowledge on the ecotoxicity of HWW, it is now necessary to continue chemical and ecotoxicological analyses in other hospitals to identify links between their characteristics (size, type of departments, period of the year, etc.) and the ecotoxicity of their effluents. Regarding chemical analyses, we recommend in particular seeking substances with strong ecotoxic potential, in addition to their level of consumption. It is also necessary to follow metabolite concentration for a better understanding about the link between composition of wastewater and ecotoxicity even if this link is not always directly possible. This statement is highlighted too by Escher and Fenner (2011) who demonstrated the need to monitor the metabolites of the substances consumed. As for ecotoxicological analyses, we recommend using a battery of bioassays that include at least three trophic levels (chronic tests) and performing bioassays on the particulate phase of effluents, which are often richer in pollutants than the dissolved phase. Following-up the assessment of toxicity during a day and a week can also provide a wealth of information.

In addition to these recommendations, performing studies combining "in-depth chemical analysis" and "ecotoxicological analysis" for the same effluent is advisable to improve the knowledge of the source of the ecotoxicity of effluents in order to make recommendations on the priority compounds for treatment or elimination from consumption in hospitals.

Lastly, although analysis of the global effluent collected at the point of discharge from the hospital provides interesting information, it is obviously now necessary to set up ecotoxocological monitoring of the effluents of each department in them, in view to obtaining better understanding of the global spatial–temporal functioning of hospitals, and to assessing the contribution of different hospital departments to global ecotoxicity more efficiently. This step is required to define effective hospital environmental management policies (replacement of toxic products by less pollutant alternative products, sterilisation by steam rather than bactericides, etc.).

References

Berto J, Rochenbach GC, Barreiros MAB, Corrêa AXR, Peluso-Silva S, Radetski CM. Physicochemical, microbiological and ecotoxicological evaluation of a septic tank/Fenton reaction combination for the treatment of hospital wastewaters. Ecotoxicol Environ Saf 2009;72:1076–81.

Besse J-P, Garric J. Human pharmaceuticals in surface waters: implementation of a prioritization methodology and application to the French situation. Toxicol Lett 2008;176:104–23.

Besse J-P, Latour J-F, Garric J. Anticancer drugs in surface waters: what can we say about the occurrence and environmental significance of cytotoxic, cytostatic and endocrine therapy drugs? Environ Int 2012;39:73–86.

Beveridge CM, Parr ACS, Smith MJ, Kerr A, Cowling MJ, Hodgkiess T. The effect of benzalkonium chloride concentration on nine species of marine diatom. Environ Pollut 1998;103:31–6.

Białk-Bielińska A, Stolte S, Arning J, Uebers U, Böschen A, Stepnowski P, et al. Ecotoxicity evaluation of selected sulfonamides. Chemosphere 2011;85:928–33.

Boillot C, Bazin C, Tissot-Guerraz F, Droguet J, Perraud M, Cetre JC, et al. Daily physicochemical, microbiological and ecotoxicological fluctuations of a hospital effluent according to technical and care activities. Sci Total Environ 2008;403:113–29.

Brausch JM, Rand GM. A review of personal care products in the aquatic environment: environmental concentrations and toxicity. Chemosphere 2011;82:1518–32.

Brown KD, Kulis J, Thomson B, Chapman TH, Mawhinney DB. Occurrence of antibiotics in hospital, residential, and dairy effluent, municipal wastewater, and the Rio Grande in New Mexico. Sci Total Environ 2006;366:772–83.

Carlsson G, Patring J, Kreuger J, Norrgren L, Oskarsson A. Toxicity of 15 veterinary pharmaceuticals in zebrafish (*Danio rerio*) embryos. Aquat Toxicol 2013;126:30–41.

Catastini C, Mullot J-U, Luis Castillo PM, Tordjman I, Cervantes P, Modelon H, et al. Recherche de molécules anticancéreuses dans des effluents hospitaliers: étude pilote. Tech Hosp 2009;714:56–61.

Chang X, Meyer MT, Liu X, Zhao Q, Chen H, Chen J-a, et al. Determination of antibiotics in sewage from hospitals, nursery and slaughter house, wastewater treatment plant and source water in Chongqing region of Three Gorge Reservoir in China. Environ Pollut 2010;158:1444–50.

Corapcioglu M, Jiang S. Colloid facilitated groundwater contaminant transport. Water Resour Res 1993;29:2215–26.

Cunningham VL, Buzby M, Huthcinson T, Mastrocco F, Parke N, Roden N. Effects of human pharmaceuticals on aquatic life: next steps. Environ Sci Technol 2006;40:3456–62.

Daniel P, Gaskell SJ, Bishop H, Campbell C, Nicholson RI. Determination of tamoxifen and biologically active metabolites in human breast tumours and plasma. Eur J Cancer Clin Oncol 1981;17:1183–9.

De Liguoro M, Di Leva V, Gallina G, Faccio E, Pinto G, Pollio A. Evaluation of the aquatic toxicity of two veterinary sulfonamides using five test organisms. Chemosphere 2010;81:788–93.

DellaGreca M, Iesce MR, Isidori M, Nardelli A, Previtera L, Rubino M. Phototransformation products of tamoxifen by sunlight in water. Toxicity of the drug and its derivatives on aquatic organisms. Chemosphere 2007;67:1933–9.

Duong HA, Pham NH, Nguyen HT, Hoang TT, Pham HV, Pham VC, et al. Occurrence, fate and antibiotic resistance of fluoroquinolone antibacterials in hospital wastewaters in Hanoi, Vietnam. Chemosphere 2008;72:968–73.

Eguchi K, Nagase H, Ozawa M, Endoh YS, Goto K, Hirata K, et al. Evaluation of antimicrobial agents for veterinary use in the ecotoxicity test using microalgae. Chemosphere 2004;57:1733–8.

Emmanuel E, Keck G, Blanchard J-M, Vermande P, Perrodin Y. Toxicological effects of disinfections using sodium hypochlorite on aquatic organisms and its contribution to AOX formation in hospital wastewater. Environ Int 2004;30:891–900.

Emmanuel E, Perrodin Y, Keck G, Blanchard JM, Vermande P. Ecotoxicological risk assessment of hospital wastewater: a proposed framework for raw effluents discharging into urban sewer network. J Hazard Mater 2005;117:1-11.

EPA ECOTOX database. (last access 01/12/12)http://www.epa.gov/ecotox/.

Escher BI, Baumgartner R, Koller M, Treyer K, Lienert J, McArdell CS. Environmental toxicology and risk assessment of pharmaceuticals from hospital wastewater. Water Res 2011;45:75–92.

Escher BI, Fenner K. Recent advances in environmental risk assessment of transformation products. Environ Sci Technol 2011;45:3835–47.

Feldmann DF, Zuehlke S, Heberer T. Occurrence, fate and assessment of polar metamizole (dipyrone) residues in hospital and municipal wastewater. Chemosphere 2008;71:1754–64.

García-Galán MJ, González Blanco S, López Roldán R, Díaz-Cruz S, Barceló D. Ecotoxicity evaluation and removal of sulfonamides and their acetylated metabolites during conventional wastewater treatment. Sci Total Environ 2012;437:403–12.

Gómez MJ, Petrović M, Fernández-Alba AR, Barceló D. Determination of pharmaceuticals of various therapeutic classes by solid-phase extraction and liquid chromatography–tandem mass spectrometry analysis in hospital effluent wastewaters. J Chromatogr A 2006;1114:224–33.

Grolimund D, Borkovec M. Release and transport of colloidal particles in natural porous media. 1. Modelling. Water Resour Res 2001;37:559–70.

Gros M, Rodríguez-Mozaz S, Barceló D. Rapid analysis of multiclass antibiotic residues and some of their metabolites in hospital, urban wastewater and river water by ultra-high-performance liquid chromatography coupled to quadrupole-linear ion trap tandem mass spectrometry. J Chromatogr A in press; http://dx.doi.org/10.1016/j.chroma.2012.12.072.

Grung M, Källqvist T, Sakshaug S, Skurtveit S, Thomas KV. Environmental assessment of Norwegian priority pharmaceuticals based on the EMEA guideline. Ecotoxicol Environ Saf 2008;71:328–40.

Halling-Sørensen B, Nors Nielsen S, Lanzky PF, Ingerslev F, Holten Lützhøft HC, Jørgensen SE. Occurrence, fate and effects of pharmaceutical substances in the environment — a review. Chemosphere 1998;36:357–93.

Han GH, Hur HG, Kim SD. Ecotoxicological risk of pharmaceuticals from wastewater treatment plants in Korea: occurrence and toxicity to *Daphnia magna*. Environ Toxicol Chem 2006;25:265–71.

Hartmann A, Golet EM, Gartiser S, Alder AC, Koller T, Widmer RM. Primary DNA damage but not mutagenicity correlates with ciprofloxacin concentrations in German hospital wastewaters. Arch Environ Contam Toxicol 1999;36:115–9.

Heberer T. Tracking persistent pharmaceutical residues in the aquatic environment: a review of recent research data. Toxicol Lett 2002;131:5-17.

Huggett DB, Brooks BW, Peterson B, Foran CM, Schlenk D. Toxicity of select beta adrenergic receptor-blocking pharmaceuticals (B-blockers) on aquatic organisms. Arch Environ Contam Toxicol 2002;43:229–35.

INERIS. Fiches de données toxicologiques et environnementales des substances chimiques; 2005 [http://www.ineris.fr/rapports-d%C3%A9tude/toxicologie-et-environnement/fiches-de-donn%C3%A9es-toxicologiques-et-environnementales- (Dernier accès 18/12/2012)].

Isidori M, Lavorgna M, Nardelli A, Pascarella L, Parrella A. Toxic and genotoxic evaluation of six antibiotics on non-target organisms. Sci Total Environ 2005;346:87–98.

Jean J, Perrodin Y, Pivot C, Trepo D, Perraud M, Droguet J, et al. Identification and prioritization of bioaccumulable pharmaceutical substances discharged in hospital effluents. J Environ Manage 2012;103:113–21.

Jjemba PK. Excretion and ecotoxicity of pharmaceutical and personal care products in the environment. Ecotoxicol Environ Saf 2006;63:113–30.

Jolibois B, Guerbet M, Vassal S. Glutaraldehyde in hospital wastewater. Arch Environ Contam Toxicol 2002;42:137–44.

Joss A, Keller E, Alder AC, Göbel A, McArdell CS, Ternes T, et al. Removal of pharmaceuticals and fragrances in biological wastewater treatment. Water Res 2005;39:3139–52.
Kidd KA, Blanchfield PJ, Mills KH, Palace VP, Evans RE, Lazorchak JM, et al. Collapse of a fish population after exposure to a synthetic estrogen. Proc Natl Acad Sci 2007;104: 8897–901.
Kosma CI, Lambropoulou DA, Albanis TA. Occurrence and removal of PPCPs in municipal and hospital wastewaters in Greece. J Hazard Mater 2010;179:804–17.
Kovalova L, McArdell CS, Hollender J. Challenge of high polarity and low concentrations in analysis of cytostatics and metabolites in wastewater by hydrophilic interaction chromatography/tandem mass spectrometry. J Chromatogr A 2009;1216:1100–8.
Kovalova L, Siegrist H, Singer H, Wittmer A, McArdell CS. Hospital wastewater treatment by membrane bioreactor: performance and efficiency for organic micropollutant elimination. Environ Sci Technol 2012;46:1536–45.
Kumar A, Xagoraraki I. Pharmaceuticals, personal care products and endocrine-disrupting chemicals in U.S. surface and finished drinking waters: a proposed ranking system. Sci Total Environ 2010;408:5972–89.
Kümmerer K. Drugs in the environment: emission of drugs, diagnostic aids and disinfectants into wastewater by hospitals in relation to other sources — a review. Chemosphere 2001;45:957–69.
Kummerer K, Erbe T, Gartiser S, Brinker L. AOX—emissions from hospitals into municipal waste water. Chemosphere 1998;36:2437–45.
Kümmerer K, Helmers E. Hospital effluents as a source for platinum in the environment. Sci Total Environ 1997;193:179–84.
Lai H-T, Hou J-H, Su C-I, Chen C-L. Effects of chloramphenicol, florfenicol, and thiamphenicol on growth of algae *Chlorella pyrenoidosa*, *Isochrysis galbana*, and *Tetraselmis chui*. Ecotoxicol Environ Saf 2009;72:329–34.
Langford KH, Thomas KV. Determination of pharmaceutical compounds in hospital effluents and their contribution to wastewater treatment works. Environ Int 2009;35:766–70.
Lenz K, Hann S, Koellensperger G, Stefanka Z, Stingeder G, Weissenbacher N, et al. Presence of cancerostatic platinum compounds in hospital wastewater and possible elimination by adsorption to activated sludge. Sci Total Environ 2005;345:141–52.
Lin AY-C, Tsai Y-T. Occurrence of pharmaceuticals in Taiwan's surface waters: impact of waste streams from hospitals and pharmaceutical production facilities. Sci Total Environ 2009;407:3793–802.
Lin AY-C, Wang X-H, Lin C-F. Impact of wastewaters and hospital effluents on the occurrence of controlled substances in surface waters. Chemosphere 2010;81:562–70.
Lin AY-C, Yu T-H, Lin C-F. Pharmaceutical contamination in residential, industrial, and agricultural waste streams: risk to aqueous environments in Taiwan. Chemosphere 2008;74:131–41.
Lindberg R, Jarnheimer P-Å, Olsen B, Johansson M, Tysklind M. Determination of antibiotic substances in hospital sewage water using solid phase extraction and liquid chromatography/mass spectrometry and group analogue internal standards. Chemosphere 2004;57:1479–88.
Liu W, Ming Y, Huang Z, Li P. Impacts of florfenicol on marine diatom *Skeletonema costatum* through photosynthesis inhibition and oxidative damages. Plant Physiol Biochem 2012;60:165–70.
Liu X, Zhang J, Yin J, Duan H, Wu Y, Shao B. Analysis of hormone antagonists in clinical and municipal wastewater by isotopic dilution liquid chromatography tandem mass spectrometry. Anal Bioanal Chem 2010;396:2977–85.
Löffler D, Ternes TA. Analytical method for the determination of the aminoglycoside gentamicin in hospital wastewater via liquid chromatography–electrospray–tandem mass spectrometry. J Chromatogr A 2003;1000:583–8.
Madden JC, Enoch SJ, Hewitt M, Cronin MTD. Pharmaceuticals in the environment: good practice in predicting acute ecotoxicological effects. Toxicol Lett 2009;185:85-101.
Mahnik S, Rizovski B, Fuerhacker M, Mader R. Determination of 5-fluorouracil in hospital effluents. Anal Bioanal Chem 2004;380:31–5.
Mahnik SN, Lenz K, Weissenbacher N, Mader RM, Fuerhacker M. Fate of 5-fluorouracil, doxorubicin, epirubicin, and daunorubicin in hospital wastewater and their elimination by activated sludge and treatment in a membrane-bio-reactor system. Chemosphere 2007;66:30–7.
Massoudieh A, Ginn TR. Modeling colloid-enhanced contaminant transport in stormwater infiltration basin best management practices. Vadose Zone J 2008;7:1215–22.
Mc Gechan M, Lewis D. Transport of particulate and colloid sorbed contaminants through soil, part 1: general principles. Biosyst Eng 2002;83:255–73.
Molander L, Ågerstrand M, Rudén C. WikiPharma — a freely available, easily accessible, interactive and comprehensive database for environmental effect data for pharmaceuticals. Regul Toxicol Pharmacol 2009;55:367–71.
Mullot J-U, Karolak S, Fontanova A, Levi Y. Modeling of hospital wastewater pollution by pharmaceuticals: first results of Mediflux study carried out in three French hospitals. Water Sci Technol 2010;62:2912–9.
Ohlsen K, Ternes T, Werner G, Wallner U, Löffler D, Ziebuhr W, et al. Impact of antibiotics on conjugational resistance gene transfer in *Staphylococcus aureus* in sewage. Environ Microbiol 2003;5:711–6.
Park J. Pharmaceuticals in the environment and management approaches in Korea. KEI; 2005 [RE-12].
Park S, Choi K. Hazard assessment of commonly used agricultural antibiotics on aquatic ecosystems. Ecotoxicology 2008;17:526–38.
Pena A, Paulo M, Silva LJG, Seifrtová M, Lino CM, Solich P. Tetracycline antibiotics in hospital and municipal wastewaters: a pilot study in Portugal. Anal Bioanal Chem 2010;396:2929–36.
Perrodin Y, Christine B, Sylvie B, Alain D, Jean-Luc B-K, Cécile C-O, et al. A priori assessment of ecotoxicological risks linked to building a hospital. Chemosphere 2013;90: 1037–46.
Sanderson H, Johnson DJ, Reitsma T, Brain RA, Wilson CJ, Solomon KR. Ranking and prioritization of environmental risks of pharmaceuticals in surface waters. Regul Toxicol Pharmacol 2004;39:158–83.
Sanderson H, Thomsen M. Comparative analysis of pharmaceuticals versus industrial chemicals acute aquatic toxicity classification according to the United Nations classification system for chemicals. Assessment of the (Q)SAR predictability of pharmaceuticals acute aquatic toxicity and their predominant acute toxic mode-of-action. Toxicol Lett 2009;187:84–93.
Santiago S, Becker van Slooten K, Chèvre N, Pardos M, Benninghoff C, Thybaud E, et al. Guide pour l'utilisation des tests écotoxicologiques, avec les daphnies, les bactéries luminescentes et les algues vertes, appliqués aux échantillons de l'environnement; 2002 [55 pp.].
Santos LHMLM, Araújo AN, Fachini A, Pena A, Delerue-Matos C, Montenegro MCBSM. Ecotoxicological aspects related to the presence of pharmaceuticals in the aquatic environment. J Hazard Mater 2010;175:45–95.
Sim W-J, Lee J-W, Lee E-S, Shin S-K, Hwang S-R, Oh J-E. Occurrence and distribution of pharmaceuticals in wastewater from households, livestock farms, hospitals and pharmaceutical manufactures. Chemosphere 2011;82:179–86.
Snell TW, DesRosiers NJD. Effect of progesterone on sexual reproduction of *Brachionus manjavacas* (Rotifera). J Exp Mar Biol Ecol 2008;363:104–9.
SOCOTEC ENVIRONNEMENT. Etude diagnostiquc des flux de pollution rejetés par les hôpiaux: Edouard Herriot de Lyon, Nord de Grenoble, de Chambéry - Compte rendu des visites préliminaires des 19 et 20 mai 1994. Lyon: Société française d'hygiène hospitalière de l'hôpital de la Croix-rousse; 1994 [1885, 42 pp.].
Stoichev T, Baptista MS, Basto MCP, Vasconcelos VM, Vasconcelos MTSD. Effects of minocycline and its degradation products on the growth of *Microcystis aeruginosa*. Ecotoxicol Environ Saf 2011;74:219–24.
Straub JO, Gysel D, Kastl U, Klemmer J, Soderegger M, Studer M. Environmental risk asessment for ancillary substances in biotechnological production of pharmaceuticals. Environ Toxicol Chem 2012;31:681–7.
Sui Q, Wang B, Zhao W, Huang J, Yu G, Deng S, et al. Identification of priority pharmaceuticals in the water environment of China. Chemosphere 2012;89:280–6.
Sun Y-x, Gu P. Determination of haloacetic acids in hospital effluent after chlorination by ion chromatography. J Environ Sci 2007;19:885–91.
Tauxe-Wuersh A, De Alencastro LP, Grandjean D, Tarradellas J. Trace determination of tamoxifen and 5-fluorouracil in hospital and urban wastewater. 2006;86:473–85.
Ternes TA. Occurrence of drugs in German sewage treatment plants and rivers. Water Res 1998;32:3245–60.
TGD. Technical Guidance Document in Support of Commission Directive 93/67/EEC on Risk Assessment for New Notified Substances and Commission Regulation (EC); 2003.
Thomas KV, Dye C, Schlabach M, Langford KH. Source to sink tracking of selected human pharmaceuticals from two hospitals and and a wastewater treatment works. J Environ Monit 2007;9:1410–8.
Tsakona M, Anagnostopoulou E, Gidarakos E. Hospital waste management and toxicity evaluation: a case study. Waste Manag 2007;27:912–20.
Vazquez-Roig P, Andreu V, Blasco C, Picó Y. Risk assessment on the presence of pharmaceuticals in sediments, soils and waters of the Pego–Oliva Marshlands (Valencia, eastern Spain). Sci Total Environ 2012;440:24–32.
Verlicchi P, Al Aukidy M, Galletti A, Petrovic M, Barceló D. Hospital effluent: investigation of the concentrations and distribution of pharmaceuticals and environmental risk assessment. Sci Total Environ 2012a;430:109–18.
Verlicchi P, Al Aukidy M, Zambello E. Occurrence of pharmaceutical compounds in urban wastewater: removal, mass load and environmental risk after a secondary treatment—a review. Sci Total Environ 2012b;429:123–55.
Verlicchi P, Galletti A, Petrovic M, Barceló D. Hospital effluents as a source of emerging pollutants: an overview of micropollutants and sustainable treatment options. J Hydrol 2010;389:416–28.
Villegas-Navarro A, Santiago MR, Perez FR, Torres RR, Abularach TD, Reyes JL. Determination of LC50 from *Daphnia magan* in treated industrial waste water and non-treated hospital effluents. Environ Int 1997;23:535–40.
Weissbrodt D, Kovalova L, Ort C, Pazhepurackel V, Moser R, Hollender J, et al. Mass flows of X-ray contrast media and cytostatics in hospital wastewater. Environ Sci Technol 2009;43:4810–7.
Wu X, Hawse JR, Subramaniam M, Goetz MP, Ingle JN, Spelsberg TC. The tamoxifen metabolite, endoxifen, is a potent antiestrogen that targets estrogen receptor α for degradation in breast cancer cells. Cancer Res 2009;69:1722–7.
Yin J, Shao B, Zhang J, Li K. A preliminary study on the occurrence of cytostatic drugs in hospital effluents in Beijing, China. Bull Environ Contam Toxicol 2010;84:39–45.
Yuan S, Jiang X, Xia X, Zhang H, Zheng S. Detection, occurrence and fate of 22 psychiatric pharmaceuticals in psychiatric hospital and municipal wastewater treatment plants in Beijing, China. Chemosphere 2013;90:2520–5.
Zgorska A, Arendarczyk A, Grabinska-Sota E. Toxicity assessment of hospital wastewater by the use of a biotest battery. Arch Environ Prot 2011;37:55–61.
Zounkova R, Kovalova L, Blaha L, Dott W. Ecotoxicity and genotoxicity assessment of cytotoxic antineoplastic drugs and their metabolites. Chemosphere 2010;81:253–60.

1.3 Synthèse

Au regard du nombre de produits différents utilisés dans les hôpitaux (e.g. détergents, surfactants, médicaments, produits de contrastes iodés, lessives, ...) très peu de contaminant ont été recherchés dans les effluents hospitaliers (297 au total dont 11 métaux). A titre de comparaison, environ 3000 médicaments sont aujourd'hui prescrits dans les hôpitaux européens. En revanche, on observe que les résidus pharmaceutiques sont les contaminants les plus recherchés dans les effluents hospitaliers (197 résidus pharmaceutiques recherchés sur 297 contaminants). Deux groupes thérapeutiques ressortent majoritairement : les antibiotiques et les antipsychotiques. Par ailleurs, même s'ils restent très peu étudiés au regard de leur diversité potentiel, on observe que les métabolites représentent une part non négligeable des contaminants recherchés (24 molécules). De plus, parmi les 34 publications scientifiques regroupées dans cette revue bibliographique, seulement 3 d'entre elles se sont intéressées à la fraction de RP adsorbées sur les particules en suspension. A chaque fois, les concentrations après désorption étaient supérieures à celles en solution.

Considérant le grand nombre de molécules différentes présentes dans les effluents hospitaliers, il est nécessaire de hiérarchiser les molécules d'intérêt. Pour cela, de nombreux auteurs utilisent un seul critère : la consommation du médicament au sein de l'hôpital. Or, il n'est pas pertinent de prendre en compte ce seul paramètre pour prioriser les molécules que ce soit pour estimer les molécules qu'on peut retrouver dans l'effluent ou que l'on cherche à évaluer l'impact environnemental potentiel. En effet, le lien entre consommation d'un médicament et présence d'un résidu pharmaceutiques dans l'effluent n'est pas du tout évident. Des paramètres comme l'excrétion, la métabolisation ou le caractère ambulatoire du traitement sont des critères à prendre également en compte pour évaluer la présence d'un résidu pharmaceutique dans l'effluent hospitalier. Dans le chapitre suivant, nous avons fait un zoom sur les résidus pharmaceutiques dans les effluents hospitaliers, sans considérer les autres substances éventuellement présentes.

Chapitre 2

Ecotoxicité des résidus pharmaceutiques des effluents hospitaliers

2.1 Enjeux et démarche

Les effluents hospitaliers sont, de manière générale, traités de la même façon que les effluents urbains c'est-à-dire évacués vers les STEP. Or ces installations ne sont pas conçues pour dégrader 100% des résidus pharmaceutiques présents avec une grande diversité et parfois des concentrations importantes dans les effluents hospitaliers. Ainsi, nous avons voulu déterminer quelles sont, au sein de l'effluent hospitalier, les molécules qui présentaient le plus grand danger écotoxicologique parmi les 197 recherchées. Pour cela, nous avons comparé les données d'écotoxicité (quand elles existaient) des molécules détectées à leurs concentrations maximales respectives déjà mesurées dans les effluents hospitaliers par divers auteurs. On obtient alors des quotients de danger (Hazard Quotient : HQ) qui nous permettent de classer les molécules présentes dans les effluents hospitaliers en fonction de leur danger écotoxicologique.

2.2 Article 2 : *Pharmaceuticals in hospital wastewater : Their ecotoxicity and contribution to the environmental hazard of the effluent*

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Pharmaceuticals in hospital wastewater: Their ecotoxicity and contribution to the environmental hazard of the effluent

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HIGHLIGHTS

- 197 Pharmaceuticals were sought in hospital wastewater.
- PNEC are available in bibliography for 150 of them.
- Calculation of 127 hazard quotients has been performed.
- Hazardousness of pharmaceuticals is greatly variable.
- 15 Compounds are very hazardous for the environment.

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ABSTRACT

Nowadays, pharmaceuticals are found in every compartment of the environment. Hospitals are one of the main sources of these pollutant emissions sent to wastewater treatment plants (WWTP) that are poorly equipped to treat these types of compounds efficiently. In this work, for each pharmaceutical compound found in hospital wastewater (HWW), we have calculated a hazard quotient (HQ) corresponding to the highest concentration measured in HWW divided by its predicted no effect concentration (PNEC). Thus we have assessed the contribution of each compound to the ecotoxicological threat of HWW taken as a whole. Fifteen compounds are identified as particularly hazardous in HWW. In future more attention should be given to their analysis and replacement in hospitals, and to their elimination in WWTPs. This work also highlights the lack of knowledge of the ecotoxicity of certain pharmaceutical compounds found in HWW at high concentrations (mg L^{-1}). In order to extend this study, it is now necessary to investigate ecotoxic risks linked to various emission scenarios, focusing in particular on dilution in the aquatic environment and the production of metabolites, especially during transit inside WWTPs.

1. Introduction

Nowadays, pharmaceuticals (PCs) are ubiquitous in the environment. Indeed, several ecosystemic compartments are contaminated by these substances: the hydrosphere (surface water (Heath et al., 2010), groundwater (Loos et al., 2010), drinking water (Gibs et al., 2007)); the geosphere (Silva et al., 2011a,b Yang et al., 2011) and the biosphere (Lajeunesse et al., 2011).

Among the different sources emitting these PCs into the environment, hospitals are particularly interesting. This is because the numerous care activities performed inside these establishments (anaesthesia, anticancer treatment, diagnosis, etc.) lead to the consumption of large quantities of PCs. This intensive activity at a single point leads to high concentrations of PCs (through excretion) in hospital wastewater (HWW), sometimes greater than in urban wastewater (Verlicchi et al., 2010).

HWW is almost always untreated before being discharged into urban wastewater networks and then into municipal wastewater treatment plants (WWTP) (Emmanuel et al., 2004) despite the fact that these plants are not designed to remove complex compounds such as PCs (Ternes, 1998; Heberer, 2002; Joss et al., 2005). Although some PCs entering WWTPs are removed (e.g. biodegradation or adsorption onto sludge), a sizeable amount is released into the environment (Verlicchi et al., 2012b).

Some of these PCs present a considerable threat for aquatic organisms even at very low concentrations. Given the great number of compounds measured in HWW (Orias and Perrodin, 2013), it is necessary to characterize their respective contributions to the hazardousness of effluents discharged into the environment, in order to rank them. This will make it possible to identify the pharmaceutical compounds on which hospital managers must focus their efforts in order to decrease their release into the

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environment and their potential impacts on aquatic ecosystems and/or on the activated sludges of WWTP.

In order to establish the compounds most concerned by the environmental hazardousness of HWW, we compare concentrations measured in HWW (Orias and Perrodin, 2013) to the available ecotoxicity data (PNEC: Predictive No Effect Concentration). A hazard quotient (HQ) is calculated for each compound, characterizing its level of involvement in the environmental hazardousness of HWW.

Finally, we make proposals in this work to improve knowledge on the environmental hazard of these compounds, and to assess the environmental risks linked to various scenarios of releasing treated HWW into the environment.

2. Materials and method

2.1. Determination of PC concentrations in HWW

Two main approaches can be used to determine the concentration of PCs in HWW: (i) a theoretical approach (Mullot et al., 2010; Escher et al., 2011) consisting in assessing the quantity of PCs that could be present in HWW (Predictive Effluent Concentration), considering various parameters such as consumption and excretion. The advantage of this method is that it takes into account every compound used in a hospital, but considerable uncertainty remains due to the parameters considered. The other approach (ii) is experimental (Verlicchi et al., 2012a; Perrodin et al., 2013) and consists in measuring the concentration of PCs directly in HWW. However, measuring every PC potentially present in HWW is very expensive. Moreover, limits of detection (LOD) are often too high to assess all ecotoxicological hazards as PNECs are regularly higher than LODs. Nevertheless, this type of approach provides real information on concentrations in the effluent.

In this work, we kept the highest concentrations of each PC already measured in HWW from a previous study (Orias and Perrodin, 2013), in order to obtain the "worst case" scenario. It should be noted that only one measure was made for several PCs. In the future, when more data is available, it could be relevant to study median values to evaluate "typical" concentrations of each PC in HWW.

2.2. Ecotoxicity of PCs in HWW (PNEC calculation)

In a previous study (Orias and Perrodin, 2013), PNEC's of PCs were calculated according to modelled ecotoxicological data using the ECOSAR method (Sanderson et al., 2003), experimental data from international databases (e.g. EPA ECOTOX, Wikipharma (Molander et al., 2009)) and also from the literature. These results are used in this study.

2.3. Involvement of each PCs in hazardousness of HWW (HQ calculation)

The involvement of PCs in the environmental hazard of HWW depends not only on its concentration in HWW but also on its ecotoxicity.

In order to identify and rank these PCs, a hazard quotient was calculated for each compound according to the equation below:

$$HQ = HWW_{max}\text{conc.}/PNEC$$

With: HWW_{max} conc.: highest concentration ever measured in HWW in $\mu g\ L^{-1}$. PNEC: Predictive No Effect Concentration in $\mu g\ L^{-1}$.

The PC with the highest HQ will be considered that most involved in the hazard of HWW.

It is noteworthy that 172 of the 198 PCs sought in the HWW were detected. Of the 172 PCs detected, data was insufficient for 34 to calculate their PNEC. Finally, only 127 HQs were calculated.

3. Results

3.1. Type of available data

In the following paragraphs, the PCs are analysed according to their distribution in therapeutic classes (Table 1). These classes are those of the ATC (Anatomic Therapeutic and Chemical) classification proposed by the World Health Organisation Collaborating Center for Drug Statistics Methodology (WHOCC, 2011).

3.1.1. PC detection in HWW (Fig. 1)

PCs from classes J (antibiotic and antiviral) and N (anaesthesia compound, antidepressants, etc.) were those most sought for and detected in HWW, with 47 and 46 PCs detected out of 60 and 47, respectively. Furthermore, 22 of the 22 compounds of class C sought (Cardio-vascular system) in HWW were detected. We also found 12 anticancer PCs (class L) of the 16 sought, 11 compounds linked to the musculoskeletal system (class M) of the 14 sought and 9 sex hormones (class G) of the 10 sought. For each group V (various), A (Alimentary and tract metabolism) and D (Dermatologicals), 7 compounds were sought with 7, 6 and 5 detected, respectively. Finally, we found 5 compounds linked to the respiratory system (class R) of the 6 sought. It is noteworthy that among the PCs sought for in HWW, no compounds from classes B (Blood and blood forming organs), H (Systemic hormonal preparations, excluding sex hormones and insulins), P (Antiparasitic products, insecticides and repellents) and S (Sensory organs) were found.

3.1.2. Available PNECs

Of the compounds detected in HWW, considering every class, not enough or no ecotoxicological data was available for 22% of them, making it impossible to calculate a PNEC. Class N contained the most compounds for which no or insufficient data was available. Indeed, 14 PCs detected in class N (of 47) did not have a calculable PNEC. Two other therapeutic classes, i.e. classes V and G, drew attention due to the lack of ecotoxicological data. In class V, 7 out of 7 compounds were detected, but sufficient experimental and modelled data for calculating a PNEC could only be found for one of them. For 3 others, only modelised data were available. Moreover, for 2 compounds of this class, detected at concentrations ranging from a hundred $\mu g\ L^{-1}$ to a $mg\ L^{-1}$, there was no data on their ecotoxicity. Concerning 22 compounds classed as veterinary compounds sought in HWW, only 5 were detected. Indeed,

Table 1
Classes and codes of Anatomic Therapeutic and Chemical classification.

Code	Content
A	Alimentary tract and metabolism
B	Blood and blood forming organs
C	Cardiovascular system
D	Dermatologicals
G	Genito-urinary system and sex hormones
H	Systemic hormonal preparations, excluding sex hormones and insulins
J	Antiinfectives for systemic use
L	Antineoplastic and immunomodulating agents
M	Musculo-skeletal system
N	Nervous system
P	Antiparasitic products, insecticides and repellents
R	Respiratory system
S	Sensory organs
V	Various

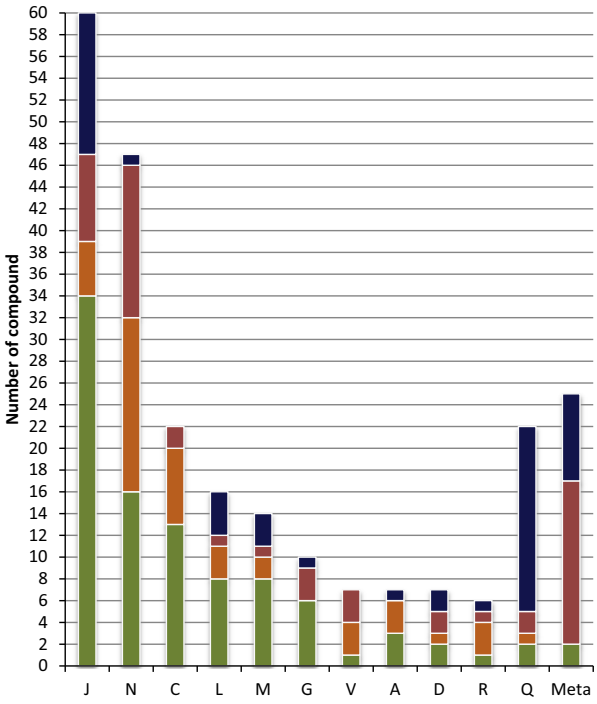

Fig. 1. Distribution of pharmaceuticals searched for in hospital wastewater (HWW) and ecotoxicity data available according to therapeutic classes (ATC classification). In green, compounds detected in HWW with an experimental PNEC available, in orange, compounds detected in HWW with only a modelised (ECOSAR) PNEC available, in red, compounds detected in HWW without PNEC available and in blue, compounds not detected in HWW. Q and Meta columns respectively represent veterinary compounds and metabolites. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

some compounds are not used exclusively for medical purposes; they can also be used for veterinary purposes. What is more, they can be formed after "parent" compounds have reacted with other compounds. Certain of the compounds found can be considered as corresponding to metabolites of compounds for human use since some molecules are structurally very similar. In addition, some compounds could belong to several categories and so being hard to classify. That is why we can find veterinary compound in HWW.

Ecotoxicological data were also lacking for classes of sex hormones in the compounds detected in HWW. Indeed, no experimental data was available for 3 of the 10 hormones found. However, the intrinsic properties of these compounds are active at very low concentrations, a fact confirmed by the PNECs of the other compounds from this class.

The class whose ecotoxicity had been studied most was class J (antibiotics and antivirals). However, 8 class J compounds detected in HWW had no calculable PNEC. These compounds included ritonavir, an antiviral identified in a previous study as particularly bioaccumulative and poorly biodegradable (Jean et al., 2012).

Considering metabolites, despite the large number of them detected in HWW, we had few data concerning their ecotoxicity. Indeed, the issue of metabolites is emerging and has not yet received the attention it deserves. That is why we chose to exclude this class from the HQ calculations.

3.2. Concentration of PCs in HWW

The range of PC concentrations found in HWW is very wide, varying from several ng L^{-1} (e.g. ethinylestradiol and toremifene) to several mg L^{-1} (e.g. iobitridol and iopamidol). However, in most cases, PCs were detected between a hundred ng L^{-1} and a hundred µg L^{-1}. We found the highest concentrations in class V (iodised contrast medium).

3.3. Ecotoxicity of PCs present in HWW

In Figs. 2a–2c, we grouped every PNEC available in the literature on the PCs sought in HWW. To facilitate reading, we organised them into 3 categories: compounds with a PNEC higher than 1 µg L^{-1} (the less ecotoxic), compounds with PNECs between 100 ng L^{-1} and 1 µg L^{-1} (intermediate ecotoxicity), and compounds with PNECs under 100 ng L^{-1} (the most ecotoxic). Compounds with a PNEC calculated on the basis of modelled data (according to ECOSAR) are represented in orange.

Finally, in appendix 1, we grouped the 77 compounds (45 human pharmaceuticals and 32 metabolites and veterinary compounds) already sought in HWW and/or detected in HWW, without an available or calculable PNEC.

3.4. Involvement of PCs in the hazard of HWW

3.4.1. HQs < 1: no ecotoxicological hazard in HWW (n = 50) (Figs. 3 and 4)

The range of calculated HQs was very wide (from 1 to 10^{-5}). Among the compounds detected in HWW presenting a low ecotoxicological hazard for aquatic organisms, we found a distribution similar to that observed for all the compounds sought. That is to say that we found almost all the classes, with the same distribution for the compounds with low hazard as in HWW (Fig. 1), with classes N and J being the main classes. It should be noted that no D compound was found in the compounds with low ecotoxicity in HWW.

3.4.2. HQs between 1 and 1000: hazardous compounds in HWW (n = 62) (Figs. 5 and 6)

Here again, we can see the same distribution of compounds searched for in HWW according to their therapeutic classes. However, we observed a lower number of G and A compounds and a higher number of D compounds in this group of hazardous compounds in HWW.

3.4.3. HQs higher than 1000: very compounds hazardous in HWW (n = 15) (Fig. 7)

The 15 PCs with a HQ > 1000 represented 33% of the hormones measured (3 of 9 hormones measured in HWW belonged to the most hazardous compounds in HWW). However, 7 (clotrimazole, lidocaine, propyphenazone, sulpiride, chlorpromazine, sulfapyridine and ethinylestradiol) of the 15 compounds detected were detected only once in HWW (Table 2). Moreover, ampicillin, estrone and estradiol were detected in two out of three measurements. The remaining compounds of these 15 most hazardous compounds were detected 5 times or more (11 times for trimethoprim) (Table 2).

According to Orias and Perrodin (2013), caffeine should be present in this group but due to a mistake in the transcription from an original article to the EPA ECOTOX database, we dropped this compound from this category. We shall discuss this in greater detail in the discussion part.

4. Discussion

4.1. PC concentrations in HWW

Firstly, when performing a detailed examination of every study carried out on the concentration of pharmaceuticals in HWW all

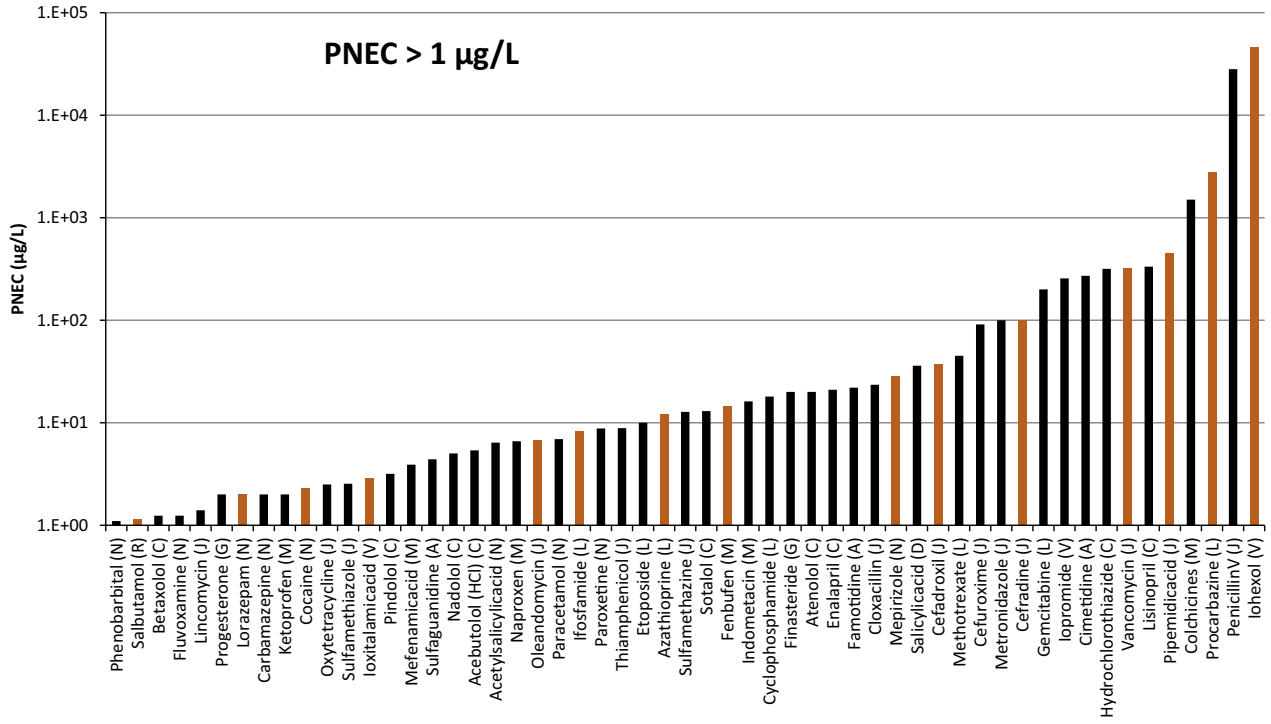

Fig. 2a. PNEC greater than 1 µg L^{-1} of pharmaceuticals searched for in HWW. In black, PNEC calculated on the basis of experimental data and in orange, PNEC calculated on the basis of modelised data (ECOSAR). Letters in brackets correspond to the therapeutic group (Table 1). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

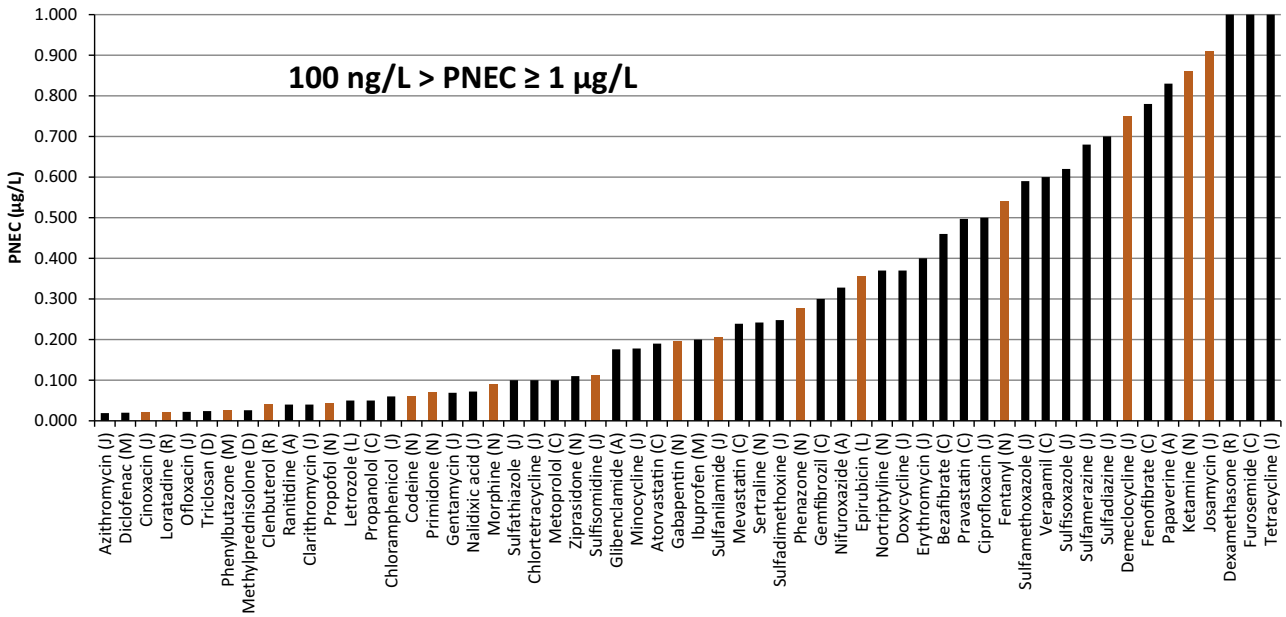

Fig. 2b. PNEC between 100 ng L^{-1} and 1 µg L^{-1} of pharmaceuticals searched for in HWW. In black, PNEC calculated on the basis of experimental data and in orange, PNEC calculated on the basis of modelised data (ECOSAR). Letters in brackets correspond to the therapeutic group (Table 1). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

over the world, we observed that they have all been performed in the north hemisphere. Indeed, no study concerning South-American, African or Oceanic hospitals was found. Also, given the socio-economic, climatic and epidemiological differences between the continents considered, the consumption of pharmaceuticals is probably not the same as that in the hospitals observed.

Moreover, the pharmaceuticals studied in HWW are often grouped by therapeutic class (e.g. antibiotics, anticancer, etc.) although these classes are human-metabolism-based. But, with the very wide range of metabolisms found in non-target organisms (e.g., vertebrates, crustaceans, algae, bacteria, etc.) in the environment, these therapeutic classes are no longer relevant. An alternative classification could group PCs according to their toxicophores (or mode of action). A toxicophore is a sub-structure inside a molecule which is responsible for a specific mode of action on metabolism (Escher and Fenner, 2011). This was suggested by Besse and

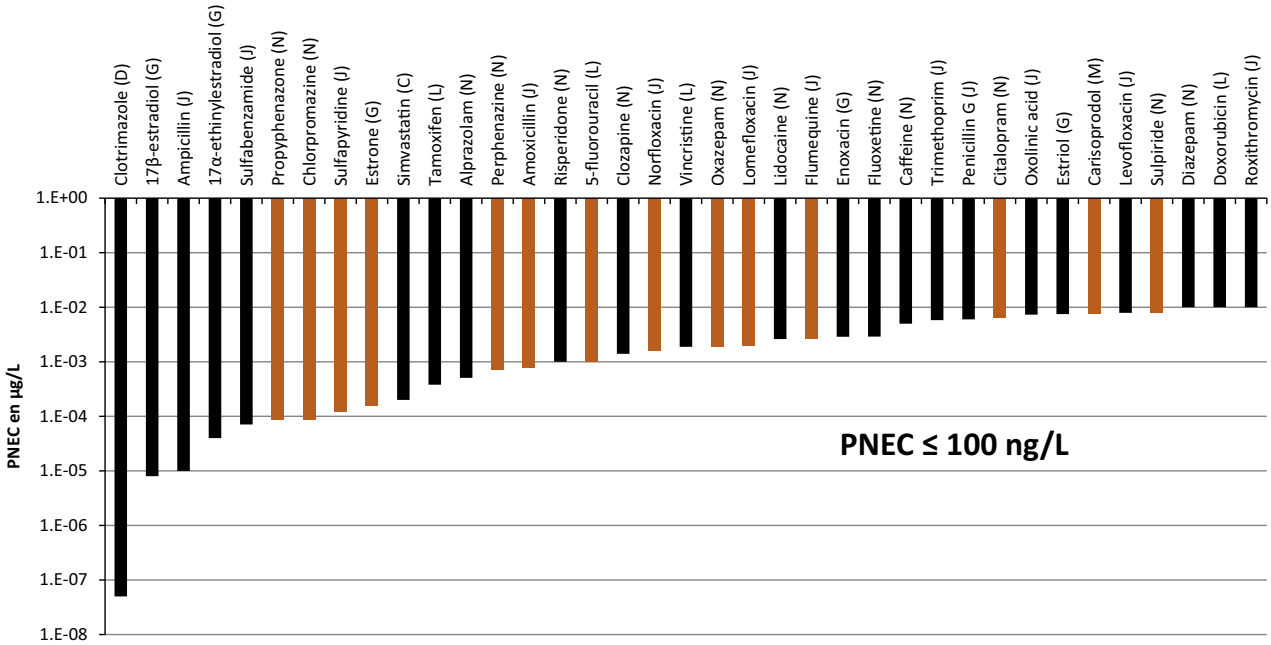

Fig. 2c. PNEC lower than 100 ng L^{-1} of pharmaceuticals searched for in HWW In black, PNEC calculated on the basis of experimental data and in orange, PNEC calculated on the basis of modelised data (ECOSAR). Letters in brackets correspond to the therapeutic group (Table 1). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

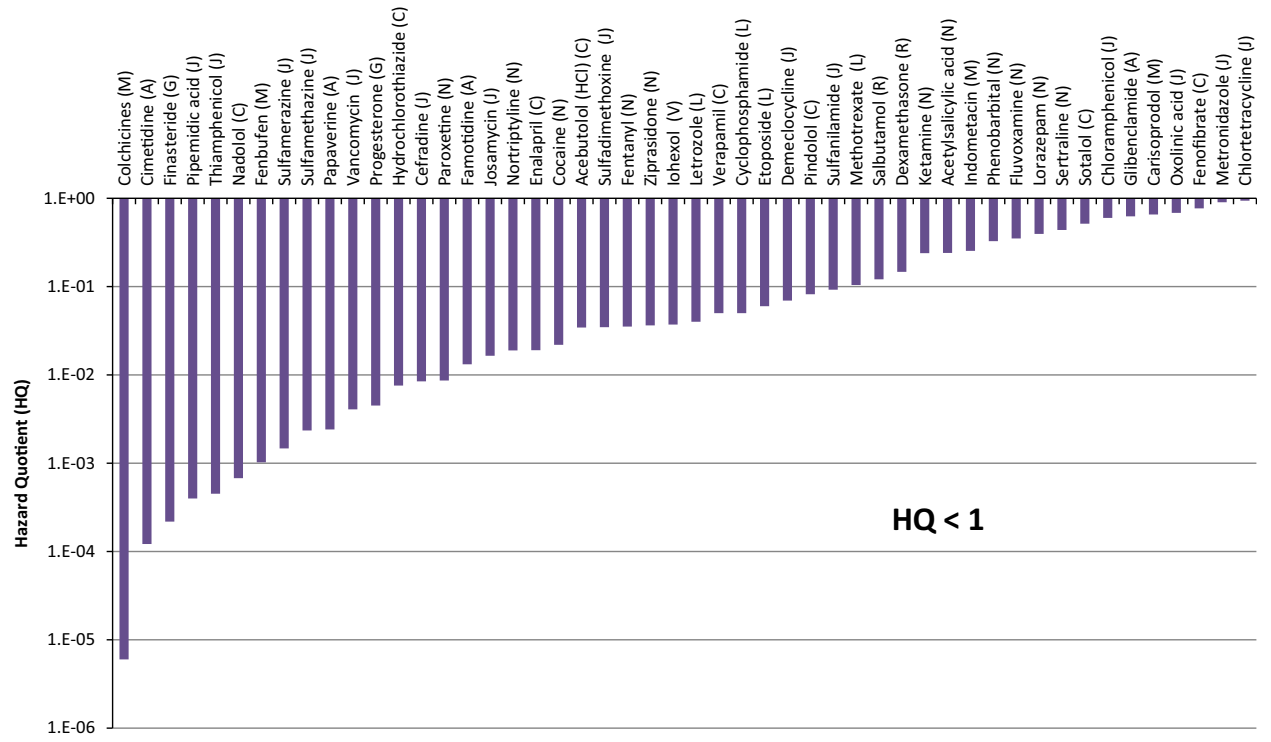

Fig. 3. Compounds with a hazard quotient (HQ) lower than 1 ($n = 50$). Letters in brackets correspond to the therapeutic group (Table 1).

Garric (2008) in their method of prioritising pharmaceuticals in surface water. This kind of classification could permit to consider every pollutant find in HWW.

Concerning the range of PC concentrations, we note that it not only varies considerably from one pharmaceutical to another but also for a specific PC when several measurements are performed. Numerous parameters could be responsible for these variations, such as the type of hospital concerned (e.g. general or psychiatric), number of beds, the country or the moment in the year when the measurement was performed (e.g. phenomenon of epidemics).

Finally, it should be borne in mind that many compounds have never been sought in HWW, essentially due to analytical difficulties (e.g. amiodarone). Indeed, the HWW matrix is very complex and measurements are not always easy. In order to remediate this limitation, several teams of chemists have recently developed an analysis method to measure increasing numbers of

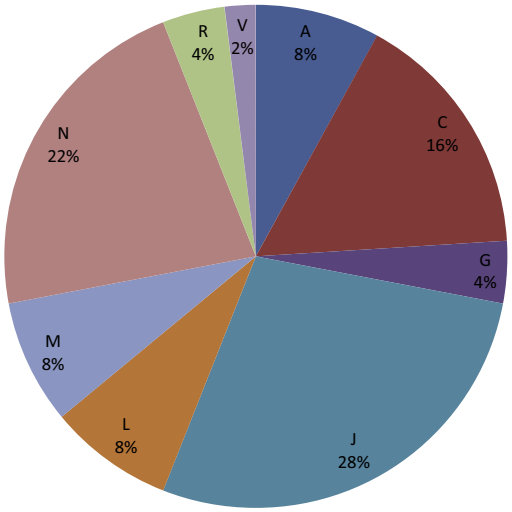

Fig. 4. Distribution of no hazardous compounds (HQ lower than 1) found in HWW according to therapeutic classes (ATC classes). (n = 50).

active ingredients in the same sample, achieving greater accuracy in HWW (Tauxe-Wuersch et al., 2006; Weissbrodt et al., 2009; Gros et al., 2013; Yuan et al., 2013).

4.2. Ecotoxicity of PCs found in HWW

Of the PCs studied in HWW (except metabolites and veterinary compounds), 46 of the PNECs were calculated with modelled data due to the lack of experimental ecotoxicological data. However, the discrepancy between experimental and theoretical data is sometimes very large (e.g. ranitidine, ampicillin). That is why we emphasize the need to perform minimal tests (batteries of bioassays with algae, daphnia or other first consumers, and fish) on each compound. One group of compounds attracts particular attention concerning the lack of ecotoxicity data, i.e. iodine contrast media (e.g. diatrizoate, iobitridol, iohexol, etc.). Of the 7 compounds of this group, only 1 has been tested on living organisms and no data exists for 3 others. This is all the more worrying considering the concentrations of these compounds detected in HWW (several mg L^{-1} for the highest concentrations).

Very little ecotoxicological information is available for another group of compounds measured in HWW: Metabolites. Indeed, of the 24 metabolites measured (17 were detected), the ecotoxicological data available was sufficient to calculate a PNEC for only 2 of them (i.e. clofibric acid and 2,2,-difluordeoxyuridine). Escher and Fenner (2011) suggest an interesting method for determining which metabolites (or "transformation products") should be studied in priority. However, it is difficult to generalize this method to every compound given that ecotoxicological data is lacking for numerous parent compounds, making it necessary to develop ecotoxicity tests for them.

4.3. PCs involved in the hazard of HWW

Some PCs are highlighted by our study as very hazardous for the environment because of their high concentration measured in HWW, or because of their ecotoxicity, or both.

Of the 15 PCs most implicated in the hazardousness of HWW, we found 3 hormones (of the 9 measured in HWW), making class G proportionally that most represented among the most hazardous compounds. It can be assumed that this is linked to the intrinsic property of these compounds which are efficient not only in the human body but also at very low concentrations in non-target organisms. Conversely, other very ecotoxic PCs are relegated to lower positions in the classification (e.g. alprazolam and citalopram) or are absent, either

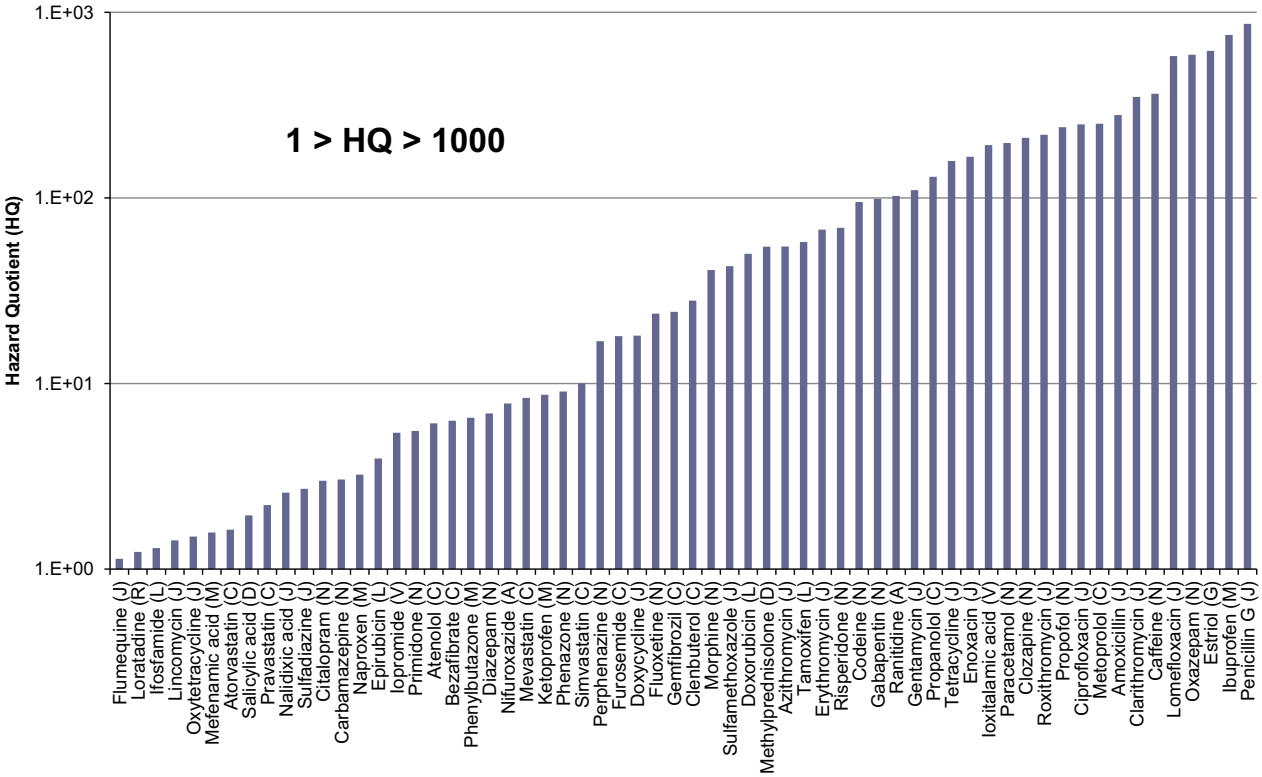

Fig. 5. Compounds with a hazard quotient (HQ) between 1 and 1000 (n = 62). Letters in brackets correspond to the therapeutic group (Table 1).

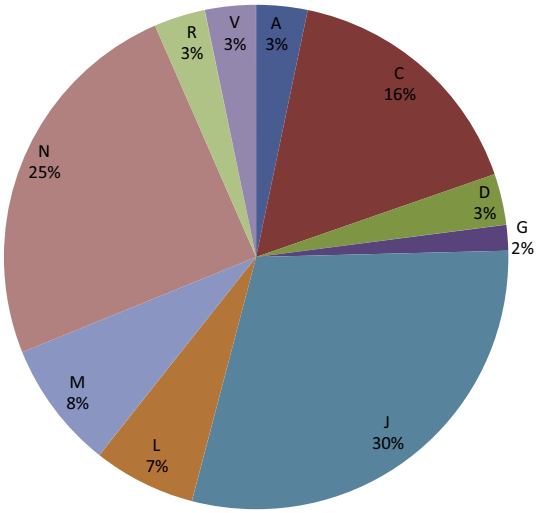

Fig. 6. Distribution of hazardous compounds (HQ greater than 1) found in HWW according to therapeutic classes (ATC classes). (n = 76).

Table 2
Number of measurements and detection of the 16 most dangerous compound in HWW.

ATC class	Compound	Measurement	Detection
D	Clotrimazole	1	1
N	Lidocaine	1	1
N	Propyphenazone	1	1
N	Sulpiride	1	1
N	Chlorpromazine	1	1
J	Sulfapyridine	2	1
G	17α-Ethinylestradiol	4	1
G	17β-Estradiol	3	2
G	Estrone	3	2
J	Ampicillin	3	2
L	5-Fluorouracil	6	5
J	Norfloxacin	8	7
J	Ofloxacin	8	7
M	Diclofenac	9	9
J	Trimethoprim	11	11

due to the low concentration measured (e.g. perphenazine) or to lack of detection (e.g. sulfapyridine).

It is noteworthy that only the direct ecotoxicity of PCs was considered in our approach to assess the hazard of PCs present in HWW. Nonetheless, the potential bioaccumulation of some PCs could be responsible for greater indirect ecotoxicity (e.g. *via* biomagnification). Tamoxifen, a potentially strongly bioaccumulative compound, perfectly illustrates this limitation. Indeed, in our study it corresponds to the 39th HQ, although its strong bioaccumulative potential should place it among the most hazardous compounds. Finally, once bioaccumulation potential can be demonstrated along with the assessment of indirect ecotoxicity, we will be able to use these two approaches to obtain a wider view of the contribution of PCs to the hazardousness of HWW.

However, concerning the involvement of PCs hazardousness in HWW, we have to say that numerous of its have been measured only one time (Table 3). More measurement in HWW is necessary in order to point out more accurately the respective contribution of each PC.

4.4. The special case of caffeine

At first sight caffeine appears to be the 5th most ecotoxic compound (PNEC = 0.05 ng L^{-1}) of those measured in HWW. In the light of this finding, we investigated the data leading to this result. The lowest available ecotoxic concentration (LAEC) is 0.05 μg L^{-1}. This value was extracted from the EPA (EPA ECOTOX) database and the study in question was an inter-laboratory study carried out by Bantle et al. (1994). During this study, 2 parameters (i.e. growth and mortality) were measured on *X. laevis* embryos exposed for 4 days to a range of caffeine concentrations. The lethal concentrations 50 (LC50 for mortality) and lowest observed effect concentrations (LOEC for development) measured in this study varied from 0.22 μg L^{-1} to 0.37 μg L^{-1} and from 0.05 to 0.125 μg L^{-1} (22 tests), respectively, according to the EPA database. However, on analysing the publication of Bantle et al. (1994) in closer detail, a mismatch of measurement unit between the database and the publication became apparent. A mistake had been made in the transcription of data from the publication to the database. Indeed, in the EPA database, the results were expressed in μg L^{-1} but in the article by Bantle et al., the values were identical except for the unit which was mg ml^{-1} (i.e. g L^{-1}). This means that the LAEC from this study was not 0.05 μg L^{-1} but 0.05 mg mL^{-1} (or 50 mg L^{-1}). This result is more consistent considering the second inter-laboratory study available concerning caffeine published by DeYoung et al. (1996). Consequently, the most sensitive organism is no longer *X. laevis*. The next LAEC was 5 μg L^{-1} (decrease in the development of a periphytic community). Finally, on the basis of an LAED 5 μg L^{-1} (DeYoung et al., 1996), the real HQ of caffeine is 364 (with a modified PNEC

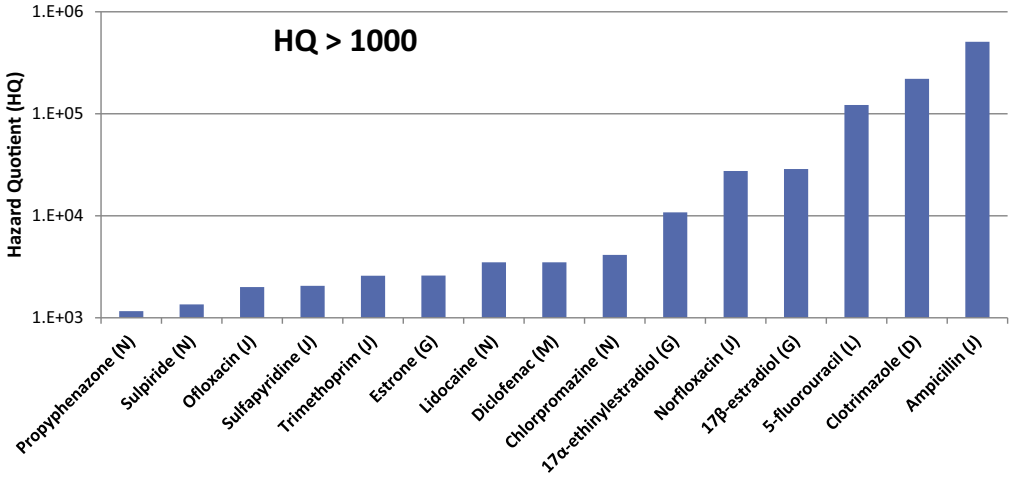

Fig. 7. Hazard quotient (HQ) of the 15 most hazardous compounds in HWW. Letters in brackets correspond to the therapeutic group (Table 1).

Table 3
Number of measurements and detection of pharmaceuticals (PCs) searched for in HWW.

	Between 10 and 15 times	Between 5 and 10 times	Between 1 and 5 times	One time
Measurements	3 PCs	24 PCs	68 PCs	101 PCs
Detections	3 PCs	16 PCs	49 PCs	103 PCs

of 0.5 μg L^{-1} (EF = 10 according to the ecotoxicity tests available for the model ecosystem)), making caffeine the 20th most hazardous compound!

5. Conclusion and perspectives

Firstly, this study highlighted the huge variability of the ecotoxicity of PCs present in HWW (PNEC between 0.05 pg L^{-1} for clotrimazole and 45.7 mg L^{-1} for iohexol).

Next, the HQ calculation allows identifying the PCs most implicated in the environmental hazardousness of HWW. According to the PCs considered, this contribution could be linked mainly to the high concentration of a compound in the effluent (e.g. 5-fluorouracil), to strong ecotoxicity (e.g. clotrimazole), or to a combination of both parameters (e.g. ethinylestradiol).

It should be noted that the most hazardous compounds do not belong to only one or two particular therapeutic classes. We found them in every class, with a slight overrepresentation of compounds linked to the nervous system (class N) (Table 2).

It will be necessary in the future to determine the cumulative ecotoxic effect of all the compounds found in HWW without omitting the potential interaction between different molecules (additivity, antagonism or synergy), in line with Boillot et al. (2008). This could be done for different effluents corresponding to different hospitals (e.g. psychiatric, oncologic, general, etc.), different locations, or according to the size of the hospital.

Regarding the characterization of compound ecotoxicity, it will be necessary to take into account indirect ecotoxicity linked to the bioaccumulative potential of certain PCs, in addition to the direct ecotoxicity considered in this study. Furthermore, the ecotoxicity tests should not be performed only with prescribed drugs (e.g. tamoxifen citrate or hydrochloride amiodarone) but also with the forms excreted (active ingredients or metabolites) after metabolisation inside the human body.

It could be also useful to perform measures upstream, ward by ward, inside different hospital departments, in order to determine the location of the point sources of the ecotoxic compounds most involved in emissions. This could permit the prioritization of the areas to be equipped with the appropriate technologies for treating such compounds (e.g. reverse osmosis, enzyme reduction targeting a special kind of compound). This kind of upstream treatment is already in place for radioactive isotopes (nuclear medicine) due to regulatory constraints.

The "hospital-end-of-pipe" approach used in this work does not allow evaluating the local risk linked to the emission of such effluents in aquatic ecosystems. Future works will consider risk assessment and the exposure of non-target organisms (PC concentrations in the environment), could be carried out to assess the risk linked to different local discharge scenarios.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chemosphere.2014.01.016.

References

Bantle, J.A., Burton, D.T., Dawson, D.A., Dumont, J.N., Finch, R.A., Fort, D.J., Linder, G., Rayburn, J.R., Buchwalter, D., Gaudet-Hull, A.M., Maurice, M.A., Turley, S.D., 1994. Fetax interlaboratory validation study: phase II testing. Environ. Toxicol. Chem. 13, 1629–1637.

Besse, J.-P., Garric, J., 2008. Human pharmaceuticals in surface waters: implementation of a prioritization methodology and application to the French situation. Toxicol. Lett. 176, 104–123.

Boillot, C., Bazin, C., Tissot-Guerraz, F., Droguet, J., Perraud, M., Cetre, J.C., Trepo, D., Perrodin, Y., 2008. Daily physicochemical, microbiological and ecotoxicological fluctuations of a hospital effluent according to technical and care activities. Sci. Total Environ. 403, 113–129.

DeYoung, D.J., Bantle, J.A., Hull, M.A., Burks, S.L., 1996. Differences in sensitivity to developmental toxicants as seen in xenopus and pimephales embryos. Bull. Environ. Contam. Toxicol. 56, 143–150.

Emmanuel, E., Keck, G., Blanchard, J.-M., Vermande, P., Perrodin, Y., 2004. Toxicological effects of disinfections using sodium hypochlorite on aquatic organisms and its contribution to AOX formation in hospital wastewater. Environ. Int. 30, 891–900.

EPA ECOTOX database, 2013. <http://www.epa.gov/ecotox/> (accessed 01.08.13).

Escher, B.I., Fenner, K., 2011. Recent advances in environmental risk assessment of transformation products. Environ. Sci. Technol. 45, 3835–3847.

Escher, B.I., Baumgartner, R., Koller, M., Treyer, K., Lienert, J., McArdell, C.S., 2011. Environmental toxicology and risk assessment of pharmaceuticals from hospital wastewater. Water Res. 45, 75–92.

Gibs, J., Stackelberg, P.E., Furlong, E.T., Meyer, M., Zaugg, S.D., Lippincott, R.L., 2007. Persistence of pharmaceuticals and other organic compounds in chlorinated drinking water as a function of time. Sci. Total Environ. 373, 240–249.

Gros, M., Rodríguez-Mozaz, S., Barceló, D., 2013. Rapid analysis of multiclass antibiotic residues and some of their metabolites in hospital, urban wastewater and river water by ultra-high-performance liquid chromatography coupled to quadrupole-linear ion trap tandem mass spectrometry. J. Chromatogr. A 1292, 173–188.

Heath, E., Kosjek, T., Farre, M., Quintana, J.B., de Alencastro, L.F., Castiglioni, S., Gans, O., Langford, K., Loos, R., Radjenović, J., Rocca, L.M., Budzinski, H., Tsipi, D., Petrovic, M., Barcelo, D., 2010. Second interlaboratory exercise on non-steroidal anti-inflammatory drug analysis in environmental aqueous samples. Talanta 81, 1189–1196.

Heberer, T., 2002. Tracking persistent pharmaceutical residues in the aquatic environment: a review of recent research data. Toxicol. Lett. 131, 5–17.

Jean, J., Perrodin, Y., Pivot, C., Trepo, D., Perraud, M., Droguet, J., Tissot-Guerraz, F., Locher, F., 2012. Identification and prioritization of bioaccumulable pharmaceutical substances discharged in hospital effluents. J. Environ. Manage. 103, 113–121.

Joss, A., Keller, E., Alder, A.C., Göbel, A., McArdell, C.S., Ternes, T., Siegrist, H., 2005. Removal of pharmaceuticals and fragrances in biological wastewater treatment. Water Res. 39, 3139–3152.

Lajeunesse, A., Gagnon, C., Gagné, F., Louis, S., Čejka, P., Sauvé, S., 2011. Distribution of antidepressants and their metabolites in brook trout exposed to municipal wastewaters before and after ozone treatment – evidence of biological effects. Chemosphere 83, 564–571.

Loos, R., Locoro, G., Comero, S., Contini, S., Schwesig, D., Werres, F., Balsaa, P., Gans, O., Weiss, S., Blaha, L., Bolchi, M., Gawlik, B.M., 2010. Pan-European survey on the occurrence of selected polar organic persistent pollutants in ground water. Water Res. 44, 4115–4126.

Molander, L., Ågerstrand, M., Rudén, C., 2009. WikiPharma – a freely available, easily accessible, interactive and comprehensive database for environmental effect data for pharmaceuticals. Regul. Toxicol. Pharmacol. 55, 367–371.

Mullot, J.-U., Karolak, S., Fontana, A., Levi, Y., 2010. Modeling of hospital wastewater pollution by pharmaceuticals: first results of Mediflux study carried out in three French hospitals. Water Sci. Technol. 62, 2912–2919.

Orias, F., Perrodin, Y., 2013. Characterisation of the ecotoxicity of hospital effluents: a review. Sci. Total Environ. 454–455, 250–276.

Perrodin, Y., Bazin, C., Bony, S., Devaux, A., Bertrand-Krajewski, J.-L., Cren-Olivé, C., Roch, A., Brelot, E., 2013. A priori assessment of ecotoxicological risks linked to building a hospital. Chemosphere 90, 1037–1046.

Sanderson, H., Johnson, D.J., Wilson, C.J., Brain, R.A., Solomon, K.R., 2003. Probabilistic hazard assessment of environmentally occurring pharmaceuticals toxicity to fish, daphnids and algae by ECOSAR screening. Toxicol. Lett. 144, 383–395.

Silva, B.F., Jelic, A., López-Serna, R., Mozeto, A.A., Petrovic, M., Barceló, D., 2011a. Occurrence and distribution of pharmaceuticals in surface water, suspended solids and sediments of the Ebro river basin, Spain. Chemosphere 85, 1331–1339.

Silva, B.F.D., Jelic, A., López-Serna, R., Mozeto, A.A., Petrovic, M., Barceló, D., 2011b. Occurrence and distribution of pharmaceuticals in surface water, suspended solids and sediments of the Ebro river basin, Spain. Chemosphere 85, 1331–1339.

Tauxe-Wuersch, A., De Alencastro, L.F., Grandjean, D., Tarradellas, J., 2006. Trace determination of tamoxifen and 5-fluorouracil in hospital and wastewaters. Int. J Environ. Anal. Chem. 86, 473–485.

Ternes, T.A., 1998. Occurrence of drugs in German sewage treatment plants and rivers. Water Res. 32, 3245–3260.

Verlicchi, P., Galletti, A., Petrovic, M., Barceló, D., 2010. Hospital effluents as a source of emerging pollutants: an overview of micropollutants and sustainable treatment options. J. Hydrol. 389, 416–428.

Verlicchi, P., Al Aukidy, M., Galletti, A., Petrovic, M., Barceló, D., 2012a. Hospital effluent: investigation of the concentrations and distribution of pharmaceuticals and environmental risk assessment. Sci. Total Environ. 430, 109–118.

Verlicchi, P., Al Aukidy, M., Zambello, E., 2012b. Occurrence of pharmaceutical compounds in urban wastewater: removal, mass load and environmental risk after a secondary treatment—a review. Sci. Total Environ. 429, 123–155.

Weissbrodt, D., Kovalova, L., Ort, C., Pazhepurackel, V., Moser, R., Hollender, J., Siegrist, H., McArdell, C.S., 2009. Mass flows of X-ray contrast media and cytostatics in hospital wastewater. Environ. Sci. Technol. 43, 4810–4817.

WHO Collaborating Centre for Drug Statistics Methodology, 2011. Guidelines for ATC classification and DDD assignment. Oslo, 2010.

Yang, Y., Fu, J., Peng, H., Hou, L., Liu, M., Zhou, J.L., 2011. Occurrence and phase distribution of selected pharmaceuticals in the Yangtze estuary and its coastal zone. J. Hazard. Mater. 190, 588–596.

Yuan, S., Jiang, X., Xia, X., Zhang, H., Zheng, S., 2013. Detection, occurrence and fate of 22 psychiatric pharmaceuticals in psychiatric hospital and municipal wastewater treatment plants in Beijing, China. Chemosphere 90, 2520–2525.

2.3 Synthèse

Ce travail a permis de mettre en évidence une liste de 15 molécules avec un quotient de danger très important (> 1000). On retrouve globalement la même diversité de molécules dans ces 15 molécules que dans celles recherchées dans les effluents hospitaliers. Cependant, notons que la classification de la dangerosité ne prend pas en compte le traitement ultérieur par la STEP. Le passage de l'effluent hospitalier à travers la STEP peut totalement modifier la hiérarchie de dangerosité de tous ces composés. En effet, l'efficacité des traitements biologiques classiques dans les STEP peut varier fortement d'un composé à un autre. Il est donc intéressant d'aller plus loin, par exemple en considérant les taux d'abattement de ces différents contaminants, parfois disponible dans la littérature. Par ailleurs, cette dangerosité ne prend en compte que l'écotoxicité directe. En effet, la bioaccumulabilité des composés n'est pas du tout prise en compte dans ce travail. Or, le transfert trophique des contaminants peut poser des problèmes majeurs pour la santé des écosystèmes voire pour celle de l'Homme. Nous développerons les enjeux liés à la bioaccumulation dans le chapitre suivant.

Chapitre 3

Transferts des résidus pharmaceutiques dans la biocœnose aquatique

3.1 Définition des concepts clés

Lorsqu'on s'intéresse au transfert des contaminants dans les organismes, trois concepts apparaissent comme fondamentaux : la bioconcentration, la bioaccumulation et la bioamplification. Bien que centraux, ces concepts sont souvent utilisés les uns à la place des autres, particulièrement en ce qui concerne les termes « bioconcentration » et « bioaccumulation ». C'est pourquoi nous avons choisi ici de préciser le sens des mots que nous allons utiliser ci-après :

- **Bioconcentration** : phénomène par lequel un organisme va concentrer une substance en concentration supérieure à celle du milieu uniquement via le milieu (respiration et diffusion cutanée). Ce milieu peut être l'eau ou le sédiment pour les organismes aquatiques ou le sol et/ou l'air pour les organismes terrestre. Ce processus est le résultat du rapport entre la vitesse de pénétration de la substance dans l'organisme (via la respiration et la diffusion cutanée) et la vitesse d'élimination (via échanges respiratoires, métabolisme et dilution par la croissance) : c'est le facteur de bioconcentration (BCF).
- **Bioaccumulation** : phénomène par lequel un organisme va concentrer une substance en concentration supérieure à celle du milieu via toutes les voies d'exposition y compris l'alimentation. Ce processus est le résultat du rapport entre la vitesse de pénétration de la substance dans l'organisme (via respiration, diffusion cutanée ET alimentation) et la vitesse d'élimination (via les échanges respiratoires, le métabolisme et la dilution par la croissance) : c'est le facteur de bioaccumulation (BAF).
- **Bioamplification** : phénomène par lequel un contaminant se retrouve en concentration plus importante dans un organisme que dans son alimentation. Ainsi, un contaminant qui est bioamplifié se retrouve en concentration toujours plus élevé chez l'organisme du maillon n+1 que chez l'organisme du maillon n.

Ces différents processus permettent aux organismes de prélever les nutriments nécessaires à leur développement dans leur environnement (e.g. vitamines, oligo-éléments ou encore acides gras essentiels). Cependant, les composés xénobiotiques sont aussi concernés par ces phénomènes. Il est alors indispensable de faire la distinction entre les différents mécanismes décrits ci-avant. En effet, il est possible qu'un contaminant puisse être bioconcentré ou bioaccumulé à l'échelle de l'organisme sans qu'il soit nécessairement bioamplifié le long des chaînes alimentaires. Ainsi, les risques pour les écosystèmes ne seront pas les mêmes si un contaminant est bioaccumulé et/ou bioamplifié.

3.2 Impacts du transfert des contaminants sur les écosystèmes

La première référence documentée à propos de la bioconcentration de contaminants dans les écosystèmes remonte à 1954 avec Foster qui relevait des teneurs en radio-phosphore (^{32}P) mille fois supérieures dans le phytoplancton que dans l'eau de la rivière. Cette rivière située dans l'état de Washington recevait les effluents de réacteurs nucléaires militaires situés en amont (Ramade, 2011). Si cette première observation du transfert des contaminants dans les organismes aquatiques n'a pas mise en évidence d'impact sanitaire retentissant, ce n'est pas le cas de la catastrophe de Minamata. Pendant la première moitié du 20$^{\text{ème}}$ siècle, l'entreprise Chisso Chemical a relargué des effluents chargés en résidus mercuriels dans la baie de Minamata, au Japon. Après méthylation dans les sédiments par le périphyton, le mercure inorganique très peu biodisponible le devient fortement et se transfert dans le réseau trophique de la baie (Figure 3.1). Plusieurs milliers de personnes sont touchées par cette intoxication à cause de la forte consommation de poissons prédateurs situés au sommet de la chaîne alimentaire (e.g. thon). Mais la bioconcentration des contaminants ne concerne pas que les éléments inorganiques.

C'est en 1960 que Hunt & Bischoff (Hunt and Bischoff, 1960) ont pour la première fois décrit le phénomène de bioaccumulation d'un contaminant chimique organique. Ces chercheurs ont mis en évidence que la concentration en dichlorodiphenyldichloroethane (DDD) dans le phytoplancton du lac Clear (Californie) était près de 360 fois plus élevée que dans l'eau. D'autres organochlorés sont bien connus pour leurs propriétés de composés bioaccumulables : les polychlorobiphényles (PCB), les dioxines, les composés perfluorés,... Aujourd'hui, on retrouve toutes ces substances de manière ubiquitaire à la surface de la planète. Grâce aux courants atmosphériques, on peut retrouver certains d'entre eux à plusieurs milliers de kilomètres de la plus proche source d'émission (e.g. PCB aux îles Kerguelen (Jaffal et al., 2011)).

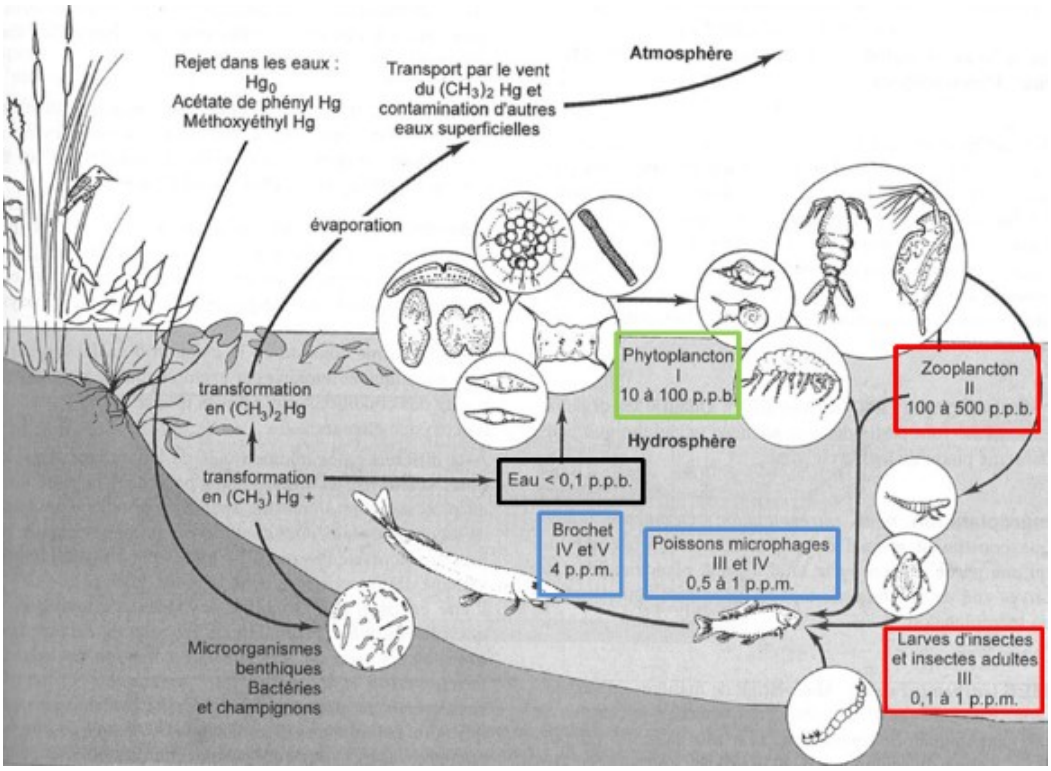

Figure 3.1 *Exemple de transfert de contaminant dans une chaîne alimentaire : Incorporation et bioamplification du mercure dans une biocœnose dulçaquicole (issu de Ramade (2002)). Encadré en noir le milieu d'exposition, en vert, les producteurs primaires, en rouge les consommateurs secondaires et en bleu les autres consommateurs.*

Historiquement, on remarque que toutes les découvertes autour de ces contaminants présents dans l'Environnement se sont toujours faites de manières conjointes avec l'évolution des techniques analytiques. La capacité d'analyse, tant qualitative que quantitative, est une problématique centrale en écotoxicologie.

3.3 Caractérisation de la bioaccumulabilité

3.3.1 Coefficient de partage

Le coefficient de partage obéit à un principe découvert 1872 qui stipule qu'un « coefficient de partition constant régie le partage d'un corps pur entre deux phases différentes » (Berthelot and Jungfleisch, 1872). C'est cette notion vieille de près de 150 ans qui aujourd'hui est fondamentale dans la caractérisation théorique de la bioaccumulabilité d'une substance. En effet, le coefficient de partage entre l'octanol et l'eau (K_{ow}) sert de base à toutes les évaluations de bioaccumulabilité des substances.

3.3.2 Relation quantitative entre structure et activité (QSAR)

En 1979, Hansch & Leo (Hansch and Leo, 1979) présentent un modèle qui, à partir de la structure de la molécule, est capable de déterminer son activité chimique et biologique : il s'agit du modèle QSAR (Quantitative Structure-Activity Relationship). Ce modèle va plus loin que le seul K_{ow} en intégrant des propriétés polaires et stériques des molécules pour en évaluer l'activité biologique. Il est également possible d'intégrer dans ce modèle des paramètres biologiques, comme illustré en figure 3.2 (e.g. affinité molécule/récepteurs, constante d'inhibition, pharmacodynamique) (Selassie et al., 2003). Grâce à ce modèle, il est possible de déterminer la bioconcentrabilité d'une substance et donc sa capacité théorique à se concentrer du milieu vers les organismes. Aujourd'hui, la modélisation (QSAR) est largement utilisée pour l'évaluation de la bioconcentrabilité d'une substance. Cependant, comme le montre la figure 3.3, les caractéristiques utilisées dans le modèle QSAR comme l'hydrophobicité ou la polarité ne suffisent pas à déterminer avec certitude la bioconcentrabilité d'une molécule. Le comportement au sein de l'organisme (i.e. métabolisation par chaque organisme considéré) et son exposition (i.e. via le milieu et/ou l'alimentation) sont également à considérer. En effet, il n'est pas possible avec ce type de modèle d'estimer la contribution de l'alimentation à la contamination de l'organisme considéré sans expérimentations préalables.

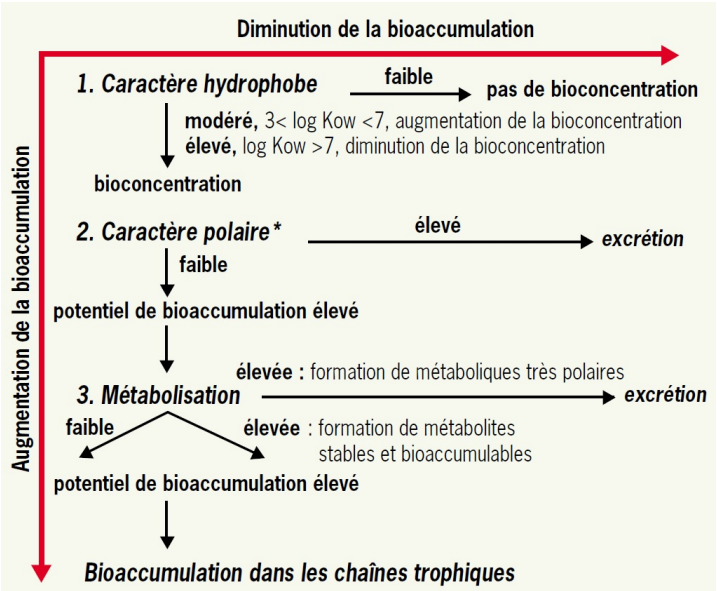

Figure 3.2 *Exemple de paramètres pris en compte par les approche QSAR pour évaluer la bioaccumulibilité d'une molécule(Adapté de Norstrom et Letcher 1996 in Abarnou and Blanchard (2000))*

3.3.3 Caractérisation expérimentale

Il existe une troisième approche pour évaluer la bioconcentrabilité ou la bioaccumulabilité d'une molécule : l'expérimentation. Cette approche étant onéreuse et complexe, on privilégie souvent la modélisation pour l'évaluation de la bioaccumulabilité. Or, si pour beaucoup de substances la bioconcentration est corrélée au K_{ow}, ce n'est pas toujours le cas (Figure 3.3). C'est pourquoi il est indispensable d'effectuer un minimum d'expérimentation pour déterminer la bioconcentrabilité réelle d'une substance.

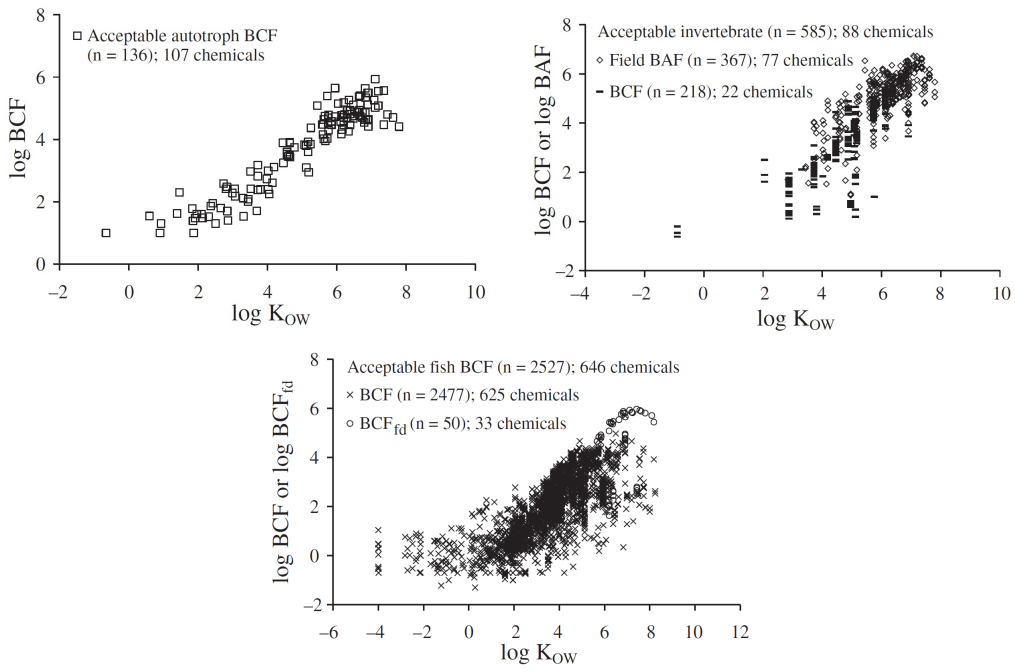

Figure 3.3 *Expression des facteurs de bioconcentration et de bioaccumulation de plusieurs contaminants déterminés expérimentalement chez des autotrophes (en haut à gauche), des invertébrés (en haut à droite) et des vertébrés (en bas au centre) en fonction des K_{ow} respectifs de ces molécules (Figures issues de Arnot and Gobas (2006))*

On remarque sur la figure 3.3 que pour les substances avec un K_{ow} supérieur à 6, les valeurs de BCF sont plus diffuses. Cette observation est confirmée par un article de Papa et al. (Papa et al., 2007) qui confirme que la linéarité des modèles QSAR pour prédire les facteurs de bioconcentration ne s'applique plus au-delà d'un K_{ow} de 6. De plus, il a été montré que les organismes classiquement utilisés en écotoxicologie (e.g. *C. reinhardtii*, *D. pulex* ou *D. rerio*) conservaient une part non négligeable de constituant cellulaires (e.g. enzymes, récepteurs, canaux ioniques ou transporteurs) en commun avec l'Homme (Gunnarson et al., 2008). Ainsi, pour les contaminants comme RP qui sont à la base conçus pour agir sur des cibles cellulaires humaines, on peut difficilement évaluer comment les organismes modèles en écotoxicologie vont réagir face à ces contaminants bien spécifiques.

3.4 Les résidus pharmaceutiques dans l'Environnement

3.4.1 Problèmatique générale

Le terme « résidu pharmaceutique » (ou « résidu médicamenteux ») est un terme générique qui désigne toute molécule, métabolisée ou non, issue d'un médicament. Il peut s'agir de :

- **Principe actif**. Un médicament est constitué de deux entités, le principe actif et les excipients. Après l'administration du médicament, la totalité du principe actif n'est pas assimilée par l'organisme et une partie variable (en fonction du médicament et du patient) est évacuée dans les urines et les fèces et in fine dans les eaux usées.
- **Métabolite biologique** (humains ou animaux dans le cas de produits vétérinaires). Lorsque le principe actif transit dans l'organisme il peut être métabolisé. C'est-à-dire que des groupements fonctionnels peuvent être ajouté ou retiré par les différents organes traversés par le principe actif.
- **Métabolite environnemental**. Lorsque les résidus médicamenteux sont émis via les eaux usées vers les STEP, une métabolisation peut se produire à cause des microorganismes présents. Une fois dans l'environnement, les organismes exposés peuvent également métaboliser ces résidus. D'autres réactions comme l'ozonation ou la photodégradation peuvent également générer ce type de métabolites.

Les premiers soupçons sur la présence de résidus pharmaceutiques dans l'Environnement sont apparus au début des années 70 (Norpeth et al. 1973 in Aherne et al. 1985). Cependant, la prise conscience de la communauté scientifique n'est pas apparue avant la fin des années 90 grâce à deux découvertes majeures : (i) la découverte de la présence de nombreux résidus pharmaceutiques dans les effluents de stations d'épuration (Ternes, 1998) (ii) la démonstration de l'effet féminisant de l'éthynilestradiol sur les poissons de rivières au Royaume Uni (Jobling et al., 1998). Une troisième découverte majeure contemporaine des deux précitées est responsable du développement des recherches autour des résidus pharmaceutiques : la découverte de résidus pharmaceutiques dans l'eau de boisson (Heberer and Stan, 1997).

Si les eaux de surface sont aujourd'hui contaminées à travers le monde (Fick et al., 2009; Loos et al., 2009) ce n'est pas le seul compartiment écosystémique atteint par la contamination par les résidus pharmaceutiques. On retrouve déjà depuis plusieurs décennies des RP dans les eaux profondes. Dès le début des années 90, on a retrouvé des concentrations en résidus pharmaceutiques de l'ordre du μg/L dans les réservoirs d'eau profondes destinées à l'eau de boisson (Eckel et al., 1993). Par ailleurs, à la même époque, vers la fin des années 80, Jacobsen & Berglind (Jacobsen and Berglind, 1988) ont mis en évidence une contamination des sédiments par l'oxytetracycline à des teneurs non négligeables (i.e. 0,1 à 4,9 mg/kg). De nombreuses études ont déjà mis en évidence l'écotoxicité des RP aux concentrations environnementale (Brodin et al., 2013; Kidd et al., 2007; Lajeunesse et al., 2011). Cependant, la présence de ces RP à de très faibles concentrations, peut également présenter un risque pour les écosystèmes via la bioaccumulation.

3.4.2 Les résidus pharmaceutiques bioaccumulables

Dans les compartiments précités, nous avons aujourd'hui de nombreuses informations qui caractérisent quantitativement et qualitativement les RP que nous retrouvons dans l'Environnement. Cependant, un dernier compartiment écosystémique reste très peu exploré en ce qui concerne la contamination par les RP : la biosphère. C'est au début des années 90 que les premières études de bioconcentration sont développée (Nelis et al., 1991). Le but de ces premières expérimentations était « d'encapsuler » un médicament vétérinaire dans la nourriture (i.e. Artémies, Cnidaires) afin d'optimiser l'absorption du médicament par les poissons. En exposant les artémies au médicament, elles l'absorbent et/ou l'adsorbent par bioconcentration et les poissons à traiter sont nourris avec ces artémies. Cependant, il faut attendre les années 2000 pour que les expérimentations sur la bioconcentration et la bioaccumulation des RP se développent (Brown et al., 2007; Kinney et al., 2008; Lai et al., 2002). Très récemment, Lajeunesse et al., (Lajeunesse et al., 2011) ont démontrés que la bioaccumulation des RP pouvait avoir une effet sur les organismes vivants dans les rivières contaminées, notamment les poissons. En effet, ils ont démontré que les teneurs en inhibiteur sélectif de la recapture de la sérotonine (e.g. fluoxetine, sertraline, citalopram,...) mesurés dans les poissons vivants dans une rivière exposée à ce type de contamination avaient un effet sur le système nerveux. Ainsi, il est aujourd'hui évident que les phénomènes de bioconcentration et de bioaccumulation des RP dans l'environnement sont à prendre en compte pour évaluer l'impact de ces contaminants tant sur la santé des écosystèmes que sur celle de l'Homme.

3.5 La priorisation des résidus pharmaceutiques

Dans les chapitres précédents, nous avons mis en exergue la diversité des molécules présentes dans les effluents hospitaliers. Ainsi, il est nécessaire de prioriser les molécules à étudier en fonction de la question scientifique à laquelle on veut répondre. Dans les paragraphes précédents, nous avons expliqué pourquoi la bioaccumulation des résidus pharmaceutiques était la problématique qui nous intéressait. La bioaccumulabilité potentielle (obtenu par la méthode QSAR) est donc un paramètre fondamental qui va être considéré pour prioriser les molécules d'intérêt. Cependant, deux autres paramètres sont également très importants à prendre en compte. D'une part, la consommation de la molécule au sein de l'hôpital. En effet, si une molécule n'est pas consommée dans l'hôpital, il n'y a aucune probabilité de la retrouver dans les effluents hospitaliers. D'autre part, l'écotoxicité des molécules doit être prise en compte.

Pour entamer les premiers travaux expérimentaux sur les 14 molécules prioritaires, nous avons choisi les molécules les plus bioaccumulables au sein de chaque groupe de « facteur aggravant » (cf. Tableau 1) :

- L' **amiodarone** pour les « lourdement consommées » .
- Le **tamoxifen** pour les « perturbateurs endocriniens » .
- Le **ritonavir** pour les « molécules potentiellement écotoxique non encore étudiée » .

Deuxième partie

CARACTERISATION DE L'ECOTOXICITE DES RESIDUS PHARMACEUTIQUES MODELES

Parmi les 14 molécules identifiées comme prioritaires (Jean et al., 2012) dans les effluents hospitaliers en ce qui concerne la bioaccumulation, nous en avons choisi trois : l'amiodarone, le ritonavir et le tamoxifen. Avant d'évaluer leur bioaccumulabilité, il est nécessaire d'évaluer au préalabale leur écotoxicité directe. En effet, pour nous affranchir des effets écotoxiques qui pourraient perturber nos mesures de bioaccumulation, il est nécessaire de prendre en compte ces résultats pour fixer la gamme de toxicité de ces molécules pour les organismes que nous allons utiliser. Ainsi, dans un **premier chapitre**, nous présentons les différents tests d'écotoxicité réalisés pour les 3 molécules modèles choisies. Dans un **deuxième chapitre**, nous tentons d'évaluer l'impact que peut présenter l'excipient sur l'écotoxicité d'un principe actif. En effet, certaines études présentents dans la littérature sont effectuées avec le médicament (principe actif + excipient). Or, dans l'Environnement, c'est le principe actif d'une part et éventuellement l'excipient d'autre part qui sont retrouvés. Enfin, dans un **troisième chapitre**, nous évaluons le risque écotoxicologique pour les écosystèmes aquatiques, dans 4 situations différentes sur le plan international, que représente le contaminant modèle dont nous avons choisi d'étudier la bioaccumulabilité : le tamoxifen.

Chapitre 4

Caractérisation préliminaire de l'écotoxicité des contaminants modèles

4.1 Introduction

Dans la première partie de ce manuscrit, nous avons mis en évidence que pour de nombreux RP, très peu, voire aucune, données écotoxicologiques n'étaient disponibles. C'est également le cas pour nos 14 molécules prioritaires. En effet, à part l'ethynilestradiol qui présente un nombre de données assez riche, et le tamoxifen pour lequel on en possède quelques-unes (Orias and Perrodin, 2013) les autres molécules ne présentent aucune donnée écotoxicologique. Par ailleurs, dans la littérature, il existe quelques données sur le tamoxifen citrate (Mater et al., 2014; Williams et al., 2007). Or, nous préférons ne pas prendre en compte ces données étant donné l'impact que le citrate (excipient) peut présenter sur l'écotoxicité du tamoxifen (cf. Chapitre 5). Ainsi, au regard de la faible littérature disponible pour nos molécules prioritaires, nous avons décidé d'effectuer une batterie de bioessais sur nos molécules modèles pour évaluer la gamme de concentrations écotoxiques pour nos organismes modèles. En effet, avant d'évaluer la bioaccumulation de ces molécules, il est nécessaire de s'assurer que les concentrations auxquelles nous travaillons sont sub-effet afin d'éviter les intéractions entre écotoxicité et bioaccumulation. Dans le tableau 4.1, nous avons rassemblé les données physico-chimiques disponibles concernant nos 3 molécules modèles : amiodarone, tamoxifen, ritonavir.

Table 4.1 *Description et propriétés physico-chimique des molécules prioritaires testées.*

Nom de la molécule	**Tamoxifen**	**Amiodarone**	**Ritonavir**
Classe thérapeutique	Anticancéreux	Antiarythmique	Antiviral
Numéro CAS	10540-29-1	1951-25-3	155213-67-5
K_{ow}	7,1	7,57	3,9
Solubilité dans l'eau (mg/L)	0,167	4,76	1,26

La batterie de bioessais réalisée est constituée de 4 tests d'écotoxicité : inhibition de croissance 72h sur *P. subcapitata*, immobilisation 48h et inhibition du broutage 48h sur *D. magna* et inhibition de luminescence (NF ISO 11348-3) 30 minutes sur *V. fisherii*.

4.2 Matériels et méthodes

4.2.1 Inhibition de la croissance de *P. subcapitata* (72h)

Les tests de croissance réalisés sur les 3 algues sélectionnées suivent les guidelines OECD N°201 (OECD, 2006). Les algues sont ensemencées en microplaques 48 puits à une concentration de 10^4 cellules par mL dans leur milieu de culture spécifique contenant différentes concentrations de tamoxifen, après 3 jours de préconditionnement dans les conditions de l'essai soit en enceinte climatique (Aralab Fitoclima D 1200), 20°C ± 1°C éclairage continu 10 000 lux. Les gammes de concentration testées sont rassemblées dans le tableau 4.2. Trois réplicats sont réalisés pour chaque concentration. En parallèle, un témoin avec la concentration la plus élevée de DMSO introduite dans les puits (0.01%) est mis en place pour s'assurer de l'absence d'effet du solvant sur la croissance algale. Pour limiter l'évaporation dans les puits durant l'expérience, pouvant modifier la concentration de tamoxifen, de l'eau ultrapure est placée dans les puits extérieurs de la plaque. Soixante-douze heures après l'inoculation le contenu des puits est homogénéisé et la densité algale est mesurée sur cellule de Malassez (2 comptages par puits) à l'aide d'un microscope Zeiss grossissement 400 x. Des concentrations d'inhibition 50% (IC50) sont ensuite calculées à l'aide du logiciel RegTox.

4.2.2 Immobilisation de *D. magna* (48h)

Les tests d'immobilisation 48h sur daphnies sont réalisés selon le protocole standard ISO (ISO, 1995). Cinq daphnies de moins de 24h sont placées dans des tubes à essai contenant 10 mL de solution contaminée (diluée avec du milieu d'élevage) selon les gammes présentées dans le tableau 4.2. Quatre réplicats par concentration sont effectués (20 daphnies par concentration au total). Afin d'éviter l'éventuelle croissance algale qui pourrait intervenir, les tubes sont incubés dans le noir à 20°C ± 1°C pendant la période d'exposition. Pour une meilleure précision, les tests sont réalisés 2 fois pour chaque contaminant et les résultats sont moyennés. Après 48h d'exposition, le nombre de daphnie immobilisée est comptabilisé dans chaque tube et les EC50 48h sont calculées en utilisant un modèle de type log-probit.

4.2.3 Inhitbition du broutage 48h sur *D. magna*

L'inhibition de l'activité de broutage sur 48h chez daphnie est mesurée sous condition statique (ni croissance algale ni renouvellement de milieu), d'après Clément and Zaid (2004). L'exposition est menée dans des béchers en verre de 250 mL contenant 150 mL de milieu d'élevage contaminé selon les gammes présentées dans le tableau 4.2. Chaque bécher est inoculé avec 75.10^6 cellules de *P. subcapitata* (500000 cellules/mL) et 5 daphnies âgées entre 3 et 4 jours. Quatre réplicats par concentration sont réalisés. Pour chaque concentration, 2 béchers sans daphnies sont utilisés comme témoin de la croissance éventuelle des algues.

Pour prévenir au maximum cette croissance, les béchers sont placés à l'obscurité à 20°C ± 1°C. Les béchers son délicatement agités trois fois par jour pour assurer une disponibilité homogène des algues dans les béchers. Les algues restantes dans le milieu sont mesurées après 24 et 48h à l'aide d'un compteur à particule (Coultronics, model Z1, seuil de taille 3.6 μm). Pour une meilleure précision, les tests sont réalisés 2 fois pour chaque contaminant et les résultats sont moyennés. L'activité de broutage est évaluée par le calcul du taux de filtration F (ml/daphnie/h) selon Gauld (1951) :

$$\mathrm{F} = (\mathrm{V/n})\ [\ln(C_0) - \ln(C_t)\ /\ \mathrm{t} - \mathrm{A}]$$

$$\mathrm{A} = [\ln(C_0) - \ln(C'_t)]\ /\ \mathrm{t}$$

Où V (mL) est le volume total du milieu, n le nombre de daphnie dans le volume V, C_0 et C_t les densités initiales et finales (cellules/mL) et t le temps d'exposition (heures). A est un facteur correctif utilisé pour prendre en compte les changements de densité algale dans le témoin (algues sans daphnies) exprimé comme la densité algale final C'_t. Aucune mortalité ne doit être observée chez les daphnies exposées.

4.2.4 Inhibition de la luminescence de *V. fisherii* (30min)

Ces tests ont été réalisés par PROVADEMSE, sous la direction de Christine BAZIN, selon la norme NF ISO 11348-3. Ce test consiste à mesurer combien une substance inhibe la luminescence de la bactérie *V. fisherii*. Pour cela, des bactéries sont exposées à une gamme de contaminant (Table 4.2) à une température de 15°C ± 1°C. Après 30 minutes, la luminescence est mesurée (analyseur Microtox M500) et est comparée au témoin.

Table 4.2 *Gammes des concentrations testées pour le tamoxifen, l'amiodarone et le ritonavir chez V. fisheri, P. subcapitata et D. magna (NT : Non testé).*

Molécule	Organisme	Concentrations testées (μg/L)									
Tamoxifen	*V. fisheri*	NT	NT	6250	9380	12500	18750	25000	38000	50000	75000
	P. subcapitata	3	6	13	25	50	100	200	400	800	1600
	D. magna	24	49	98	195	391	781	1563	3125	6250	12500
Amiodarone	*V. fisheri*	NT	NT	6250	9380	12500	18750	25000	38000	50000	75000
	P. subcapitata	185	370	739	1479	2958	5916	11831	23663	47325	94650
	D. magna	462	924	1849	3697	7395	14789	29578	59156	118313	236625
Ritonavir	*V. fisheri*	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
	P. subcapitata	391	781	1563	3125	6250	12500	25000	50000	100000	200000
	D. magna	391	781	1563	3125	6250	12500	25000	50000	100000	200000

4.3 Résultats

Dans le tableau suivant (Tableau 4.3), nous avons réuni l'ensemble des résultats des tests d'écotoxicité obtenus.

Table 4.3 *Synthèse des résultats des tests d'écotoxicité effectués pour le tamoxifen, l'amiodarone et le ritonavir chez V. fisheri, P. subcapitata et D. magna (NT : Non testé ; NA : Non applicable).*

Molécule	Test d'écotoxicité	EC50 (mg/L)	Intervalle de confiance 95% (mg/L)
Tamoxifen	*V. fisheri* 30 min	8,2	7,31 - 8,34
	P. subcapitata 72h	0,702	0,681- 0,787
	D. magna 48h immobilisation	0,696	0,660 - 0,830
	D. magna 48h broutage	0,104	0,084 - 0,124
Amiodarone	*V. fisheri* 30 min	12,80	11,98 - 13,76
	P. subcapitata 72h	0,870	0,670 - 1,173
	D. magna 48h immobilisation	> 28	NA
	D. magna 48h broutage	0,497	0,366 - 0,669
Ritonavir	*V. fisheri* 30 min	NT	NA
	P. subcapitata 72h	> 50	NA
	D. magna 48h immobilisation	> 50	NA
	D. magna 48h broutage	> 50	NA

4.3.1 Ecotoxicité du ritonavir

Nous ne sommes pas parvenus à mettre en évidence la moindre écotoxicité du ritonavir, aux concentrations auxquelles nous avons effectué les tests.

4.3.2 Ecotoxicité de l'amiodarone

L'amiodarone présente une écotoxicité relative chez tous les organismes testés. Chez Ps, on observe une EC50 de 870 μg/L avec un intervalle de confiance à 95% relativement élevé (670 - 1173 μg/L). Chez Dm, nous ne sommes pas parvenu à mettre en évidence d'effet sur la mortalité ($>$ 28000 μg/L) à 48h alors que sur le paramètre broutage, nous remarquons une EC50 relativement basse de 497 μg/L (IC95 : 366 - 669 μg/L). Enfin, l'écotoxicité de l'amiodarone vis-à-vis de Vf est relativement faible avec une EC50 pour l'inhibition de la luminescence de 12800 mg/L (IC95 : 11980 - 13760 μg/L).

4.3.3 Ecotoxicité du tamoxifen

C'est avec le tamoxifen que nous obtenons les niveaux d'écotoxicité les plus forts (EC50 les plus faibles), quel que soit l'organisme testé ou le paramètre observé. *D. magna* est l'organisme

le plus sensible au tamoxifen avec des EC50 respectives pour la mortalité et le broutage de 696 μg/L (IC95 : 660 - 830 μg/L) et de 104 μg/L (IC95 : 84 - 124 μg/L). Les algues sont les organismes les plus sensibles au tamoxifen après les daphnies avec une EC50 de 702 μg/L (IC95 : 681 - 787 μg/L) et les bactéries marines les moins touchées avec une EC50 de 8200 μg/L (IC95 : 7310 - 8340 μg/L).

4.4 Discussion

4.4.1 Ecotoxicité des contaminants modèles

N'observant pas d'effet ni chez les algues ni chez les daphnies (mortalité), nous avons choisi de ne pas effectuer de tests supplémentaires pour le ritonavir. Par ailleurs, nous n'avons pas choisi de tester des concentrations supérieures à 200 mg, cette dernière valeur étant déjà bien au-delà de la solubilité du ritonavir dans l'eau (1,6 mg/L). Ces résultats sont surprenants car cette molécule avait été sélectionnée pour sa forte écotoxicité potentielle, en plus de sa bioaccumulabilité. Dans la littérature, nous n'avons trouvé aucune donnée sur l'écotoxicité du ritonavir. Cette absence de donnée est certainement due à sa faible écotoxicité. En effet, même si une absence d'écotoxicité est un résultat, il est très difficile de le faire publier. Ainsi, les tests d'écotoxicité de ce type de contaminant ne sont jamais visibles.

Parmi les trois molécules testées, c'est le tamoxifen qui présente la plus forte écotoxicité, tous tests confondus. Par ailleurs, c'est la seule molécule avec laquelle nous pouvons faire des comparaisons avec les résultats obtenus dans la littérature car c'est la seule molécule qui a déjà été testée. Les résultats que nous avons obtenus sont relativement cohérents avec ce que nous avons pu observer dans la littérature (Mater et al., 2014; DellaGreca et al., 2007). Par exemple, DellaGreca et al. (2007), ont effectués des tests d'écotoxicité sur 3 invertébrés : *Brachionus calyciflorus*, *Thamnocephalus platyurus* et *Daphnia magna*. Les EC50 obtenues dans ces travaux sont respectivement de 0,97, 0,40 et 1,53 mg/L et nous avons trouvé une valeur d'EC50 immobilisation de 0,696 mg/L pour *Daphnia magna*. Cette équipe a également effectué des tests chroniques avec le tamoxifen sur *Brachionus calyciflorus* et *Cerodaphnia dubia* avec des valeurs d'EC50 logiquement plus basses, respectivement 0,25 et 0,081 mg/L. Mater et al. (2014) se sont également intéressés à l'écotoxicité que le tamoxifen pouvait présenter pour les algues. Mais, ils ont utilisés du tamoxifen citrate. Nous verrons pourquoi, dans le chapitre suivant, nous ne pouvons pas réellement comparer les résultats d'écotoxicité qu'ils ont obtenus avec les nôtres.

4.4.2 Valeurs nominales et réelles

Lors de nos expériences préliminaires, nous n'avons pas effectué de suivis analytiques de nos solutions. Ceci est un point faible de nos travaux car de nombreux processus peuvent intervenir pendant la phase d'exposition pouvant causer une modification de la concentration nominale de l'exposition réelle des organismes, même à très court terme. Cette perturbation de la concentration réelle d'exposition peut être causée par plusieurs paramètres :

- **Biologique** : la dégradation métabolique et/ou l'absorption des contaminants par les organismes exposés peut diminuer la concentration nominale.
- **Chimique** : les modifications de pH influencent l'ionisation de certains composés modifiant ainsi la biodisponibilité de ces derniers.
- **Physique** : parmi les phénomènes physiques qui peuvent diminuer la concentration nominale, on trouve la photodégradation ou encore l'adsorption (e.g. sur la verrerie).

Ainsi, de manière à rendre plus robuste les tests d'écotoxicité, il serait pertinent à l'avenir d'effectuer un suivi minimum dans les milieux d'exposition. Cela permettrait également de mieux valoriser les travaux d'écotoxicologie de manière générale.

4.4.3 Complémentarité des tests écotoxicologiques

Dans nos travaux, nous avons utilisé un test peu commun dans les études d'écotoxicité : l'impact des contaminants sur le broutage des daphnies. Ce test, bien qu'aiguë au même titre que la mortalité 48h, a su montrer son intérêt dans nos travaux avec une sensibilité particulière par rapport au test de mortalité. L'utilisation de paramètres complémentaires chez le même organisme permet d'évaluer l'écotoxicité sur plusieurs fonctions de l'organisme, éventuellement plus sensibles à tel ou tel contaminant. Ce type de démarche est également réalisable chez les organismes unicellulaires comme les algues ou les bactéries où on peut observer l'impact des contaminants sur des activités enzymatiques, par exemple. Chez les algues, il serait également intéressant d'étudier l'écotoxicité de ces contaminants sur d'autres types d'algues utilisant des fonctions métaboliques différentes des algues vertes unicellulaires, par exemple chez des algues vertes flagellées ou chez des diatomées. Pour les invertébrés, il serait pertinent d'exposer des organismes avec des modes de vie différents de type benthique, par exemple, ou d'autres invertébrés que des vertébrés comme des mollusques. Les informations apportées par les tests chez ces organismes seraient particulièrement intéressantes pour des molécules comme les composés très lipophiles (comme ceux étudiés ici) qui ont tendance à s'adsorber sur les sédiments et/ou à se stocker dans les organismes riches en lipides comme les mollusques.

4.5 Conclusion et synthèse

Les données d'écotoxicité que nous avons obtenues pour le ritonavir et l'amiodarone sont les premières et les seules existantes sur le plan international à notre connaissance.
Notons cependant qu'il est nécessaire d'approfondir les tests d'écotoxicité avec le ritonavir et l'amiodarone, notamment avec des tests chroniques et sur d'autres espèces et/ou d'autres paramètres biologiques pour mieux caractériser l'écotoxicité de ces contaminants. Par ailleurs, à l'avenir, il serait judicieux de prévoir de suivre l'exposition réelle des organismes testés pour une plus grande robustesse des résultats produits. Enfin, nos résultats montrent que le tamoxifen est la molécule modèle la plus toxique parmi les 3 sélectionnées. De surcroît, c'est la seule qui a déjà été détectée dans l'Environnement.

Chapitre 5

Ecotoxicité comparative de deux médicaments modèles et de leurs principes actifs respectifs

5.1 Enjeux et démarche

Dans l'Environnement, les RP que nous retrouvons sont rarement le médicament sous sa forme initiale (principe actif + excipient) mais peuvent plutôt correspondre au principe actif et/ou à des métabolites. Il n'est donc pas cohérent, d'un point de vue écotoxicologique, d'effectuer des tests avec des substances que nous avons peu de chance de retrouver dans l'Environnement. Ainsi, dans les travaux que nous présentons dans ce chapitre, nous avons voulu évaluer l'effet des excipients sur l'écotoxicité de deux molécules modèles : l'amiodarone et le tamoxifen. Le ritonavir ne fait pas partie de cette étude car il est utilisé sans excipient. Ces travaux ont été menés en collaboration avec une équipe de l'UPR de Gif sur Yvette pour purifier l' « amiodarone HCl » en « amiodarone », indisponible dans le commerce.

5.2 Article 3 : *Comparative ecotoxicity of tamoxifen citrate and amiodarone hydrochloride and their respective active ingredients*

Article à soumettre

Comparative ecotoxicity of tamoxifen citrate and amiodarone hydrochloride and their respective active ingredients

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Abstract

Nowadays, pharmaceutical compounds (PCs) are ubiquitous in aquatic ecosystems. These molecules can threat ecosystems because of their ecotoxicity. To assess this latter, ecotoxicity tests have already been carried out by different authors, but most of the time with original drugs (active ingredient + excipient). But, in the environment, we find either one or the other. Moreover, excipient can modify the ecotoxicity of the active principle, increasing its bioavailability, for example. In this work, we performed three acute bioassays (Daphnia magna mortality, Pseudokirchneriella sucapitata growth and Vibrio fisheri bioluminescence) aiming to assess impact of excipient on ecotoxicity of active principle. These bioassays were carried out with four molecules: tamoxifen, tamoxifen citrate, amiodarone and amiodarone hydrochloride. We observed that tamoxifen were less ecotoxic than tamoxifen citrate, but we have not put in evidence ecotoxicity differences between amiodarone and amiodarone hydrochloride. Besides, ecotoxicity tests carried out with amiodarone showed a lower ecotoxicity than tamoxifen.

Keyword

Pharmaceuticals, excipients, ecotoxicity, tamoxifen citrate, amiodarone hydrochloride, *Daphnia magna*, *Pseudokirchneriella subcapitata*, *Vibrio fisheri*.

1. Introduction

Today, pharmaceutical compounds (PCs) are ubiquitous in aquatic ecosystems and continuously emitted into the environment. Three main sources of emission are identified: (i) urban effluents (Langford and Thomas, 2009); (ii) manure spreading and aquaculture (Burridge et al., 2010; Kolodziej et al., 2004) ; (iii) pharmaceutical factories effluents (Collado et al., 2014). But, PCs are molecules designed for being biologically actives and can potentially threat development of exposed organisms. Thus, ecosystems functioning can be significantly disturbed, even at low PCs concentration (Kidd et al., 2007). Otherwise, drugs are generally composed of two different

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parts: (i) active ingredient which is the molecule needed for a giving treatment and which have an effect on organism to cure (ii) and excipients which will have different roles (e.g. preservation, solubilization, taste,…) depending on their nature. In the environment, PCs found are rarely original drugs (excipient + active ingredient) but rather, in one hand, excipients, and in the other hand, active ingredients incompletely assimilated by treated organisms or metabolites resulting from metabolization of drugs inside treated body or in the Environment.

For this reason, we decided to study in one hand active ingredient ecotoxicity, in other hand original drug ecotoxicity for two model drugs and then to compare obtained results.

Concerning organisms used for tested ecotoxicity of models substances, we chose to work with: *Pseudokirchneriella subcapitata* (Ps), a microalgae belonging to Chlorophyceae family, classically used in ecotoxicology, *Daphnia magna* (Dm), freshwater planktonic macroinvertebrate belonging to Cladocera family, also classically used in ecotoxicology and finally a marine bacteria, *Vibrio fisheri* (Vf). In this way, we asses ecotoxicity on three organisms from three different trophic levels and living in freshwater and marine water.

Concerning models substances, we choose to study two drugs: (i) amiodarone chlorhydrate, a class III antiarrhythmic drug, used for heart disease treatment; (ii) tamoxifen citrate, an antioestrogenic anticancer drug used for breast cancer treatment. These drugs have been chosen because they contain excipients with different functions: hydrochloride has to preserve and to stabilize active ingredient amiodarone and citrate has enhance assimilation of tamoxifen by increasing its solubility (and so its bioavailability).

In this article, are shown exposure results of three tested organisms to the four presented substances (tamoxifen, tamoxifen citrate, amiodarone and amiodarone chlorhydrate). Results are then discussed in order to learn firsts lessons about necessity to take into account the real nature of compound emitted and found into environment.

2. Material and method

2.1. Algal cultures

Pseudokirchneriella subcapitata (Ps), green algae (*Chlorophyceae*), was cultivated under 3000 lux (16 h/day) at 20 ± 1 °C in oligo L.C. medium(AFNOR, 1980). Media and flasks were autoclaved (120°C, 1 bar, 20 min) before inoculation.

2.2. Rearing of daphnids

Daphnids (*Daphnia magna*) were reared in M4 medium (Elendt and Bias, 1990). Thirty neonates daphnids were kept in 2L glass flasks at 20± 1 °C under 500 lux (16 h/day); they were fed with a solution of *P. subcapitata* (10^6–10^7 cells/daphnid) added daily to the culture flasks. Neonates were collected daily and used in tests or discarded. Half of the medium was renewed once a week. Mother daphnids were discarded after 1month and new cultures were initiated with neonates obtained by parthenogenesis.

2.3. Chemicals

Tamoxifen (CAS N° 10540-29-1), tamoxifen citrate (CAS N° 54965-24-1) and amiodarone hydrochloride (CAS N° 19774-82-4) used in this study were purchased from Sigma-Aldrich Chemical (ST. QUENTIN FALLAVIER, France). Stock solutions were prepared in DMSO with concentration of 18.6 mM for tamoxifen (6.91g/L of tamoxifen and 10.48 g/L of tamoxifen citrate) and 29.33 mM for amiodarone (18.93 g of amiodarone/L and 20 g of amiodarone HCl/L) and then kept at -20°C in glass vials. For each exposure, test solutions were freshly prepared by proportional successive dilutions of an aliquot of the stock solutions.

2.4. Purification of amiodarone hydrochloride

A solution of Amiodarone hydrochloride (2.5 g, 3.76 mmol) in dichloromethane (50 mL) was washed with a saturated aqueous $NaHCO_3$ solution (100 mL). The aqueous layer was extracted three times with dichloromethane (3x15 mL). The organic layers were combined, washed with brine, dried overs Na_2SO_4 and evaporated to afford Amiodarone base as pale yellow oil (2.245 g, 3.47 mmol, 92%).^{1}H NMR (300 MHz, $CDCl_3$): 8.20 (s, 2H), 7.53-7.39 (m, 2H), 7.35-7.20 (m, 2H), 4.14 (t, 2H, J = 6.8 Hz), 3.09 (t, 2H, J = 6.6 Hz), 2.84 (t, 2H, 7.5 Hz), 2.72 (q, 4H, J = 7.2 Hz), 1.77 (quint., 2H, J = 7.7 Hz), 1.36 (hex, 2H, J = 7.3 Hz), 1.12 (t, 6H, J = 7.2 Hz), 0.91. ^{13}C NMR (75 MHz, $CDCl_3$): 187.8 (Cq), 166.1 (Cq), 161.5 (Cq), 153.7 (Cq), 140.7 (2CH), 138.3 (Cq), 126.4 (Cq), 124.7 (CH), 123.9 (CH), 121.1 (CH), 115.9 (Cq), 111.1 (CH), 90.9 (2Cq), 71.4 (CH_2), 51.1 (CH_2), 47.7 ($2CH_2$), 30,0 (CH_2), 28.2 (CH_2), 22.6 (CH_2), 13.8 ($2CH_3$), 12.0 (CH_3). M.S. (ESI, m/z): 646.0 $[M+H]^+$, HRMS: calcd. For $C_{25}H_{30}I_2NO_3^+$ 646.0310 found 646.0335.

2.5. Ecotoxicity tests

2.5.1. Algal growth tests

The growth tests performed were selected according to OECD guideline N° 201 (OECD, 2006) on *Pseudokirchneriella subcapitata*. Algae were seeded on 48 well microplates at a concentration of 10^4 cells per mL in their specific culture medium in contact with different concentrations of pharmaceuticals after 3 days preconditioning under the test conditions, i.e. in a climatic chamber (Aralab Fitoclima D 1200), 20 ± 1°C under continuous lighting of 10 000 lux. The range of tested concentrations went from 0 to 100 mg/L for amiodarone and amiodarone HCl and between 3 and 1600 µg/L for tamoxifen and tamoxifen citrate. Three replicates were prepared for each concentration. In parallel, a control with the highest concentration of DMSO introduced in the wells (0.5%) was prepared to ensure the absence of effect of the solvent on algal growth. To limit evaporation in the wells during the experiment, susceptible to modify the tamoxifen concentration, ultrapure water was placed in the outer wells of the plate. Seventy two hours after inoculation the content of the wells was homogenized and algal density was measured with a particle counter (Coultronics, model Z1, threshold size 3.6 µm). In order to have a better accuracy, these tests were done twice for each molecule and all values for each concentration were averaged. Inhibition concentrations 50% (IC50) were then calculated using the RegTox software.

2.5.2. Daphnid mortality tests

The daphnid mortality test was carried out according to the ISO standard protocol (ISO, 1995). Five neonate daphnids (<24 h) were transferred into a glass test tube containing 10 ml of a tamoxifen solution freshly prepared in the rearing medium. The tested concentrations were include between 0 to 250 mg/L for amiodarone and amiodarone HCl and between 0.024 and 12.5 mg/L for tamoxifen and tamoxifen citrate. Four replicate tubes were tested per concentration (20 daphnids). In order to prevent algal growth, tubes were incubated in the dark during the exposure period. In order to have a better accuracy, these tests were done twice for each molecule and all values for each concentration were averaged. After 24h and 48h of exposure, the number of dead daphnids in each tube was recorded and 48h-EC50 was calculated using a probit model.

2.5.3. Bioluminescence inhibition on *Vibrio fisheri*

These tests were performed by PROVADEMSE, directed by Christine BAZIN, according to the standard NF ISO 11348-3. This test consist in measure how much a substance inhibit luminescence of bacteria *V. fisherii*. For that, bacteria were exposed to a range on contaminant at 15°C ± 1°C. After 30 minutes, luminescence were measured (analyzer Microtox M500) and compared to the control unexposed.

2.5.4. Solvents ecotoxicity

For all ecotoxicity experiments, the DMSO ecotoxicity was tested, considering the highest exposure concentration.

3. Results

Any ecotoxicity of DMSO was detected for all tested organisms at exposure concentrations (0.5% of DMSO for Ps and Dm and 1% for Vf).

3.1. Ecotoxicity of amiodarone HCl and amiodarone

3.1.1. Inhibition of algal growth

For each tested compound, amiodarone and amiodarone hydrochloride, 3 tests of algal growth were performed. Inhibition growth of these tests were "pooled" in order to obtain only one IC50 (50% inhibition concentration) for each compound (Table 1).

Considering confidence intervals of EC50 for amiodarone and amiodarone HCl, we can say that amiodarone is as much ecotoxic as amiodarone HCl.

3.1.2. Dahpnids mortality tests

For each studied compound, amiodarone and amiodarone HCl, 3 mortality tests were performed. In experiment conditions, we couldn't put into evidence ecotoxicity of these two compounds regarding daphnids mortality. Indeed, we never reach 100% of mortality with range of tested concentrations which expend until 250 mg/L. Beyond this value, solvent burden in solution will

be toxic for organisms and will lead to daphnids mortality. However, with our results, we can say that EC50 is higher than 28 mg/L. Thus, acute ecotoxicity of amiodarone HCl and its active ingredient can be consider as low to daphnids.

3.1.3. Bioluminescence inhibition of *Vibrio fisheri*

Assays have shown a much lower ecotoxicity for both tested molecules with EC50 30min of 12.8 mg/L and 13.6 mg/L.

Table 1: Effect concentrations (EC in µg/L) of amiodarone and amiodarone HCl on tested organisms with interval confidence 95% (IC95% in µg/L; NA: Non applicable).

	Amiodarone		Amiodarone HCl	
	(µg of amiodarone /L)			
	EC50	IC 95%	EC50	IC 95%
P. subcapitata 72h	870[a]	[670 - 1173]	1068[a]	[799 - 1415]
D. magna 48h	> 28000	NA	> 28000	NA
V. fisheri 30 min	12800[b]	[11980- 13760]	13610[b]	[11460- 15420]

3.2. Ecotoxicity of tamoxifen citrate and tamoxifen

For all tested organisms, we put into evidence an ecotoxicity of tamoxifen citrate higher than for tamoxifen alone. For Ps, we observed EC50 of 702 and 509 µg/L respectively for tamoxifen and tamoxifen citrate. For Dm, the gap between ecotoxicity of these two compounds is much higher with respectively EC50 of 696 and 201 µg/L for tamoxifen and tamoxifen citrate. Finally, for Vf, we have the same order of gap than for Dm with EC50 four times higher for tamoxifen (CE50 = 8,2 mg/L) than for tamoxifen citrate (CE50 = 2,1 mg/L) (Table 2).

Table 2: Effect concentrations (EC in µg/L) of tamoxifen and tamoxifen citrate on tested organisms with interval confidence 95% (IC95%: µg/L).

	Tamoxifen		Tamoxifen citrate	
	(µg of tamoxifen /L)			
	EC50	IC 95%	EC50	IC 95%
P. subcapitata 72h	702[c]	[681 - 787]	509[e]	[470 - 558]
D. magna 48h	696[c]	[660 - 830]	201[f]	[166 - 250]
V. fisheri 30 min	8200[d]	[7310 - 8340]	2118[g]	[1663 - 2567]

4. Discussion

4.1. Indirect ecotoxicity of citrate

In this work, we have shown that, active ingredient ecotoxicity and drug ecotoxicity are not necessary equal. Indeed, tamoxifen citrate was more ecotoxic for all tested organisms than tamoxifen. In Medicine, citrate is used as an excipient with tamoxifen for increasing its solubility in blood and so enhanced its assimilation by body. It is strongly likely that citrate played the same role inside our ecotoxicity tests. In 2006, Carlsson et al. demonstrated that excipients, when released in the environment, could be harmful for ecosystems. Nevertheless, citrate is not one of these potentially harmful excipient. Indeed, ecotoxicity tests have already been performed with citrate. Ecotoxicity values are much higher than concentration of excipient used in our tests with LOEC from 80 to 485 mg/L for unicellular algae and 1.5 g/l for *D. magna* (US EPA ECOTOX Database). Thus, higher ecotoxicity of tamoxifen citrate is likely due to to effect of citrate on bioavailability of tamoxifen.

Concerning amiodarone HCl, we couldn't put into evidence excipient effect on ecotoxicity. Hydrochloride is an excipient used for preservation and stability of active ingredient. Its strong acidity (pKa < 0) had no effect on tested organisms because of its low concentration in exposure medium leading to a pH close to neutrality. We can also wondering about bioavailability of amiodarone chlorhydrate. Indeed, this latter could be increased by turning active ingredient into its salt, leading to an increase of the compound in blood. However at physiological pH, amiodarone is principally observed as protoned form (salt), implying no bioavailability difference between the free amine and its salt.

4.2. Ecotoxicological risks of amiodarone and tamoxifen

Before this work, any ecotoxicity tests had been performed about amiodarone. Well, even if rarely measured and never measured in environment, this contaminant have been identified as presenting ecotoxicological risks for environment because of its potential bioaccumulability (Jean et al., 2012) and because of emitted quantity to environment (Escher et al., 2011). Indeed, Escher et al. (2011) have detected amiodarone inside an hospital effluent at concentration up to 800 ng/L and Jean et al., (2012) have reported theoretical bioconcentration up to 10^6 depending on pH. Otherwise, amiodarone have recently been detected inside wastewater treatment plants (WWTP) effluents at concentration up to 1 µg/L (Fedorova et al., 2014) and inside WWTP sludge at content close to 300 ng/g. It is now necessary to increase knowledge about chronic ecotoxicity of these molecules in order to make a better assessment of ecotoxicological risks linked to the

presence of this PC in ecosystems. For that, perform chronic ecotoxicity bioassays is a first step unavoidable.

Concerning tamoxifen (active ingredient without citrate), we demonstrated in previous works that its ecotoxicity, regarding environmental concentrations (4 rivers analyzed in the world) could lead in some situation to significant ecotoxicological risks (Orias et al., 2015). Hence the interest to have a good ecotoxicity characterization of environmental contaminants.

5. Conclusion and perspectives

Our work demonstrate the interest to take into account excipient effect during ecotoxicity characterization of drugs. Indeed, we saw that with tamoxifen, ecotoxicity can be highly modified by excipient.

Otherwise, in this study, we performed the first ecotoxicity tests on amiodarone, a lack regretted by several authors (Escher et al., 2011; Le Corre et al., 2012). It is now necessary to deepen the characterization of the ecotoxicity of amiodarone by making chronic ecotoxicity tests. Moreover, it is also necessary to develop environmental measurements of amiodarone still too rare.

Results of the last ecotoxicity value of tamoxifen citrate confirm conclusions of recent works concerning in-depth characterization of ecotoxicological risks of tamoxifen (Orias et al, 2015). However, chronic tests on tamoxifen are still expected in order to have accurate this characterization.

Direct ecotoxicity of these compound getting better characterize, it is now interesting to improve knowledge about indirect ecotoxicity linked to these PCs, because of its strong potential to bioaccumulate in biota. Indeed, tamoxifen and amiodarone are a part of molecules identified as especially bioaccumulative (Jean et al., 2012).

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References

AFNOR, 1980. Détermination de l'inhibition de la croissance de Scenedesmus subspicatus par une substance.

Burridge, L., Weis, J.S., Cabello, F., Pizarro, J., Bostick, K., 2010. Chemical use in salmon aquaculture: A review of current practices and possible environmental effects. Aquaculture 306, 7–23. doi:10.1016/j.aquaculture.2010.05.020

Carlsson, C., Johansson, A.-K., Alvan, G., Bergman, K., Kühler, T., 2006. Are pharmaceuticals potent environmental pollutants?: Part II: Environmental risk assessments of selected pharmaceutical excipients. Sci. Total Environ. 364, 88–95. doi:10.1016/j.scitotenv.2005.06.036

Collado, N., Rodriguez-Mozaz, S., Gros, M., Rubirola, A., Barceló, D., Comas, J., Rodriguez-Roda, I., Buttiglieri, G., 2014. Pharmaceuticals occurrence in a WWTP with significant industrial contribution and its input into the river system. Environ. Pollut. 185, 202–212. doi:10.1016/j.envpol.2013.10.040

Elendt, B.P., Bias, W.R., 1990. Trace nutrient deficiency in Daphnia magna cultured in standard medium for toxicity testing. Effects of the optimization of culture conditions on life history parameters of D. magna. Water Res. 24, 1157–1167. doi:10.1016/0043-1354(90)90180-E

Escher, B.I., Baumgartner, R., Koller, M., Treyer, K., Lienert, J., McArdell, C.S., 2011. Environmental toxicology and risk assessment of pharmaceuticals from hospital wastewater. Water Res. 45, 75–92. doi:10.1016/j.watres.2010.08.019

Fedorova, G., Golovko, O., Randak, T., Grabic, R., 2014. Storage effect on the analysis of pharmaceuticals and personal care products in wastewater. Chemosphere 111, 55–60. doi:10.1016/j.chemosphere.2014.02.067

ISO, 1995. Qualité de l'eau - Détermination de la mobilité de Daphnia magna Straus, Cladocera, Crustacea, essai de toxicité aiguë.

Jean, J., Perrodin, Y., Pivot, C., Trepo, D., Perraud, M., Droguet, J., Tissot-Guerraz, F., Locher, F., 2012. Identification and prioritization of bioaccumulable pharmaceutical substances discharged in hospital effluents. J. Environ. Manage. 103, 113–121. doi:10.1016/j.jenvman.2012.03.005

Kidd, K.A., Blanchfield, P.J., Mills, K.H., Palace, V.P., Evans, R.E., Lazorchak, J.M., Flick, R.W., 2007. Collapse of a fish population after exposure to a synthetic estrogen. Proc. Natl. Acad. Sci. 104, 8897–8901. doi:10.1073/pnas.0609568104

Kolodziej, E.P., Harter, T., Sedlak, D.L., 2004. Dairy wastewater, aquaculture, and spawning fish as sources of steroid hormones in the aquatic environment. Environ. Sci. Technol. 38, 6377–6384.

Langford, K.H., Thomas, K.V., 2009. Determination of pharmaceutical compounds in hospital effluents and their contribution to wastewater treatment works. Environ. Int. 35, 766–770. doi:10.1016/j.envint.2009.02.007

Le Corre, K.S., Ort, C., Kateley, D., Allen, B., Escher, B.I., Keller, J., 2012. Consumption-based approach for assessing the contribution of hospitals towards the load of pharmaceutical residues in municipal wastewater. Environ. Int. 45, 99–111. doi:10.1016/j.envint.2012.03.008

Montes, R., Rodríguez, I., Casado, J., López-Sabater, M.C., Cela, R., n.d. Determination of the cardiac drug amiodarone and its N-desethyl metabolite in sludge samples. J. Chromatogr. A. doi:10.1016/j.chroma.2015.03.024

OECD, 2006. OECD GUIDELINES FOR THE TESTING OF CHEMICALS : Freshwater Alga and Cyanobacteria, Growth Inhibition Test.

Orias, F., Bony, S., Devaux, A., Durrieu, C., Aubrat, M., Hombert, T., Wigh, A., Perrodin, Y., 2015. Tamoxifen ecotoxicity and resulting risks for aquatic ecosystems. Chemosphere 128, 79–84. doi:10.1016/j.chemosphere.2015.01.002

US EPA ECOTOX Database http://cfpub.epa.gov/ecotox/ last access 23/03/15

5.3 Synthèse

On remarque que l'excipient du tamoxifen, le citrate, a un effet amplificateur sur l'écotoxicité du tamoxifen. L'hydrochloride en revanche n'a pas d'effet significatif sur l'écotoxicité de l'amiodarone. Ces travaux montrent que l'excipient peut jouer un rôle significatif sur l'écotoxicité d'un principe actif. De plus, cela confirme que le tamoxifen est la molécule la plus écotoxique parmi les molécules prioritaires présélectionnées. C'est pourquoi nous allons évaluer le risque de la présence de cette molécule dans les écosystèmes aquatiques.

Chapitre 6

Evaluation du risque écotoxicologique du tamoxifen

6.1 Enjeux et démarche

Entre les données obtenues à travers nos différents tests d'écotoxicité et les données disponibles dans la littérature, nous sommes en mesure d'établir une PNEC consolidée du tamoxifen. En comparant cette valeur aux concentrations déjà mesurées dans différentes rivières sur le plan international (MEC), il est possible d'évaluer le risque écotoxicologique que représente le tamoxifen pour les écosystèmes aquatiques concernés. Le résultat de ce travail est présenté dans l'article suivant.

6.2 Article 4 : *Tamoxifen ecotoxicity and resulting risks for aquatic ecosystems*

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Tamoxifen ecotoxicity and resulting risks for aquatic ecosystems

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HIGHLIGHTS

- First ecotoxicity tests of tamoxifen on algae.
- A PNEC of tamoxifen for continental aquatic ecosystems of 81 ng L^{-1}.
- Risk quotients of tamoxifen calculate for four situations.
- Tamoxifen could present a significant environmental risk for aquatic ecosystems.

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ABSTRACT

Tamoxifen, a drug used to treat cancer, is regularly found in hydrosystems at concentrations of several hundred ng L^{-1}. To characterize its ecotoxicity, we implemented a battery of bioassays on organisms belonging to 3 different trophic levels: *Pseudokirchneriella subcapitata*, *Chlorella vulgaris* and *Chlamydomonas reinhardtii*, for primary producers, *Daphnia magna* (immobilization, grazing and reproduction) for primary consumers, and *Danio rerio* for secondary consumers (embryotoxicity test). In view of the results obtained and the ecotoxicity values of tamoxifen available in the literature, we established a PNEC (Predictive No Effect Concentration) equal to 81 ng L^{-1} for continental water. This PNEC allowed us to calculate Risk Quotients (RQ) for 4 continental hydrosystems in 4 different countries in which measures of tamoxifen had already been performed on surface waters. In two of the situations studied, RQs were higher than 1, reaching a maximum of 2.6. These results show the need to deepen the characterization of ecotoxicological risks linked to the discharge of tamoxifen in surface waters. In addition, we propose applying this approach to other drug residues detected in the environment.

1. Introduction

Drugs and their residues are now recognized as ubiquitous in ecosystems: the hydrosphere (surface water (Heath et al., 2010), deep water (Loos et al., 2010), drinking water (Gibs et al., 2007)), the geosphere (Silva et al., 2011; Yang et al., 2011) and the biosphere (via bioconcentration, bioaccumulation and bioamplification phenomena) (Lajeunesse et al., 2011; Zenker et al., 2014). The main sources of these generalized contaminations are: (i) urban effluents (domestic and hospital) (Kümmerer, 2009; Verlicchi et al., 2010) treated by sewage plants (WWTP) that are often poorly adapted to ensure the total degradation of these compounds ((Ternes, 1998); (Heberer, 2002); (Joss et al., 2005)); (ii) pharmaceutical plants (Larsson et al., 2007; Collado et al., 2014); and (iii) domestic livestock breeding and aquaculture (Halling-Sørensen et al., 1998; Lalumera et al., 2004).

Among the many drugs emitted into the environment, our attention was drawn to tamoxifen. It is an anti-estrogenic compound used to treat hormone-dependent breast cancer. This compound has been detected in hospital (Langford and Thomas, 2009) and urban effluents (Zhou et al., 2009) at concentrations reaching 740 ng L^{-1}, as well as in surface waters at concentrations reaching 212 ng L^{-1} (Zhang et al., 2013). This compound has also been found in the sediment compartment (212–431 ng g^{-1}; (Yang et al., 2011)). However, only few ecotoxicity data are available concerning tamoxifen (see Table 4).

So, in the first part of this work, tamoxifen toxicity was characterized for organisms representing different trophic levels of aquatic ecosystems (primary producers, primary consumer and secondary consumer): (i) three different algae species (growth inhibition tests); (ii) A microcrustean (acute, sub-chronic

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and chronic ecotoxicity tests); (iii) A fish species (embryotoxicity test).

Then, the PNEC (Predictive No Effect Concentration) of tamoxifen was calculated for continental hydrosystems on the basis of the toxicity results obtained in this study and the data available in the literature.

Lastly, the evaluation of ecotoxicological risks related to the presence of tamoxifen in the environment was then established by comparing the PNEC established with Measured Environmental Concentrations (MEC) in different situations described in the literature (Roberts and Thomas, 2006; Coetsier et al., 2009; Yang et al., 2011; López-Serna et al., 2012), which allowed formulating a Risk Quotient (RQ) for each situation studied.

2. Materials and methods

2.1. Algal cultures

Pseudokirchneriella subcapitata (Ps), *Chlorella vulgaris* (Cv) and *Chlamydomonas reinhardtii* (Cr), 3 green algae (*Chlorophyceae*), were cultivated under 3000 lux (16 h d^{-1}) at 20 ± 1 °C in oligo L.C. medium (AFNOR, 1980) (for Ps and Cv) and in TAP (TRIS acetic acid phosphate) medium (for Cr) (Gorman and Levine, 1965). Media and flasks were autoclaved (120 °C, 1 bar, 20 min) before inoculation.

2.2. Rearing of daphnids

Daphnids (*Daphnia magna*) were reared in M4 medium (Elendt and Bias, 1990). Thirty individuals were kept in 2 L glass flasks at 20 ± 1 °C under 500 lux (16 h d^{-1}); they were fed with a solution of *P. subcapitata* (10^6–10^7 cells/daphnid) added daily to the culture flasks. Neonates were collected daily and used in tests or discarded. Half of the medium was renewed once a week. Mother daphnids were discarded after 1 month and new cultures were initiated with neonates obtained by parthenogenesis.

2.3. Chemicals

Tamoxifen (CAS Number 10540-29-1) and all the chemicals used in this study were purchased from Sigma–Aldrich Chemical (ST. QUENTIN FALLAVIER, France). A stock solution of tamoxifen was prepared in DMSO (50 mM or 18.575 g L^{-1}) and kept at −20 °C. For each exposure, test solutions were freshly prepared by proportional successive dilutions of an aliquot of the stock solutions so that DMSO concentration was always less than 0.01% (v/v) in the tested solutions.

2.4. Algal growth tests

The growth tests performed on the 3 algae were selected according to OECD guideline N° 201 (OECD, 2006). The algae were seeded on 48 well microplates at a concentration of 10^4 cells per mL (Cv, Ps) or 10^3 cells per mL (Cr) in their specific culture medium in contact with different concentrations of tamoxifen after 3 d preconditioning under the test conditions, i.e. in a climatic chamber (Aralab Fitoclima D 1200), 20 ± 1 °C under continuous lighting of 10000 lux. The range of tamoxifen concentrations tested was as follows: 0, 10, 50, 100, 200, 300, 400, 500, 600, 800 and 1600 µg L^{-1}. Three replicates were prepared for each concentration. In parallel, a control with the highest concentration of DMSO introduced in the wells (0.01%) was prepared to ensure the absence of effect of the solvent on algal growth. To limit evaporation in the wells during the experiment, susceptible to modify the tamoxifen concentration, ultrapure water was placed in the outer wells of the plate. Seventy-two hours after inoculation the content of the wells was homogenized and algal density was measured using a Malassez cell counter (2 counts per well) and a Zeiss microscope, magnification ×400. Inhibition concentrations 50% (IC50) were then calculated using the RegTox software.

2.5. Daphnid toxicity tests

2.5.1. Acute toxicity: immobilization test

The daphnid immobilization test was carried out according to the ISO standard protocol (ISO, 1995). Five neonate daphnids (<24 h) were transferred into a glass test tube containing 10 ml of a tamoxifen solution freshly prepared in the rearing medium. The concentration range tested was: 0, 100, 200, 400, 800, 1600, 3200, 6400, and 12800 µg L^{-1}. Four replicate tubes were tested per concentration (20 daphnids). In order to prevent algal growth, tubes were incubated in the dark during the exposure period. After 24 h and 48 h of exposure, the number of immobilized daphnids in each tube was recorded and 48 h-EC50 was calculated using a probit model.

2.5.2. Subchronic toxicity: grazing activity

Daphnia grazing activity was measured under static conditions (no algal growth) over a period of 48 h, according to Clément and Zaid (2004). The experiment was carried out in 250 ml glass beakers containing 150 ml of rearing medium, five daphnids (aged between 3 and 4 d at the start of test) and 500000 *P. subcapitata* cells/ml. Fives replicates per treatment were tested with the following tamoxifen concentration range: 0, 5, 25, 50, 100 and 150 µg L^{-1}. For each concentration tested, two beakers without daphnids were used as controls to evaluate possible algal growth. All the beakers were placed in the dark at 20 ± 1 °C and were gently shaken three times a day to ensure mixing of the algal suspensions in order to improve cell availability for daphnids. Residual algal densities were measured after 24 and 48 h using a particle counter (Coultronics, model Z1, threshold size 3.6 µm). Daphnid grazing activity was evaluated by calculating the filtration rate (F, in ml/daphnid/h) according to Gauld (Gauld, 1951):

$$F = (V/n)[\ln(C_0) - \ln(\mathrm{Ct})/t - A]$$

$$A = [\ln(C_0) - \ln(C'_t)]/t$$

where V (ml) is the total volume of medium, n the number of daphnids in volume V, C_0 and Ct are initial and final algal densities (cells/mL) and t the exposure duration time (duration of experiment in hours). A is a correction factor used to take into account changes in the algae density controls (algae without daphnids) expressed as the final algal density C'_t.

No mortality was observed during the experiments, whatever the tamoxifen concentration.

2.5.3. Chronic toxicity: reproduction test

To test the chronic toxicity of tamoxifen on the reproduction of *D. magna*, we referred to guideline 211 of the OECD (OECD, 1998). The daphnids aged less than 24 h were placed for 21 d in 100 mL beakers containing 80 mL of rearing medium (1 daphnid per beaker and 10 beakers per concentration) contaminated as a function of the following range of tamoxifen concentrations: 0, 5, 15, 30, 60 and 120 µg L^{-1}. The daphnids were fed once a week with an algal suspension of *P. subcapitata* so that algal density was in the region of 10^6 cells mL^{-1}. The medium was renewed twice a week. The number of young daphnids produced was counted daily.

2.6. Fish embryo test

The fish embryo test used here as an alternative to the 96 h fish acute toxicity assay was carried out according to OECD guidelines,

Table 1
Measured Environmental Concentrations (MEC) of tamoxifen in various situation.

Country	Rivers	Concentrations (ng L^{-1})		References
		Min	Max	
Spain	Ebro River	12.4	20.1	López-Serna et al. (2012)
China	Yangtze Estuary	120	129	Yang et al. (2011)
UK	Lower Tyne River	27	212	Roberts and Thomas (2006)
France	Gardon River	<5.8	25	Coetsier et al. (2009)

with the embryos immersed in the test solutions before cleavage of the blastodisc, i.e. less than 4 hpf (OECD, 2013). Zebrafish (*Danio rerio*) embryos (20 per concentration) were exposed in the dark for 96 h at 26 °C to tamoxifen prepared from a stock solution made in DMSO (18.575 g L^{-1}) then diluted in ISO water. The range of tamoxifen concentrations studied in ISO water was 50, 100, 200, 300, 400, 500, 600, 1000, 1400 and 1850 μg L^{-1}. A negative control consisted in exposing embryos to ISO water alone, and the experimental design included a solvent control (DMSO 0.01% v/v in ISO water). The positive control corresponded to embryo exposure to 3.7 mg L^{-1} of 3,4-dichloroaniline (3,4-DCA). Embryos were observed every day for 4 d and the dead ones were removed and counted.

2.7. Calculation of Risk Quotients (RQ)

The simplest and most widespread method for qualifying ecotoxicological risk linked to the exposure of an aquatic ecosystem to a given pollutant (in this case tamoxifen) is the calculation of a risk quotient. This quotient corresponds to the ratio of the "pollutant concentration present in the environment", for the scenario studied, divided by the concentration of the same pollutant "with no effect on the target ecosystem" of the scenario. If the RQ value is higher than "1", it is considered that the risk is significant. The greater is the distance of RQ value above "1", the higher is the risk. On the contrary, if the RQ value is lower than "1" the risk is considered to be non significant. Depending on the context and objective of the study, the concentration of the pollutant in the environment can be: (i) predicted, notably by modeling, in which case the term Predictable Environmental Concentration (PEC) is used; (ii) measured directly in the environment of the target ecosystem. In this case, the term Measured Environment Concentration (MEC) is used. In the framework of the present study, we worked with the MEC values obtained by different authors in different countries (Spain, UK, China and France) for different hydrosystems polluted by tamoxifen (Roberts and Thomas, 2006; Yang et al., 2011; López-Serna et al., 2012; Zhang et al., 2013) (Table 1).

The "no effect on the target ecosystem" can be determined using the PNEC (Predictive No Effect Concentration) values available in international databases, or established for the requirements of the study. The PNEC value of tamoxifen, absent from international databases, was calculated according to the rules set out in the Technical Guidance Document of the European Chemical Bureau (European Chemical Bureau (ECB) 2003):

$$PNEC = LAED/EF$$

with PNEC being the Predictive No Effect Concentration, LAED the Lowest Available Ecotoxic Data, and EF the Extrapolation Factor.

This calculation with the results of the ecotoxicity tests performed in the framework of this work, added to the ecotoxicological data available in the literature, makes it possible to calculate a robust PNEC value.

Finally, we calculated the risk quotients corresponding to the four situations found in the literature on the basis of four corresponding MEC values, and the PNEC value formulated, using the following formula:

$$RQ_x = MEC_x/PNEC$$

with MEC_x being the Measured Environmental Concentration in context "*x*", RQ_x the Risk Quotient in context *x*, and PNEC the Predictive No Effect Concentration.

3. Results

3.1. Algal growth tests

The ecotoxicity tests were used to calculate three IC50s for each species of algae (Concentration for 50% inhibition) grouped in Table 2.

The IC50 values present the same order of magnitude for the three species of algae. However, the response of *P. subcapitata* was more variable than the two other species of algae tested, with a much wider confidence interval.

3.2. Daphnid tests

3.2.1. Immobilization test

Detailed data are not provided for the sake of concision and clarity. After 24 h, we observed a significant increase in the immobilization of the daphnia as from the first exposure to tamoxifen concentrations but which never exceeded 60%, thus preventing the calculation of an EC50. However, after 48 h exposure, 95% immobilization was measured starting from 800 μg L^{-1} and 100% at 3200 μg L^{-1} tamoxifen, respectively. This result permitted calculating an EC50 (Immobilization) at 48 h of 210 μg L^{-1} (IC95%: 130–400 μg L^{-1}). Detailed data are provided in Appendix 1.

3.2.2. Grazing test

In this experiment we tested the effect of tamoxifen on the algal grazing activity of *D. magna* by measuring the rate of filtration of the daphnia exposed to an increasing concentration of tamoxifen after 24 and 48 h. The results are described in Fig. 1.

After 24 h exposure, we observed a significant impact on grazing starting from 50 μg L^{-1} tamoxifen. The average rate of filtration decreased in relation to the control by 27% at 50 μg L^{-1}, 22% at 75 μg L^{-1}, 45% at 100 μg L^{-1}, to reach 76% at 200 μg L^{-1}. These results permitted calculating an EC50 at 24 h of 104 μg L^{-1} (IC 95%: 84–124 μg L^{-1}).

Over the period 24–48 h, we first observed a slight hormesis effect on the filtration rate at 25 μg L^{-1} (+18%). Afterwards, the only significant difference of grazing with the control appeared at 200 μg L^{-1}, with an average of 40% inhibition.

It is noteworthy that the doubling of the filtration rate between 24 h and 48 h was due to the growth of the daphnia which is quite considerable at this stage of their development.

3.2.3. Reproduction test

A significant reduction of the reproduction rate was only observed after the daphnia were exposed to 120 μg L^{-1} tamoxifen (Table 3).

Table 2
Tamoxifen inhibition concentration 50% (IC50) for the three algae species Ps, Cv, et Cr with their confidence interval at 95% ($n = 10$).

Algal species	IC50 (μg L^{-1})	Confidence interval 95%
Ps	980	830–1360
Cv	610	540–660
Cr	470	430–510

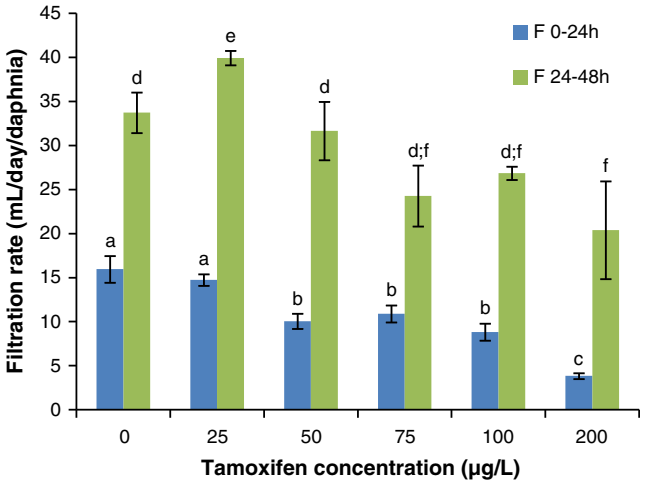

Fig. 1. Filtration rate (F: mL/day/daphnid) of daphnids exposed to tamoxifen (µg L^{-1}) ± standard deviation. Letters indicate a significant difference between concentrations (Student-*t* test, *p* value = 0.05; *n* = 3).

3.3. Fish embryo test

Fig. 2 illustrates the level of mortality encountered in zebrafish embryos. The positive control corresponding to the embryos exposed to 3,4-DCA exhibited an average of 90% mortality. Exposure to tamoxifen did not lead to any significant increase in embryo mortality compared to the control for final tamoxifen concentrations between 0.05 and 1.85 mg L^{-1}. Furthermore, time to hatching was not delayed, with hatchability remaining unchanged, and no major abnormalities were observed whatever the tamoxifen concentration.

3.4. Risk quotient (RQ)

In the framework of this study, four additional organisms (three micro-algae, an invertebrate and a vertebrate) were tested for comparison to data previously available in the literature (Table 4). The ecotoxicity of tamoxifen had already been tested on *D. magna* but we tested two new endpoints (i.e. grazing activity and reproduction). All these data allow us to specify a robust PNEC for tamoxifen in aquatic ecosystems. In particular, the ecotoxicity tests performed on micro-algae provided data for an additional trophic level (primary producers), permitting the reduction of the extrapolation factor from 50 to 10 according to the TGD rules (European Chemical Bureau (ECB), 2003). Thus a PNEC value of tamoxifen of 81 ng L^{-1} was obtained by using the LAED 810 ng L^{-1} for *C. dubia* (Della Greca et al., 2007) with the following calculation: PNEC = 810/10 = 81 ng L^{-1}.

The risk quotients calculated with this new PNEC for different rivers studied in the international literature (Table 4) are presented in Table 5.

This table highlights a significant ecotoxicological risk linked to tamoxifen in the cases of the Yangtze and Lower Tyne rivers, contrary to the cases of the Ebro and Gardon rivers where the RQ is lower than 1.

4. Discussion

4.1. Toxicity of tamoxifen for the different organisms tested

We noted, initially, that under our experimental conditions the 50% inhibition effect produced by tamoxifen on algal growth requires concentrations higher by several orders of magnitude (2000–6000 times the IC50) than those found up to now in the environment. The result of the algae test did not therefore presage an effect of tamoxifen on the compartment of primary producers, at least on the species considered in our test. The low variability of the IC 50 value obtained for each of the 3 species indicated the good reproducibility of the test. It was nonetheless noteworthy that the culture media used (LC and TAP) were enriched with nutritive substances capable of stimulating growth and masking possible toxic effects. It might be interesting to perform a growth test in a more oligotrophic medium (e.g. an ISO medium) to go further in the assessment of the risk of tamoxifen for the algal compartment.

Regarding the reproduction of *D. magna*, the concentrations for which an effect was measured in our works remained fairly high (absence of effect below 120 µg L^{-1}). Previous studies show that daphnia reproduction can, however, be inhibited by lower concentrations. Indeed, Della Greca et al. (2007), obtained an EC50 for the reproduction of *Cerodaphnia dubia* of 0.81 µg L^{-1} tamoxifen. This

Table 3
Number of daphnids produced during 21 d of tamoxifen exposure to increasing tamoxifen concentrations. (a ≠ b Student-*t* Test; *p* value = 0.05).

Tamoxifen concentration (µg L^{-1})	0	5	15	30	60	120
Number of neonates per mother	64.22[a]	59.70[a; b]	62.70[a]	63.90[a]	62.67[a]	55.44[b]
Standard deviation	5.38	4.32	6.09	5.76	3.35	6.27

Table 4
Ecotoxicity data for tamoxifen available in literature and in this study.

Reference	Species		Parameter	Endpoint	Value (µg L^{-1})
EPA ECOTOX DATABASE	*A. tonsa*	Invertebrate	Development	LOEC	49
	X. laevis	Vertebrate	Reproduction (atretic follicle stage)	LOEC	3.7
Della Greca et al. (2007)	*B. calyciflorus*	Invertebrate	Mortality	LC50 24 h	970
			Reproduction	EC50 48 h	250
	T. platyurus	Invertebrate	Mortality	LC50 24 h	400
	D. magna	Invertebrate	Immobilization	EC50 24 h	1530
	C. dubia	Invertebrate	Reproduction	EC50 7 d	0.81
Sun et al. (2007)	*O. latipes*	Vertebrate	Plasmatic vitelogenin	LOEC	1
This study	*P. subcapitata*	Algae	Growth inhibition	IC50	980
	C. culgaris	Algae	Growth inhibition	IC50	610
	C. reinhardtii	Algae	Growth inhibition	IC50	470
	D. magna	Invertebrate	Immobilization	EC50 48 h	210
			Filtration rate	EC50 24 h	104
			Reproduction		
	D. rerio	Vertebrate	Embryo lethality		

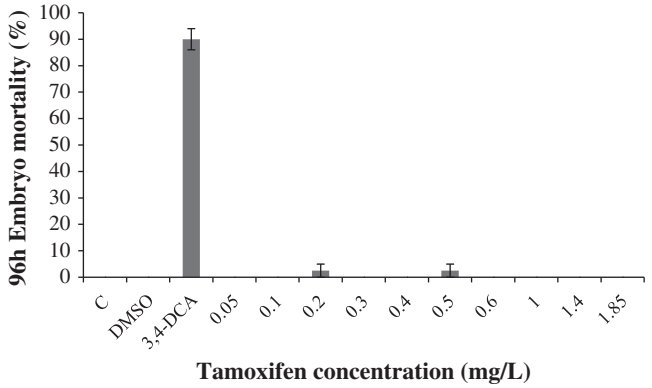

Fig. 2. Percentage of mortality in zebrafish embryos after 96 h of exposure. Each bar corresponds to the mean of 3 different experimentations ± SEM.

Table 5
Risk quotients calculated for the 4 sites studied.

Country	River	Risk quotient Min–Max
Spain	Ebro River	0.153–0.248
China	Yangtze Estuary	1.48–1.59
UK	Lower Tyne River	0.33–2.6
France	Gardon River	<0.07–0.3

difference of sensitivity between the two species of daphnia nonetheless requires verification by other tests. It is noteworthy that the measure of the effect of tamoxifen on daphnia grazing demonstrated that this parameter is more sensitive than the two other tested here. Indeed, we observed significant effects as from 24 h of exposure at relatively low concentrations (50 $\mu g\,L^{-1}$). Bengtsson et al. (2004) also suggested the importance of considering the impact of contaminants on sub-lethal parameters (e.g. grazing, locomotion, etc.).

The lower effect on reproduction after 48 h exposure could have been due to the adsorption of part of the tamoxifen on organic matter accumulating in the medium (i.e. carapace residues, senescent algae, etc.). This adsorption of dissolved tamoxifen could also explain the absence of effect measured in the "chronic daphnia" experiment. The high algal density could have led to the "sequestration" of tamoxifen making it less bioavailable for the daphnia.

Regarding tamoxifen toxicity towards fish embryos, Sun et al. (2007) observed significant effects on hatchability and time to hatching of Japanese medaka exposed to tamoxifen in the concentration range of 125–625 $\mu g\,L^{-1}$, but for a longer exposure time (14 d) than in the present work and with daily renewal of the exposure medium. In another study carried out on sea urchin, Pagano et al. (2001) showed both embryo mortality and developmental defects in a concentration range similar to that used in our study, but with tamoxifen citrate, which is known to be more bioavailable than tamoxifen.

4.2. Ecotoxicological effects linked to tamoxifen

Before our works, no data on the ecotoxicity of tamoxifen for photosynthetic organisms was available in spite of their fundamental role in ecosystem functioning. In addition, only crustaceans (*Brachionus calyciflorus* and *Cerodaphnia dubia*) had undergone chronic tests. However, other ecotoxicity tests have been performed with a compound close to tamoxifen, namely tamoxifen citrate (Williams et al., 2007; Mater et al., 2014). This drug (active principle + excipient) is prescribed to patients with breast cancer. The chemical form of tamoxifen citrate is more soluble in water than that of tamoxifen. This increase in solubility is accompanied with an increase in the bioavailability of tamoxifen, implying consequences for its ecotoxicity. Thus the ecotoxicity values of the drug and its active principle can be potentially different. That is why the ecotoxicity values obtained by these authors were not used to calculate the PNEC of tamoxifen. It is noteworthy that another value from the literature was not used to calculate the PNEC, i.e. ecotoxicity data on *S. Purpuratus* (US EPA database), since the latter is a marine species and we only focused on freshwater species in our calculation of PNEC for continental hydrosystems.

Pharmacodynamics must also be taken into account in the case of drugs. When a patient undergoes medicinal treatment, they are administered the active principle and excipients (e.g. tamoxifen citrate). However, the patient does not excrete the drug (active principle + excipients) but the active principle and its metabolites (e.g., tamoxifen-OH or endoxifen for tamoxifen). Thus, what is found in urban discharges and, finally, in the environment, is the active principle, its metabolites and possibly transformation products. Therefore the ecotoxicity tests performed on drugs (active principles + excipient), are not pertinent as such for assessing ecotoxicological risks linked to a drug. According to us, they should be performed on active ingredients AND their metabolites. Such tests can also be used only in the case where it is proven that the excipient can be excreted.

The data available concerning tamoxifen concentrations measured in the hydrosystems of different countries (Table 4) allowed the calculation of 4 risk quotients (RQ) with two higher than 1 (Table 5). Indeed, in China and United Kingdom, highest RQ's calculated are respectively 1.59 and 2.6. It should be noted that tamoxifen has been little subject to detection in the environment given the scarcity of tamoxifen measures in comparison to other pharmaceutical residues (e.g. diclofenac, ibuprofen, paracetamol, etc.).

Furthermore it should be remembered that the calculation of the PNEC used here (European Chemical Bureau (ECB), 2003) does not take into account indirect ecotoxicity linked to bioaccumulation. However, recent works have identified tamoxifen as being especially bioaccumulable and poorly biodegradable (Jean et al., 2012). Lastly, it is noteworthy that we did not calculate the RQ for sediments in spite of the availability of an environmental measure since no ecotoxicity test on sedimentary organisms (e.g. the ostracod *Heterocypris incongurens* and the dipterous insect *Chironomus riparus*) has ever been performed.

5. Conclusion

The present work first permitted enriching knowledge on the ecotoxicity of tamoxifen. A relatively strong value of its PNEC for freshwater ecosystems (81 $ng\,L^{-1}$) was calculated using the ecotoxicity data obtained. Using this basis, and following a study of the different contexts of contamination taken from the international literature, it was shown that tamoxifen could present a significant ecotoxicological risk for local aquatic ecosystems. In one of the situations studied (Roberts and Thomas, 2006), the Risk Quotient calculated was close to 3.

On the methodological level, in order to improve ecotoxicity tests for very hydrophobic compounds ($\log K_{ow} > 4$), it seems necessary to improve the experimental protocols of the usual standards, in particular for hydrophobic compounds. For example, regarding the chronic ecotoxicity tests, such as the 21-d *D. magna* test, daphnia could be left to develop in a medium without algae. Daphnia could then be fed in isolation during the time required in a medium saturated in algae without contaminant. Thus the contaminant would be less adsorbed on the algae and the daphnia would be exposed to a concentration close to the nominal concentration. Another possibility would be to increase the frequency of renewal of the exposure medium at the beginning of the experi-

ment to "saturate" the adsorption sites of the contaminant on the walls of the containers used.

It is now necessary to implement this type of approach for other pharmaceutical residues present at significant levels in the environment. Indeed, we lack the minimum ecotoxicity data required to assess the risks they present in various contexts (e.g. small and large rivers, major rivers, estuaries, etc.).

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Appendix A. Supplementary material

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References

AFNOR, 1980. Détermination de l'inhibition de la croissance de Scenedesmus subspicatus par une substance.

Bengtsson, G., Hansson, L.-A., Montenegro, K., 2004. Reduced grazing rates in Daphnia pulex caused by contaminants: Implications for trophic cascades. Environ. Toxicol. Chem. 23, 2641–2648.

Clément, B., Zaid, S., 2004. A new protocol to measure the effects of toxicants on daphnid–algae interactions. Chemosphere 55, 1429–1438.

Coetsier, C.M., Spinelli, S., Lin, L., Roig, B., Touraud, E., 2009. Discharge of pharmaceutical products (PPs) through a conventional biological sewage treatment plant: MECs vs PECs? Environ. Int. 35, 787–792.

Collado, N., Rodriguez-Mozaz, S., Gros, M., Rubirola, A., Barceló, D., Comas, J., Rodriguez-Roda, I., Buttiglieri, G., 2014. Pharmaceuticals occurrence in a WWTP with significant industrial contribution and its input into the river system. Environ. Pollut. 185, 202–212.

Della Greca, M., Iesce, M.R., Isidori, M., Nardelli, A., Previtera, L., Rubino, M., 2007. Phototransformation products of tamoxifen by sunlight in water. Toxicity of the drug and its derivatives on aquatic organisms. Chemosphere 67, 1933–1939.

Elendt, B.P., Bias, W.R., 1990. Trace nutrient deficiency in *Daphnia magna* cultured in standard medium for toxicity testing. Effects of the optimization of culture conditions on life history parameters of *D. magna*. Water Res. 24, 1157–1167.

European Chemical Bureau (ECB), I. I. 2003. Technical Guidance Document (TGD) in support of Commission Directive 93/67/EEC on Risk Assessment for new notified substances, Commission Regulation (EC) No 1488/94 on Risk Assessment for existing substances and Directive 98/8/EC of the European Parliament and of the Council concerning the placing of biocidal products on the market.

Gauld, D.T., 1951. The grazing rate of planktonic copepods. J. Mar. Biol. Assoc. U. K. 29, 695–706.

Gibs, J., Stackelberg, P.E., Furlong, E.T., Meyer, M., Zaugg, S.D., Lippincott, R.L., 2007. Persistence of pharmaceuticals and other organic compounds in chlorinated drinking water as a function of time. Sci. Total Environ. 373, 240–249.

Gorman, D.S., Levine, R.P., 1965. Cytochrome f and plastocyanin: their sequence in the photosynthetic electron transport chain of Chlamydomonas reinhardi. Proc. Natl. Acad. Sci. 54, 1665–1669.

Halling-Sørensen, B., Nors Nielsen, S., Lanzky, P.F., Ingerslev, F., Holten Lützhøft, H.C., Jørgensen, S.E., 1998. Occurrence, fate and effects of pharmaceutical substances in the environment – a review. Chemosphere 36, 357–393.

Heath, E., Kosjek, T., Farre, M., Quintana, J.B., de Alencastro, L.F., Castiglioni, S., Gans, O., Langford, K., Loos, R., Radjenović, J., Rocca, L.M., Budzinski, H., Tsipi, D., Petrovic, M., Barcelo, D., 2010. Second interlaboratory exercise on non-steroidal anti-inflammatory drug analysis in environmental aqueous samples. Talanta 81, 1189–1196.

Heberer, T., 2002. Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: a review of recent research data. Toxicol. Lett. 131, 5–17.

ISO, 1995. Qualité de l'eau - Détermination de la mobilité de *Daphnia magna* Straus, Cladocera, Crustacea, essai de toxicité aiguë.

Jean, J., Perrodin, Y., Pivot, C., Trepo, D., Perraud, M., Droguet, J., Tissot-Guerraz, F., Locher, F., 2012. Identification and prioritization of bioaccumulable pharmaceutical substances discharged in hospital effluents. J. Environ. Manage. 103, 113–121.

Joss, A., Keller, E., Alder, A.C., Göbel, A., McArdell, C.S., Ternes, T., Siegrist, H., 2005. Removal of pharmaceuticals and fragrances in biological wastewater treatment. Water Res. 39, 3139–3152.

Kümmerer, K., 2009. The presence of pharmaceuticals in the environment due to human use – present knowledge and future challenges. J. Environ. Manage. 90, 2354–2366.

Lajeunesse, A., Gagnon, C., Gagné, F., Louis, S., Čejka, P., Sauvé, S., 2011. Distribution of antidepressants and their metabolites in brook trout exposed to municipal wastewaters before and after ozone treatment – evidence of biological effects. Chemosphere 83, 564–571.

Lalumera, G.M., Calamari, D., Galli, P., Castiglioni, S., Crosa, G., Fanelli, R., 2004. Preliminary investigation on the environmental occurrence and effects of antibiotics used in aquaculture in Italy. Chemosphere 54, 661–668.

Langford, K.H., Thomas, K.V., 2009. Determination of pharmaceutical compounds in hospital effluents and their contribution to wastewater treatment works. Environ. Int. 35, 766–770.

Larsson, D.G.J., de Pedro, C., Paxeus, N., 2007. Effluent from drug manufactures contains extremely high levels of pharmaceuticals. J. Hazard. Mater. 148, 751–755.

Loos, R., Locoro, G., Comero, S., Contini, S., Schwesig, D., Werres, F., Balsaa, P., Gans, O., Weiss, S., Blaha, L., Bolchi, M., Gawlik, B.M., 2010. Pan-European survey on the occurrence of selected polar organic persistent pollutants in ground water. Water Res. 44, 4115–4126.

López-Serna, R., Petrović, M., Barceló, D., 2012. Occurrence and distribution of multi-class pharmaceuticals and their active metabolites and transformation products in the Ebro River basin (NE Spain). Sci. Total Environ.

Mater, N., Geret, F., Castillo, L., Faucet-Marquis, V., Albasi, C., Pfohl-Leszkowicz, A., 2014. In vitro tests aiding ecological risk assessment of ciprofloxacin, tamoxifen and cyclophosphamide in range of concentrations released in hospital wastewater and surface water. Environ. Int. 63, 191–200.

OECD, 1998. OECD GUIDELINES FOR TESTING OF CHEMICALS: *Daphnia magna* Reproduction Test.

OECD, 2006. OECD GUIDELINES FOR THE TESTING OF CHEMICALS: Freshwater Alga and Cyanobacteria, Growth Inhibition Test.

OECD, 2013. OECD GUIDELINES FOR TESTING OF CHEMICALS: Fish Embryo Acute Toxicity (FET) Test.

Pagano, G., de Biase, A., Deeva, I.B., Degan, P., Doronin, Y.K., Iaccarino, M., Oral, R., Trieff, N.M., Warnau, M., Korkina, L.G., 2001. The role of oxidative stress in developmental and reproductive toxicity of tamoxifen. Life Sci. 68, 1735–1749.

Roberts, P.H., Thomas, K.V., 2006. The occurrence of selected pharmaceuticals in wastewater effluent and surface waters of the lower Tyne catchment. Sci. Total Environ. 356, 143–153.

Silva, B.F.d., Jelic, A., López-Serna, R., Mozeto, A.A., Petrovic, M., Barceló, D., 2011. Occurrence and distribution of pharmaceuticals in surface water, suspended solids and sediments of the Ebro river basin, Spain. Chemosphere 85, 1331–1339.

Sun, L., Zha, J., Spear, P.A., Wang, Z., 2007. Tamoxifen effects on the early life stages and reproduction of Japanese medaka (Oryzias latipes). Environ. Toxicol. Pharmacol. 24, 23–29.

Ternes, T.A., 1998. Occurrence of drugs in German sewage treatment plants and rivers. Water Res. 32, 3245–3260.

US EPA database. http://www.epa.gov/ecotox/; last access 01/03/14.

Verlicchi, P., Galletti, A., Petrovic, M., Barceló, D., 2010. Hospital effluents as a source of emerging pollutants: an overview of micropollutants and sustainable treatment options. J. Hydrol. 389, 416–428.

Williams, T.D., Caunter, J.E., Lillicrap, A.D., Hutchinson, T.H., Gillings, E.G., Duffell, S., 2007. Evaluation of the reproductive effects of tamoxifen citrate in partial and full life-cycle studies using fathead minnows (Pimephales Promelas). Environ. Toxicol. Chem. 26, 695–707.

Yang, Y., Fu, J., Peng, H., Hou, L., Liu, M., Zhou, J.L., 2011. Occurrence and phase distribution of selected pharmaceuticals in the Yangtze Estuary and its coastal zone. J. Hazard. Mater. 190, 588–596.

Zenker, A., Cicero, M.R., Prestinaci, F., Bottoni, P., Carere, M., 2014. Bioaccumulation and biomagnification potential of pharmaceuticals with a focus to the aquatic environment. J. Environ. Manage. 133, 378–387.

Zhang, J., Chang, V.W.C., Giannis, A., Wang, J.-Y., 2013. Removal of cytostatic drugs from aquatic environment: a review. Sci. Total Environ. 445–446, 281–298.

Zhou, J.L., Zhang, Z.L., Banks, E., Grover, D., Jiang, J.Q., 2009. Pharmaceutical residues in wastewater treatment works effluents and their impact on receiving river water. J. Hazard. Mater. 166, 655–661.

6.3 Synthèse

En ne s'intéressant qu'au risque écotoxicologique direct (hors bioaccumulation), on observe que le tamoxifen présente déjà un risque pour les écosystèmes aquatiques. En effet, à plusieurs endroits dans le monde où le tamoxifen a été détecté, le quotient de risque est supérieur à 1.

Dans la partie suivante de ce manuscrit, nous allons mesurer dans quelle mesure le tamoxifen, déjà identifié comme écotoxique, peut se transférer et se concentrer dans une chaîne alimentaire expérimentale.

Troisième partie

ETUDE EXPERIMENTALE DU TRANSFERT TROPHIQUE D'UN RESIDU PHARMACEUTIQUE MODELE

Il est aujourd'hui possible d'évaluer la bioconcentrabilité d'une molécule sur la base de ses propriétés physico-chimiques. Cependant, la bioaccumulation ou la bioamplification sont plus difficiles à aborder par la seule modélisation. Ainsi, pour répondre à la problématique de transfert des RP dans les réseaux trophiques, nous avons mis en place une chaîne alimentaire artificielle et cloisonnée. Nous avons choisi trois espèces modèles représentatives de 3 niveaux trophiques différents (Figure 6.1) que nous avons alternativement exposées ou non au contaminant via l'alimentation et/ou via le milieu (l'eau en l'occurrence) :

- Un **producteur primaire** : *Pseudokirchneriella subcapitata*, une algue unicellulaire de la famille des Chlorophycées.
- Un **consommateur primaire** : *Daphnia magna*, un crustacé de la famille des Cladocères.
- Un **consommateur secondaire** : *Danio rerio*, un poisson tropical de la famille des Cyprinidés.

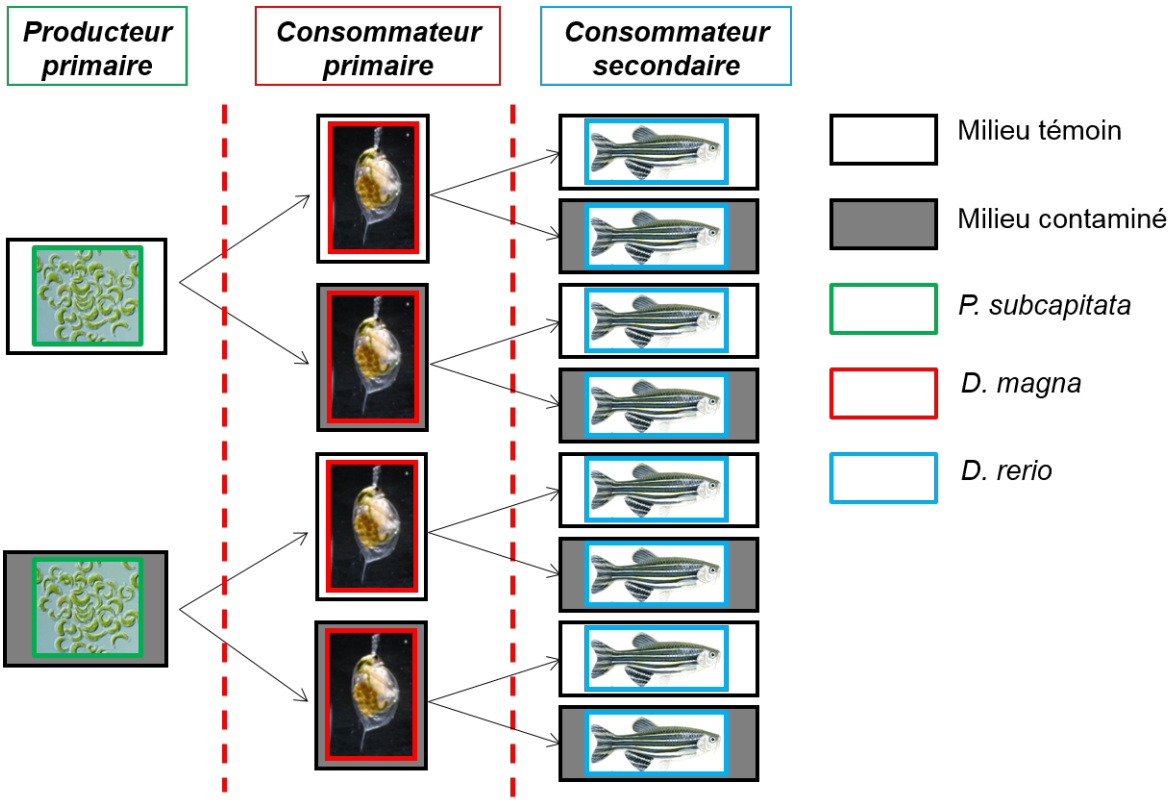


Figure 6.1 *Schéma conceptuel de la chaîne alimentaire artificielle étudiée*

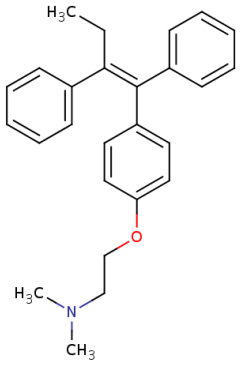
Une fois établie notre chaîne alimentaire modèle, il a fallu déterminer un contaminant modèle. Nous avons choisi le tamoxifen pour 3 raisons majeures : (i) il fait partie de la liste des 14 composés prioritaires (ii) il a déjà été détecté plusieurs fois dans l'Environnement, (iii) il est analysable dans l'eau (chromatographie) et dans les organismes (isotopie).

Le problème majeur auquel nous avons été confrontés au moment d'entamer nos travaux sur la bioaccumulation du tamoxifen était d'ordre analytique. En effet, les protocoles d'extraction et d'analyse sur des matrices biologiques complexes (e.g. algues, cuticule, tissu,...) sont rarement disponibles pour les résidus pharmaceutiques. Au moment d'entamer nos études, ces protocoles n'existaient pas pour le tamoxifen. Au moment où ce manuscrit est rédigé, ces protocoles n'existent toujours pas pour les cellules algales. Ainsi, nous avons développé une autre méthode analytique pour étudier la bioaccumulation du tamoxifen dans toutes les matrices biologiques susceptible d'être exposées dans l'environnement. C'est grâce aux isotopes stables que nous sommes parvenus à surmonter notre contrainte analytique.

Laurent SIMON de l'équipe E3S de l'UMR LEHNA a été un acteur majeur dans le développement de cette méthode. En effet, le tamoxifen existe sous une forme enrichie en ^{15}N sans que ses propriétés biologiques ou physico-chimiques ne soient modifiées (Tableau 6.1). Par ailleurs, au sein des organismes vivants, il existe un ratio naturel entre les différents isotopes stables de l'azote (i.e. $^{15}N/^{14}N$). Ainsi, lorsqu'un organisme est exposé au tamoxifen ^{15}N, nous avons supposé que le ratio $^{15}N/^{14}N$ naturel pouvait être modifié en faveur du ^{15}N. Au regard des différentes contraintes liées à la nature de notre molécule modèle et de notre molécules analytique, les gammes de concentrations d'exposition ont été guidées par les critères suivants :

- **Ne pas dépasser la solubilité** du tamoxifen (167 μg/L) pour ne pas qu'il y ait de précipitation qui pourrait perturber la mesure de bioconcentration.
- **Ne pas induire de toxicité** pour les organismes pour éviter la confusion d'effet entre écotoxicité directe et bioconcentration et/ou bioaccumulation.
- **Ne pas être trop basses** pour que les concentrations soient mesurables par HPLC dans l'eau et par isotopie dans les organismes.

Table 6.1 *Structure et caractéristiques physico-chimiques du tamoxifen*



Nom :	Tamoxifen
N°CAS :	10540-29-1
Classe thérapeutique :	Antinéoplasiques (Classe C)
Solubilité dans l'eau :	167 μg/L
Masse molaire :	371,5146 $\pm$ 0,0233 g/mol
K_{ow} :	7,1
pKa :	8,6

Par ailleurs, au cours de toutes les expérimentations présentées dans cette partie, nous avons fait un effort particulier pour limiter la présence de plastique dans les contenants d'exposition. Cet effort a été particulièrement important pour la dernière expérience où des poissons ont été exposés 21 jours au tamoxifen (Figure 6.2). En effet, nous avons utilisé des aquariums en verre monobloc pour éviter la présence de joint en caoutchouc ou en silicone, nous avons chauffé la chambre d'exposition plutôt que l'eau dans laquelle baignaient les poissons pour limiter l'adsorption sur d'éventuels dispositifs de chauffage immergés et enfin nous avons effectué plusieurs renouvellements du milieu pour maintenir la concentration d'exposition la plus proche possible de la concentration nominale.

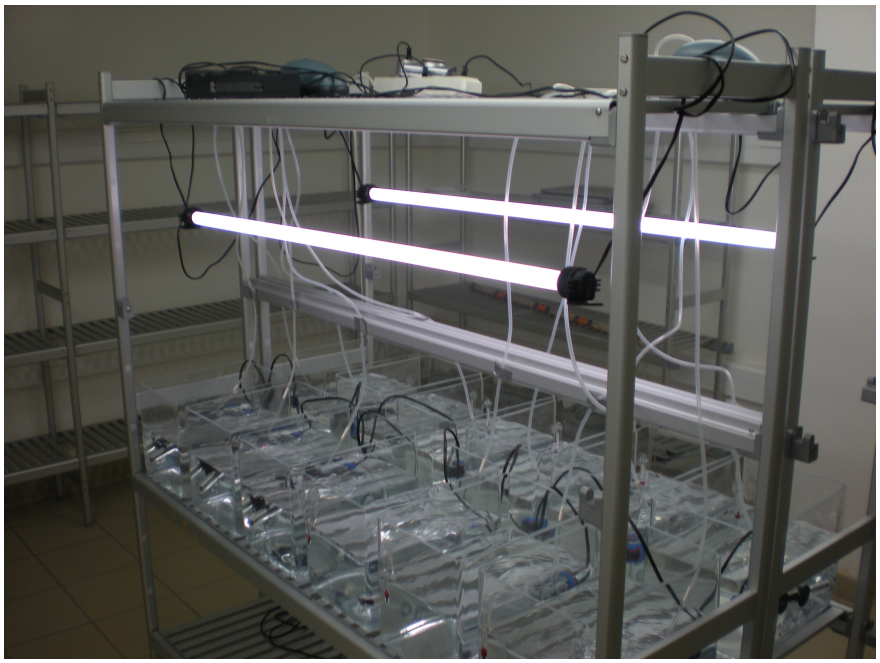


Figure 6.2 *Photographie de l'expérience « bioconcentration poisson » installée à l'Ecoaquatron, sur le site de la Doua à Villeurbanne.*

Dans les chapitres suivants, nous présentons les résultats obtenus concernant la bioconcentration et la bioaccumulation du tamoxifen ^{15}N mesurées grâce à notre méthode isotopique dans les trois organismes présentés ci-avant.

Chapitre 7

Bioconcentration du ^{15}N tamoxifen chez *Pseudokirchneriella subcapitata*

7.1 Enjeux et démarche

Les algues unicellulaires sont le premier maillon de la chaîne alimentaire dans de nombreux écosystèmes aquatiques. Ainsi, nous avons choisi ce premier organisme pour répondre à notre problématique de transfert des RP au sein des écosystèmes. Dans ces premiers travaux, nous avions deux objectifs : (i) déterminer si notre méthode d'analyse utilisant les isotopes stables était viable (ii) déterminer la dynamique de bioconcentration du tamoxifen chez une algue unicellulaire. Pour cela, nous avons exposé des algues 5 jours à plusieurs concentrations en tamoxifen : 1, 10 et 100 μg/L. La teneur dans les algues est ensuite mesurée après quelques minutes d'exposition, à 12h, 24h, 48h, 144h et 168h afin de connaitre la dynamique de bioconcentration dans ces organismes.

7.2 Article 5 :*Experimental assessment of the bioconcentration of ^{15}N-tamoxifen in* Pseudokirchneriella subcapitata

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Experimental assessment of the bioconcentration of ^{15}N-tamoxifen in *Pseudokirchneriella subcapitata*

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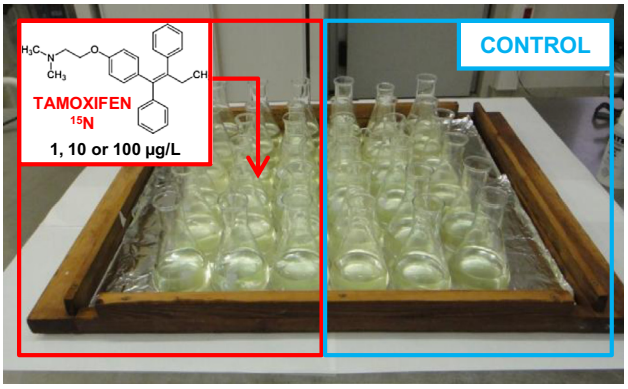
HIGHLIGHTS

- We study the bioconcentration of tamoxifen in algae at several concentrations.
- Tamoxifen concentrations tested are 1, 10 and 100 µg L^{-1}.
- We use stable isotopes (^{14}N/^{15}N) in order to follow the bioconcentration.
- We find a bioconcentration factor up to 27000.

GRAPHICAL ABSTRACT



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ABSTRACT

Nowadays, pharmaceutical compounds (PC) are ubiquitous in aquatic ecosystems. In addition to direct ecotoxicity, the bioconcentration of PC in organisms is a phenomenon which could have an impact on the whole ecosystem. In order to study this phenomenon, we exposed unicellular algae (*Pseudokirchneriella subcapitata*) to ^{15}N-tamoxifen, an anticancer drug labelled with a stable nitrogen isotope used as a tracer. By measuring ^{15}N enrichment over time, we were able to measure the increase of tamoxifen content in algae. This enrichment was measured by an elemental analyser coupled with an isotopic ratio mass spectrometer (EA–IRMS). Algal cells were exposed for 7 d to 3 concentrations of tamoxifen: 1, 10 and 100 µg L^{-1}. Our result shows a high bioconcentration in algae from the first minutes of contact. The highest bioconcentration factor measured is around 26500. We also observe that bioconcentration is not linked to the exposure concentration. This study is the first to use stable isotopes in order to monitor PCs in aquatic organisms such as algae. The use of stable isotopes in ecotoxicology offers interesting perspectives in the field of contaminant transfer in organisms and along the trophic web.

1. Introduction

Nowadays, pharmaceuticals (PCs) are ubiquitous in aquatic ecosystems all over the world: surface water (Roberts and Thomas, 2006; Farré et al., 2008), groundwater (Fick et al., 2009), drinking water (Touraud et al., 2011), sediments (Silva et al., 2011) and biota (Zenker et al., 2014). The main routes of ecosystem pollution by PCs include: pharmaceutical manufacturing (formulation, production, etc.) (Sanchez et al., 2011), manure spreading and fish breeding (Halling-Sørensen et al., 1998; Lalumera et al., 2004), and the discharge of urban effluents (from households and hospitals) (Kümmerer, 2009; Verlicchi et al., 2010). In recent years, improvements made to analytic methods have made it possible to detect

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PCs at ever lower concentrations. At these environmental concentrations, the risk for ecosystems generally is not from direct ecotoxicity but from indirect ecotoxicity via the bioconcentration phenomenon. The PCs generally measured are the most consumed in a given area (Calamari et al., 2003; Wiegel et al., 2004; Loos et al., 2009) while numerous other parameters may be more relevant than drug consumption. Among these parameters are excretion rate of a given drug, PC persistence and bioaccumulability. Indeed, PCs and other emerging ecotoxic contaminants can lead to bioconcentrations high enough to endanger the organisms exposed (Lajeunesse et al., 2011). In a previous study, Jean et al. (2012) determined 14 priority PCs in hospital wastewater on the basis of three criteria: bioaccumulability, biodegradability and their consumption inside hospitals.

These priority PCs include tamoxifen (N° CAS 10540-29-1) which has already been measured in aquatic ecosystems at amounts ranging from a few ng L^{-1} to several hundred ng L^{-1} in surface water, and also at several hundred ng L^{-1} in sediments (Roberts and Thomas, 2006; Coetsier et al., 2009; Yang et al., 2011; López-Serna et al., 2012). Tamoxifen is an anticancer drug used to treat breast cancer. Considering the physicochemical properties of tamoxifen, it can theoretically bioconcentrate 385 000 times in organisms (Jean et al., 2012). Thus it is necessary to study how this kind of contaminant is transferred along the trophic chain.

In this paper, we present the results of an experimental study of the bioconcentration of a pharmaceutical compound, tamoxifen, in the unicellular algae *Pseudokirchneriella subcapitata* (Chlorophyceae), an organism commonly used in ecotoxicology. To our knowledge, this is the first time that isotopic chemistry rather than analytical chemistry and radioactivity has been used to monitor a PC. Indeed, tamoxifen, and PCs in general, are very difficult if not impossible to analyse in a biological matrix using classical analysis methods (e.g. HPLC–MS/MS), especially due to the extraction step. They require large quantities of biomass (at least several mg) and there is often a significant loss of contaminant which prevents working at concentrations close to environmental ones. In order to study bioconcentration, we used ^{15}N tamoxifen which has the same chemical behaviour as classical tamoxifen and which is usually used as a standard in analytical chemistry. Consequently, we could use elementary analysis associated with mass spectrometry (EA–IRMS) to monitor the presence of tamoxifen in algae due to its enrichment by ^{15}N. Thus we were not limited by the nature of the matrix and the sample mass was limited to 1 mg down to 50 µg of dry biomass depending on the nitrogen content.

In this work, we used ^{15}N tamoxifen in order to observe how algae, the first link in numerous food chains, adsorbs and/or absorbs this compound through time. To do this, we exposed algal solutions to 1, 10 and 100 µg L^{-1} of ^{15}N tamoxifen for seven days.

2. Materials and methods

2.1. Algal culture

P. subcapitata (Ps), a green algae (Chlorophyceae), was cultivated under 3000 lux 16 h d^{-1} at 20 ± 1 °C in oligo L.C. medium (AFNOR, 1980). The media and flasks were autoclaved (120 °C, 1 bar, 20 min) before reinoculation. In order to calculate the algal biomass in each replicate, we multiplied the algal density measured with a particle counter (Coultronics, model Z1, threshold size 3.6 µm) by the average weight of an algal cell (20 pg/cell) proposed by the OECD guideline (OECD, 2006).

2.2. Toxicant

Tamoxifen $^{15}N^{13}C_2$ (see Fig. 1) was purchased from Sigma–Aldrich chemical (ST QUENTIN FALAVIER, FRANCE), prepared in methanol (1 g L^{-1}) and kept in glass ampoule at −20 °C.

2.3. Algal exposure to ^{15}N tamoxifen

We managed this exposure in three steps corresponding to the three concentrations tested with a control in each step: (i) 0–100 µg L^{-1}; (ii) 0–10 µg L^{-1} and (iii) 0–1 µg L^{-1}. The exposure medium corresponded to the culture medium but diluted 5 times to enhance the nitrogen contrast. For the concentrations of 100 and 10 µg L^{-1} tested, we directly introduced 15 µL and 1.5 µL of stock solution (1 g L^{-1}), respectively. However, for the 0–1 µg L^{-1} step, we introduced 1.5 mL from a solution with a concentration of 100 µg L^{-1} (in water) in each contaminated Erlenmeyer flask in order to obtain a concentration of 1 µg L^{-1}. It should be noted that the highest concentration of methanol in experiments is 0.01%. For each step, we had 18 contaminated replicas (with concentrations of 1, 10 and 100 µg L^{-1}) and 18 controls without contaminant. Each replica correspond to an Erlenmeyer flask containing 150 mL of medium with an algal density of 600 000 cells mL^{-1} ($90 \cdot 10^6$ cells all in all). The 36 Erlenmeyer flasks were placed on a rotation table at 3000 lux, 20 °C ± 1 °C, with a photoperiod of 16 h d^{-1}. Immediately after exposure, at 12 h, 24 h, 48 h, 144 h and 168 h, three contaminated Erlenmeyer flasks and three controls were removed. We measured the pH and algal density was determined with a particle counter (Coultronics, model Z1, threshold size 3.6 µm). Then, the algae were centrifuged for 20 min at 3000 rpm (1600 G) in glass tubes. The pellets were then transferred inside a plastic 1 mL Eppendorf tube and plunged into liquid nitrogen for five minutes. Finally, the Eppendorf tubes were placed in a lyophilizer for 48 h at −85 °C and 0.01 mbar.

2.4. ^{15}N measurements

After freeze-drying, the algae samples were weighed at 1 mg in tin capsules before analysis. Nitrogen concentration and $^{15}N/^{14}N$ ratio were measured using an isotope ratio mass spectrometer (Isoprime 100, Isoprime Ltd., Manchester, UK) coupled in continuous flow with an elemental analyser (FlashEA 1112 Thermo Electron, Milan, Italy). F-aspartic acid was used to calibrate the N concentration measurements. For the isotope ratio measurements, a two-point normalization of the values measured was performed using international reference material IAEA-N1 and IAEA-305 following IUPAC guidelines. The $^{15}N/^{14}N$ ratio were expressed as $\delta^{15}N$ in ‰ relative to atmospheric N_2 (Air):

$$\delta^{15}N = \left(^{15}N/^{14}N_{sample}/^{15}N/^{14}N_{Air} - 1\right) \times 10^3$$

and converted to atomic fraction (i.e. atomic%, Coplen, 2011):

$$x(^{15}N) = (^{15}N/^{15}N_{sample})/(1 + ^{15}N/^{14}N_{sample})$$

In-house standards calibrated against IAEA-N1 and IAEA-N2 were interspersed within the samples and the standard deviations for the replicate analyses were lower than 0.2‰.

2.5. Calculation of excess ^{15}N and tamoxifen concentration

Excess ^{15}N concentration, [^{15}N] was calculated from the difference between the ^{15}N fraction ($x(^{15}N)$) of algae exposed to

Name:	Tamoxifen
N°CAS:	10540-29-1
Water solubility:	167 µg/L
Kow:	7.1
pKa:	8.6

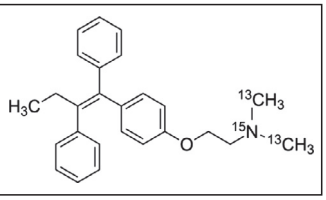

Fig. 1. Physico-chemical properties and topological formula of tamoxifen.

^{15}N-tamoxifen and the ^{15}N fraction ($x(^{15}N)_{control}$) measured in the control treatment, without exposure, as follows:

$$[^{15}N] = (x(^{15}N) - x(^{15}N)_{control}) \cdot [N],$$

where [N] is the N concentration in the sample.

As ^{15}N-tamoxifen was the only ^{15}N tracer source in the experiment, and assuming no significant dissociation of tamoxifen by chemical reactions or metabolic pathways (under our experimental conditions (DellaGreca et al., 2007)), the tamoxifen concentration was calculated from the ^{15}N concentration:

$$[\text{Tamoxifen}](\mu g \cdot g^{-1}) = [^{15}N]/15 \cdot M_{\text{Tamoxifen}} \cdot 10^6.$$

with $M_{\text{tamoxifen}}$ (374.49 g mol^{-1}) being the molar mass of [^{13}C, ^{15}N]-tamoxifen.

A detection limit of tamoxifen in algae of 0.5 μg g^{-1} was calculated using this equation from the detection limit of ^{15}N/^{14}N ratio difference, which is less than 0.2‰.

2.6. Bioconcentration factor calculation

Bioconcentration factors (BCF) are calculated according to the following equation:

$$\text{BCF} = \frac{\text{Tamoxifen content in algae (g kg}^{-1})}{\text{Medium tamoxifen concentration (g kg}^{-1})}$$

3. Results

3.1. Evolution of algal biomass and pH during experiments

Fig. 2 shows the kinetics of the algal biomass through time. Algal growth began with a classically observed exponential phase before stabilisation at 6 d when all the nutrients had been consumed. It is noteworthy that we observed no effect of tamoxifen on algal growth in our experiments.

Fig. 3 shows the evolution of pH during the experiments. Regarding pH, we observed that alkalinity peaked at 9.2 at 48 h, before decreasing slightly and stabilising between 8.2 and 8.5. Interestingly, at 48 h, during the peak, variability was particularly high compared to the other points.

3.2. Kinetics of ^{15}N tamoxifen content in algae

3.2.1. Algae exposed to 100 μg L^{-1} (Fig. 4)

As soon as the first minutes of exposition, we observed a high tamoxifen content in the algae (up to 1000 μg g^{-1} DW namely 10 000 times more than the nominal medium concentration). Then, during the first 24 h, we observed a rapid increase of the algae tamoxifen content which peaked at 24 h with a maximum of

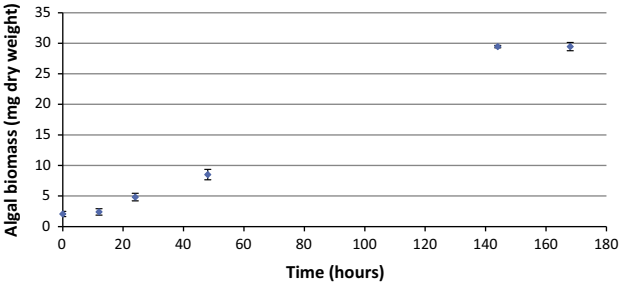

Fig. 2. Algal biomass (mg dry weight) through time (h). Each point represents the average biomass for all treatments and at each time (the three controls and 1, 10 and 100 μg L^{-1}, thus 6 data for each time). Error bars show standard deviation.

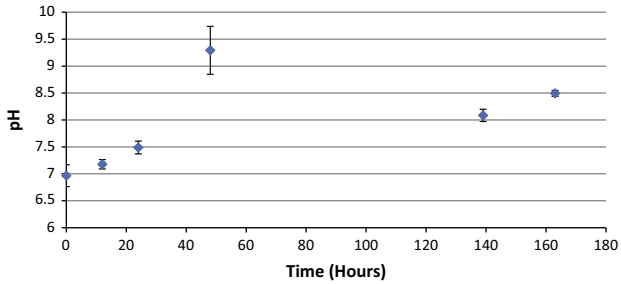

Fig. 3. Evolution of pH through time (h). Each point represents the average biomass for all treatments and at each time (the three controls and 1, 10 and 100 μg L^{-1}, thus 6 data for each time). Error bars show standard deviation.

26 000 times more tamoxifen in the algae (2.67 mg g^{-1} DW) than in water. Finally, in the third part of the curve, the content decreased until concentrations stabilized but at still high contents in the algae (between 553 and 527 μg g^{-1} DW).

3.2.2. Algae exposed to 10 μg L^{-1} (Fig. 5)

In this treatment, we can observe nearly the same kinetics of bioconcentration of tamoxifen. Indeed, we have a great content during the first minutes of contact (96.9 μg g^{-1} DW), then a peak (around 220 μg g^{-1} DW) and finally a stable content (between 55 and 59 μg g^{-1} DW). It should be noted that the content in algae, whatever the time, is approximately 10 times lower in algae exposed to 100 μg L^{-1}.

3.2.3. Algae exposed to 1 μg L^{-1} (Fig. 5)

For the "1 μg L^{-1}" treatment (Fig. 6), the variability around the average values is greater than for the "10 μg L^{-1}" and "100 μg L^{-1}" treatments. Moreover, the factor 10 that have be seen between "10 μg L^{-1}" and "100 μg L^{-1}" is higher here (around 25). However, despite the high variability, the shape of kinetics of tamoxifen in algae at 1 μg L^{-1} is similar compared "10 μg L^{-1}" treatment with a progressive increase from 4 μg g^{-1} at $t = 0$ to approximately 7 μg g^{-1} between 12th and 48th h. At the end of the experiment, tamoxifen content in algae remain constant around 2 μg g^{-1}.

3.3. Mass balance and BCF calculation

As shown in Fig. 7, we calculated the mass balance through time to determine how much tamoxifen injected in each replica was fixed on or in the algae. This mass balance was calculated by multiplying the average algal biomass and the average tamoxifen content at each concentration and at each time. We observed that the mass balance was slightly overestimated (up to 20%), especially for the "10 μg L^{-1}" treatment, maybe because of a difference compared to the nominal concentration. However, for the "1 μg L^{-1}"

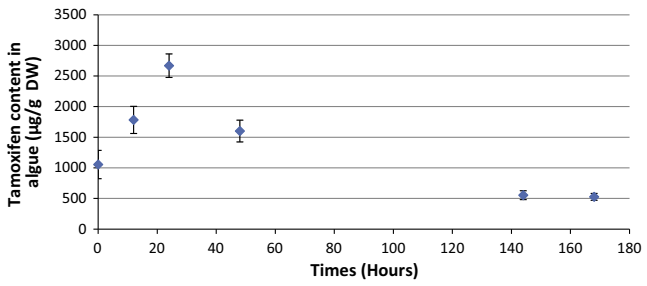

Fig. 4. Kinetics of the tamoxifen content in algae (μg tamoxifen/g dry weight) exposed to 100 μg L^{-1} during 6 d. Error bars show standard deviation.

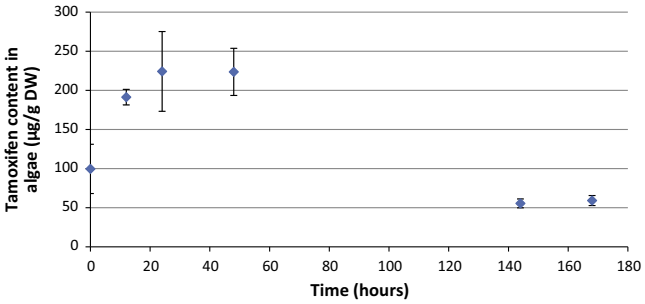

Fig. 5. Kinetics of the tamoxifen content in algae (µg tamoxifen/g dry weight) exposed to 10 µg L^{-1} during 6 d. Error bars show standard deviation.

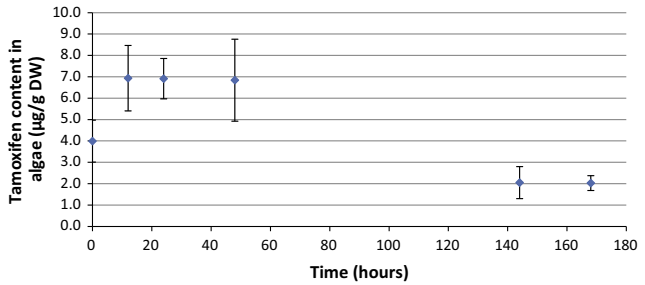

Fig. 6. Kinetics of the tamoxifen content in algae (µg tamoxifen/g dry weight) exposed to 1 µg L^{-1} during 6 d. Error bars show standard deviation.

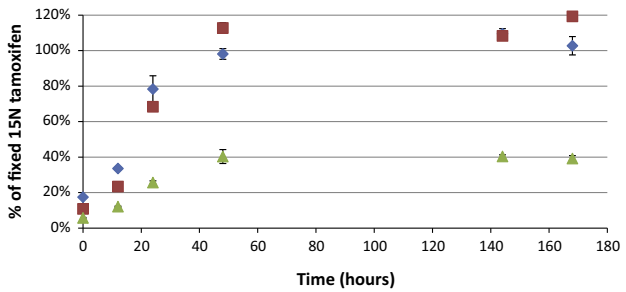

Fig. 7. Percentage of nominal tamoxifen fixed through time for the concentrations tested: with 100 µg L^{-1} shown by blue diamonds, 10 µg L^{-1} by red squares and 1 µg L^{-1} by green triangles. Error bars show standard deviation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

treatment, we obtained only 40% of the nominal content fixed in the algae. This can be explained by the fact that the algae in this treatment were exposed to an intermediate dilution. During this intermediate step, we may have lost part of the tamoxifen introduced (e.g. by adsorption). We think that the concentration adsorbed on the glass was the same at each concentration but that it represented a larger fraction compared to the dose of 1 µg L^{-1}. That is why we were unable to detect this "loss of adsorption" in the other treatments.

These results showed that the bioconcentration of tamoxifen in the algae was at equilibrium from 48 h to the end of the experiment. Thus it was possible to establish experimental bioconcentration factors (BCF) for each concentration tested. Indeed, BCF are calculable only when the contaminant considered is at equilibrium between the medium and the organism considered. In our case, BCF corresponds to the 3 maximum contents for 1, 10 and 100 µg L^{-1}, respectively, i.e. "6900", "22 400" and "26 700", respectively.

4. Discussion

4.1. Bioconcentration kinetics of tamoxifen in Pseudokirchneriella subcapitata

Whatever the treatment, 1, 10 or 100 µg L^{-1} of tamoxifen, we observed significant ^{15}N enrichment through time, especially at higher tamoxifen concentrations. This ^{15}N enrichment was directly due to the adsorption and/or absorption of ^{15}N labelled tamoxifen on and/or inside the algae cells. We can describe the bioconcentration kinetics observed in our work in four steps:

(i) Step 0: Very high bioconcentration during the first minutes of exposure.
(ii) Step 1: A progressive increase of bioconcentration until reaching a peak during the first 24 h.
(iii) Step 2: A decrease until stabilisation during the next 24 h.
(iv) Step 3: A stabilisation of the tamoxifen content in the algae during the final days.

These steps were accentuated more or less according to treatment, but the general shape was the same for all the concentrations tested. Correa-Reyes et al. (2007) studied the bioconcentration kinetics of nonylphenols in a marine microalgae (*Isochrysis galbana*) and showed that bioconcentration was even faster for this kind of contaminant. It reached a maximum stable content after only two hours of exposure. In order to explain the dynamics observed in our work, several hypotheses are possible:

(a) Adsorption and desorption beyond the p*K*a of tamoxifen, which is 8.4. The pH of 8.4 was reached between 48 h and 72 h. However, if this desorption were due to tamoxifen ionisation, the decrease of tamoxifen content in the algae should have occurred simultaneously with this change of pH. But this was not what we observed as there was a lag of 24 h. Moreover, using the mass balancer, we observed that 100% of the tamoxifen had fixed in and/or on algae. Thus no desorption of tamoxifen occurred beyond the p*K*a.
(b) Adsorption, absorption then depuration. When examining only the evolution of algal content in the algae, it could be plausible that the tamoxifen was first adsorbed, then progressively absorbed and metabolised by the algae since the algal tamoxifen content decreased. However, tamoxifen is a very stable molecule (DellaGreca et al., 2007). Moreover, it is unlikely that the cellular mechanism of the algae is efficient enough to metabolise a human pharmaceutical like tamoxifen, especially in such a short time. Degradation could also have been due to photolysis. However, DellaGreca et al. (2007) demonstrated that this process was very long under natural light (only 50% conversion after 30 d sunlight exposition).
(c) Adsorption and/or absorption and then dilution in the biomass. It is this hypothesis which seems the most probable explanation for these bioconcentration dynamics in the algae. Bioconcentration at $t = 0$ was so fast that we assume the phenomenon involved was adsorption. Then, the bioconcentration process was slower during step 1. It could have occurred by progressive adsorption on the cell wall and/or absorption inside the algal cells. It could also have been simply due to an increase in pH that increased the bioconcentration.

It is noteworthy that none of our interpretations consider that tamoxifen was metabolised. However, even though metabolisation was improbable due to the fact that it is a very stable molecule

(DellaGreca et al., 2007), we cannot totally exclude partial metabolisation during our experiments (e.g., hydrolysis, photolysis, depuration, etc.).

4.2. Theoretical and experimental BCF

The theoretical bioconcentration factors (BCF) of tamoxifen modelled by QSAR methodology were established according to pH and varied within 11 900 (pH 7) and 385 000 (pH 9) (Jean et al., 2012). We observed that in our work the maximal BCF measured was around 26 600 and so within the range modelled. *P. subcapitata* is not, a priori, a "target organism" of tamoxifen (contrary to human being). This was confirmed by the fact that "algal tamoxifen content/tamoxifen concentration in water" ratios remained almost stable when increasing the tamoxifen concentration in water (22 423 at 10 $\mu g\ L^{-1}$ and 26 681 at 100 $\mu g\ L^{-1}$). However, this relation can be modified if the organism is a "target" of the molecule considered. Jin et al. (2012) studied the bioconcentration of prometryne, a herbicide, on *Chlamydomonas reinhardtii* (unicellular algae), and observed that the BCF decreased when the concentration of prometryne in the medium increased.

Our study is the first to examine the bioconcentration dynamics of a pharmaceutical compound (PC), in this case an anticancer drug, in a microalgae. We found only a few other studies in the literature that focused on contaminants in algae (Wang et al., 1996; Jin et al., 2012). Indeed, Correa-Reyes et al. (2007) studied the bioconcentration of nonylphenols, a type of organic molecule used in particular as a dispersant, on *I. galbana*, and found a maximum BCF of 6940. For tetrachloroethylene, another organic compound used in several industrial process, the BCFs were lower, between 100 and 300 (Wang et al., 1996). The only study to our knowledge dealing with the bioconcentration of PCs in a unicellular algae is that of Vernouillet et al. (2010), in which the BCF of carbamazepine, an antiepileptic, was observed to be almost nil (BCF = 2). The bioconcentration of other PCs (flumequine, oxytetracycline, oxolinic acid) on aquatic plants has been studied (Delépée et al., 2004) but the plants used were bryophytes. The comparison of BCF between molecules and/or organisms is nonetheless still very difficult in each case because these values vary greatly according to the organism, molecule and experimental conditions considered (e.g. light, pH, etc.) (Voutsas et al., 2002; Delépée et al., 2004).

However, classically, high adsorption on the beaker walls can occur with hydrophobic compounds such as tamoxifen. Moreover, the inaccuracy of the tools used could also be responsible for a shift from the nominal concentration. All these phenomena leading to the reduction of the dissolved tamoxifen concentration could be responsible for underestimating the experimental BCF. But, in previous studies (data not shown), we've already observed a total disappearance in few days of dissolved tamoxifen in the medium containing algae, while in beakers without algae, tamoxifen concentration was still high.

Besides, the theoretical BCF calculated by QSAR takes into account only the influence of pH. But we observed that the BCFs did not evolve correlatively to the pH during algal growth, especially between 24 and 48 h. It should be noted that we could not verify if the highest experimental BCF observed here was the highest experimental BCF possible. Indeed, after 48 h, all the tamoxifen injected was inside and/or on the algae, thereby limiting bioconcentration. It is reasonable to assume that if we had injected a higher quantity of tamoxifen, the maximum BCF would have been higher and closer to the maximum BCF calculated by QSAR (385 000) and perhaps even higher.

Finally, Lajeunesse et al. (2011) recently demonstrated that even for poorly bioaccumulable compounds, the bioaccumulation of such compounds present in concentrations of around $ng\ L^{-1}$ in water could have an effect on ecosystem organisms like fishes. Thus it is legitimate to question the impact of highly bioaccumulable compounds like tamoxifen, which have already been detected in the environment at concentrations higher than 200 $ng\ L^{-1}$. Zenker et al. (2014) also thought that better consideration of compound bioaccumulability in ecotoxicity assessments is fundamental when considering the ubiquity of contaminants in our environment.

4.3. A multi-advantage approach

In order to study the bioconcentration of organic molecules (in opposition to metals), classical measuring methods use either radioactive isotopes (Meredith-Williams et al., 2012) or analytic chemistry (e.g. LC–MS/MS (Garcia et al., 2012) or HPLC/MS (Vernouillet et al., 2010). The first leads to evident risks for both the scientist's health and that of the studied organisms. The second is quite expensive, extractions are time consuming and protocols in the biological matrix with exposure to environmental concentrations are available only for very few pharmaceuticals.

In our study, we used stable isotopes to quantify tamoxifen adsorbed and/or absorbed in algal cells. Stable isotopes have long been used as tracers in several fields: biogeochemical cycles, plant physiology (Hama et al., 1983), trophic relations inside ecosystems (Foulquier et al., 2010), contaminant degradation and transfer processes (Badia-Fabregat et al., 2014; Colabuono et al., 2014), and so forth. Stable isotopes can also be used in human physiology, notably in order to track the fate of pharmaceuticals in the body (Fan et al., 2012) and in palaeoenvironmental (Pucéat et al., 2003; Bajpai et al., 2013) and in paleoecological studies (Amiot et al., 2010).

To our knowledge, the method we used had only been used once before to track the bioconcentration of a contaminant (i.e. tetrachloroethylene) in an organism (i.e. marine unicellular algae) through time (Wang et al., 1996). This analytic method presents several advantages compared to radioactive isotopes and analytical chemistry. Indeed, the drawbacks linked to the use of radioactive isotopes (i.e. safety, impact on living organisms) are eliminated with this method. In addition, bulk carbon or nitrogen isotope analysis by EA–IRMS is generally 1 order of magnitude cheaper than extraction and analysis using a chromatographic method such as HPLC–MS on complex matrix. Moreover, it requires lower sample masses (typically 50 μg to 1 mg compared to several mg by HPLC–MS), which is a considerable advantage for researchers working with small organisms and microorganisms. The two main advantages of our method are: (i) no loss of matter since it does not include an extraction step; (ii) our method is not limited by the nature of the matrix and so any kind of sample can be analysed (e.g. sediment, biological matrix, etc.).

However, this method has certain disadvantages when studying the fate of a molecule. The detection limit using stable isotope labelling is generally higher (ppm range) than using HPLC–MS (ppb range) because of the natural minor isotope abundance (for example, $^{15}N/^{14}N$ is close to 0.003677 in natural substance). The other main disadvantage is linked more to the compound to be monitored than to the methodology itself: in-depth knowledge of the metabolisation routes and metabolites of the compound studied are necessary in order to know what is being measured. Indeed, as it is an indirect measurement (enrichment of the matrix with a stable isotope) one must be sure that not only the labelled part of the molecule is measured. Thus, if the compound is metabolised during the experiment, the enrichment measured could also be due to the presence of the labelled atom alone or inside a metabolite. Otherwise, in order to amplify the isotopic ratio and so improve the accuracy and the resolution of the analysis, the exposure medium must be as nitrogen poor as possible, if the labelled atom used is nitrogen.

5. Conclusion

Our works demonstrated that tamoxifen is a highly bioaccumulable compound. Indeed, the BCF measured in *P. subcapitata* was always higher than 6900. The highest BCF measured under our experimental conditions was around 26600. But 100% of the tamoxifen injected was contained in the algae after 48 h exposure. Therefore it is probable that if more tamoxifen had been injected, the highest BCF would have been even higher. However, no toxic effects of tamoxifen on algal growth were observed under our experimental conditions (maximum 100 $\mu g\ L^{-1}$ during 7 d).

The analysis method used, isotopic chemistry, presents numerous advantages compared to the other methods usually used for these kinds of studies. It requires very little biomass and it is indifferent to the nature of the matrix studied.

It is now necessary to observe the fate of ^{15}N tamoxifen not only in other algal species but also on other organisms like invertebrates and fishes. Consequently, it will be possible to improve knowledge on the transfer of contaminants in trophic webs.

It could also be interesting in the future to study the fate of other bioaccumulative contaminants with characteristics compatible with isotopic chemistry. Diclofenac, acetaminophen and carbamazepine could be interesting candidates (only one nitrogen atom) for a shift from chemical ecotoxicology to isotopic ecotoxicology.

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References

AFNOR, 1980. Détermination de l'inhibition de la croissance de Scenedesmus subspicatus par une substance.
Amiot, R., Buffetaut, E., Lécuyer, C., Wang, X., Boudad, L., Ding, Z., Fourel, F., Hutt, S., Martineau, F., Medeiros, M.A., Mo, J., Simon, L., Suteethorn, V., Sweetman, S., Tong, H., Zhang, F., Zhou, Z., 2010. Oxygen isotope evidence for semi-aquatic habits among spinosaurid theropods. Geology 38, 139–142.
Badia-Fabregat, M., Rosell, M., Caminal, G., Vicent, T., Marco-Urrea, E., 2014. Use of stable isotope probing to assess the fate of emerging contaminants degraded by white-rot fungus. Chemosphere 103, 336–342.
Bajpai, S., Holmes, J., Bennett, C., Mandal, N., Khosla, A., 2013. Palaeoenvironment of Northwestern India during the late Cretaceous Deccan volcanic episode from trace-element and stable-isotope geochemistry of intertrappean ostracod shells. Global Planet. Change 107, 82–90.
Calamari, D., Zuccato, E., Castiglioni, S., Bagnati, R., Fanelli, R., 2003. Strategic survey of therapeutic drugs in the Rivers Po and Lambro in Northern Italy. Environ. Sci. Technol. 37, 1241–1248.
Coetsier, C.M., Spinelli, S., Lin, L., Roig, B., Touraud, E., 2009. Discharge of pharmaceutical products (PPs) through a conventional biological sewage treatment plant: MECs vs PECs? Environ. Int. 35, 787–792.
Colabuono, F.I., Barquete, V., Taniguchi, S., Ryan, P.G., Montone, R.C., 2014. Stable isotopes of carbon and nitrogen in the study of organochlorine contaminants in albatrosses and petrels. Mar. Pollut. Bull. 83, 241–247.
Correa-Reyes, G., Viana, M.T., Marquez-Rocha, F.J., Licea, A.F., Ponce, E., Vazquez-Duhalt, R., 2007. Nonylphenol algal bioaccumulation and its effect through the trophic chain. Chemosphere 68, 662–670.
Delépée, R., Pouliquen, H., Le Bris, H., 2004. The bryophyte Fontinalis antipyretica Hedw. bioaccumulates oxytetracycline, flumequine and oxolinic acid in the freshwater environment. Sci. Total Environ. 322, 243–253.
DellaGreca, M., Iesce, M.R., Isidori, M., Nardelli, A., Previtera, L., Rubino, M., 2007. Phototransformation products of tamoxifen by sunlight in water. Toxicity of the drug and its derivatives on aquatic organisms. Chemosphere 67, 1933–1939.
Fan, T.W.M., Lorkiewicz, P.K., Sellers, K., Moseley, H.N.B., Higashi, R.M., Lane, A.N., 2012. Stable isotope-resolved metabolomics and applications for drug development. Pharmacol. Ther. 133, 366–391.
Farré, M., Petrovic, M., Gros, M., Kosjek, T., Martinez, E., Heath, E., Osvald, P., Loos, R., Le Menach, K., Budzinski, H., De Alencastro, F., Müller, J., Knepper, T., Fink, G., Ternes, T.A., Zuccato, E., Kormali, P., Gans, O., Rodil, R., Quintana, J.B., Pastori, F., Gentili, A., Barceló, D., 2008. First interlaboratory exercise on non-steroidal anti-inflammatory drugs analysis in environmental samples. Talanta 76, 580–590.
Fick, J., Söderström, H., Lindberg, R.H., Phan, C., Tysklind, M., Larsson, D.G.J., 2009. Contamination of surface, ground, and drinking water from pharmaceutical production. Environ. Toxicol. Chem. 28, 2522–2527.
Foulquier, A., Simon, L., Gilbert, F., Fourel, F., Malard, F., Mermillod-Blondin, F., 2010. Relative influences of DOC flux and subterranean fauna on microbial abundance and activity in aquifer sediments: new insights from 13C-tracer experiments. Freshwater Biol. 55, 1560–1576.
Garcia, S.N., Foster, M., Constantine, L.A., Huggett, D.B., 2012. Field and laboratory fish tissue accumulation of the anti-convulsant drug carbamazepine. Ecotoxicol. Environ. Saf. 84, 207–211.
Halling-Sørensen, B., Nors Nielsen, S., Lanzky, P.F., Ingerslev, F., Holten Lützhøft, H.C., Jørgensen, S.E., 1998. Occurrence, fate and effects of pharmaceutical substances in the environment – a review. Chemosphere 36, 357–393.
Hama, T., Miyazaki, T., Ogawa, Y., Iwakuma, T., Takahashi, M., Otsuki, A., Ichimura, S., 1983. Measurement of photosynthetic production of a marine phytoplankton population using a stable 13C isotope. Mar. Biol. 73, 31–36.
Jean, J., Perrodin, Y., Pivot, C., Trepo, D., Perraud, M., Droguet, J., Tissot-Guerraz, F., Locher, F., 2012. Identification and prioritization of bioaccumulable pharmaceutical substances discharged in hospital effluents. J. Environ. Manage. 103, 113–121.
Jin, Z.P., Luo, K., Zhang, S., Zheng, Q., Yang, H., 2012. Bioaccumulation and catabolism of prometryne in green algae. Chemosphere 87, 278–284.
Kümmerer, K., 2009. The presence of pharmaceuticals in the environment due to human use – present knowledge and future challenges. J. Environ. Manage. 90, 2354–2366.
Lajeunesse, A., Gagnon, C., Gagné, F., Louis, S., Čejka, P., Sauvé, S., 2011. Distribution of antidepressants and their metabolites in brook trout exposed to municipal wastewaters before and after ozone treatment – evidence of biological effects. Chemosphere 83, 564–571.
Lalumera, G.M., Calamari, D., Galli, P., Castiglioni, S., Crosa, G., Fanelli, R., 2004. Preliminary investigation on the environmental occurrence and effects of antibiotics used in aquaculture in Italy. Chemosphere 54, 661–668.
Loos, R., Gawlik, B.M., Locoro, G., Rimaviciute, E., Contini, S., Bidoglio, G., 2009. EU-wide survey of polar organic persistent pollutants in European river waters. Environ. Pollut. 157, 561–568.
López-Serna, R., Petrović, M., Barceló, D., 2012. Occurrence and distribution of multi-class pharmaceuticals and their active metabolites and transformation products in the Ebro River basin (NE Spain). Sci. Total Environ.
Meredith-Williams, M., Carter, L.J., Fussell, R., Raffaelli, D., Ashauer, R., Boxall, A.B.A., 2012. Uptake and depuration of pharmaceuticals in aquatic invertebrates. Environ. Pollut. 165, 250–258.
OECD. 2006. OECD guidelines for the testing of chemicals: Freshwater Alga and Cyanobacteria, Growth Inhibition Test.
Pucéat, E., Lécuyer, C., Sheppard, S.M.F., Dromart, G., Reboulet, S., Grandjean, P., 2003. Thermal evolution of Cretaceous Tethyan marine waters inferred from oxygen isotope composition of fish tooth enamels. Paleoceanography 18, 1029.
Roberts, P.H., Thomas, K.V., 2006. The occurrence of selected pharmaceuticals in wastewater effluent and surface waters of the lower Tyne catchment. Sci. Total Environ. 356, 143–153.
Sanchez, W., Sremski, W., Piccini, B., Palluel, O., Maillot-Maréchal, E., Betoulle, S., Jaffal, A., Aït-Aïssa, S., Brion, F., Thybaud, E., Hinfray, N., Porcher, J.-M., 2011. Adverse effects in wild fish living downstream from pharmaceutical manufacture discharges. Environ. Int. 37, 1342–1348.
Silva, B.F.d., Jelic, A., López-Serna, R., Mozeto, A.A., Petrovic, M., Barceló, D., 2011. Occurrence and distribution of pharmaceuticals in surface water, suspended solids and sediments of the Ebro river basin, Spain. Chemosphere 85, 1331–1339.
Touraud, E., Roig, B., Sumpter, J.P., Coetsier, C., 2011. Drug residues and endocrine disruptors in drinking water: Risk for humans? Int. J. Hyg. Environ. Health 214, 437–441.
Verlicchi, P., Galletti, A., Petrovic, M., Barceló, D., 2010. Hospital effluents as a source of emerging pollutants: an overview of micropollutants and sustainable treatment options. J. Hydrol. 389, 416–428.
Vernouillet, G., Eullaffroy, P., Lajeunesse, A., Blaise, C., Gagné, F., Juneau, P., 2010. Toxic effects and bioaccumulation of carbamazepine evaluated by biomarkers measured in organisms of different trophic levels. Chemosphere 80, 1062–1068.
Voutsas, E., Magoulas, K., Tassios, D., 2002. Prediction of the bioaccumulation of persistent organic pollutants in aquatic food webs. Chemosphere 48, 645–651.
Wang, X., Harada, S., Watanabe, M., Koshikawa, H., Sato, K., Kimura, T., 1996. Determination of bioconcentration potential of tetrachloroethylene in marine algae by 13C. Chemosphere 33, 865–877.
Wiegel, S., Aulinger, A., Brockmeyer, R., Harms, H., Löffler, J., Reincke, H., Schmidt, R., Stachel, B., von Tümpling, W., Wanke, A., 2004. Pharmaceuticals in the river Elbe and its tributaries. Chemosphere 57, 107–126.
Yang, Y., Fu, J., Peng, H., Hou, L., Liu, M., Zhou, J.L., 2011. Occurrence and phase distribution of selected pharmaceuticals in the Yangtze Estuary and its coastal zone. J. Hazard. Mater. 190, 588–596.
Zenker, A., Cicero, M.R., Prestinaci, F., Bottoni, P., Carere, M., 2014. Bioaccumulation and biomagnification potential of pharmaceuticals with a focus to the aquatic environment. J. Environ. Manage. 133, 378–387.

7.3 Synthèse

Au cours de ces travaux, nous avons, tout d'abord, pour la première fois mis en évidence la fiabilité de notre méthode analytique avec cependant certains biais qui seront explicités dans la dernière partie de ce document. Nous avons mis en évidence une dynamique de bioconcentration très rapide du tamoxifen au sein des cellules algales et obtenu des BCF toujours supérieurs à 6900 dans nos conditions expérimentales. Il est maintenant nécessaire d'étudier cette dynamique chez des organismes de différents niveaux trophiques. Par ailleurs, connaissant désormais la dynamique chez les algues, nous pouvons utiliser ces données pour étudier l'impact du régime alimentaire dans la bioaccumulation d'organisme consommant ces algues. C'est ce que nous allons présenter dans la partie suivante avec *Daphnia magna*.

Chapitre 8

Bioaccumulation du ^{15}N tamoxifen chez *Daphnia magna*

8.1 Enjeux et démarche

Après avoir étudié la dynamique de bioconcentration du tamoxifen chez les algues, nous pouvons désormais étudier le rôle de ce maillon chez les daphnies qui vont les consommer. Pour cela, nous avons exposé alternativement des daphnies à une alimentation (algues) contaminée ou non dans un milieu contaminé ou non. Nous avons testé deux concentrations dans l'eau : 5 et 50 μg/L. Quotidiennement et pendant 5 jours, les daphnies de ces trois concentrations d'exposition étaient alimentées par des algues contaminées (une seule teneur testée) ou non. Avant et après chaque alimentation, le ratio ^{15}N/^{14}N des daphnies a été mesuré pour évaluer l'enrichissement lié à la bioconcentration ou à la bioaccumulation du tamoxifen.

8.2 Article 6 : *Respective contributions of diet and medium to the bioaccumulation of pharmaceutical compounds in the first levels of an aquatic trophic web*

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RESEARCH ARTICLE

Respective contributions of diet and medium to the bioaccumulation of pharmaceutical compounds in the first levels of an aquatic trophic web

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Abstract Nowadays, pharmaceuticals (PCs) are ubiquitous in aquatic ecosystems. It is known that these compounds have ecotoxic effects on aquatic organisms at low concentrations. Moreover, some of them can bioaccumulate inside organisms or trophic webs exposed at environmental concentrations and amplify ecotoxic impacts. PCs can bioaccumulate in two ways: exposure to a medium (e.g., respiration, diffusion, etc.) and/or through the dietary route. Here, we try to assess the respective contributions of these two forms of contamination of the first two levels of an aquatic trophic web. We exposed *Daphnia magna* for 5 days to 0, 5, and 50 μg/L ^{15}N-tamoxifen and then fed them with control and contaminated diets. We used an isotopic method to measure the tamoxifen content inside the daphnids after several minutes' exposure and every day before and after feeding. We found that tamoxifen is very bioaccumulative inside daphnids (BCF up to 12,000) and that the dietary route has a significant impact on contamination by tamoxifen (BAF up to 22,000), especially at low concentrations in medium.

Keywords Bioconcentration · Bioaccumulation · Pharmaceutical compound · Tamoxifen · Stable isotopes

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Introduction

Nowadays, pharmaceutical compounds (PCs) are ubiquitous in aquatic ecosystems. Indeed, PCs are found in surface waters (Blair et al. 2013; Fick et al. 2009; Kolpin et al. 2002), groundwater (Barnes et al. 2008; Loos et al. 2010), coastal zone (Claessens et al. 2013; Thomas and Hilton 2004), sediment (Schultz et al. 2010; Vazquez-Roig et al. 2012), and in biota (Zenker et al. 2014) all over the world. They are found at concentrations ranging from several picrograms per liter to several milligrams per liter (Loos et al. 2009). Among these PCs, bioaccumulative PCs potentially threaten aquatic ecosystems due to their bioaccumulation inside organisms and along trophic webs (Lajeunesse et al. 2011). These compounds often have a high octanol/water partition coefficient K_{ow}.

Tamoxifen, an anticancer drug used to treat breast cancer, is one of these bioaccumulative PCs (Jean et al. 2012). Tamoxifen is a hydrophobic molecule, having high octanol/water partition coefficients with log K_{ow} up to 7.1. QSAR models predicted bioconcentration factors between 1650 and 385,000 (Jean et al. 2012). This molecule has already been detected numerous times in the water (several hundred ng/L) and sediments of aquatic ecosystems (Roberts and Thomas 2006; Coetsier et al. 2009; Yang et al. 2011; López-Serna et al. 2012). In previous works, we demonstrated experimentally that tamoxifen was highly bioconcentrated in the first level of the aquatic trophic web, with substantial accumulation in unicellular algae when they are exposed to dissolved tamoxifen in water (Orias et al. 2015). The consumption of contaminated food sources (i.e., aquatic primary producers exposed to dissolved tamoxifen) might therefore constitute an important route of exposure for primary consumers.

The aim of this work is to determine experimentally the transfer of tamoxifen between a primary producer (photosynthetic algae) and a primary consumer (*Daphnia magna*), and to assess

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the respective contributions of exposure through feeding and through surrounding water to tamoxifen bioaccumulation in the first levels of a simplified freshwater food chain. Daphnids were exposed simultaneously to a medium and to a diet (i.e., microalgae), both contaminated by tamoxifen. We assess the respective contribution of each route to the bioaccumulation of tamoxifen by comparing these organisms to control organisms exposed only via diet or only via water or not exposed at all.

Because of analytic difficulties in measuring PCs within complex biological matrices, tamoxifen transfers between water, algae, and daphnids were quantified by stable isotopic labelling with ^{15}N using [^{15}N]-tamoxifen and by monitoring the evolution of ^{15}N contents inside daphnids through time.

Materials and methods

Algal cultures and rearing of daphnids

Algal culture (*Pseudokirchneriella subcapitata*) and daphnid (*Daphnia magna*) rearing conditions were described in previous works (Clément and Zaid 2004).

Chemicals

Tamoxifen-$^{13}C_2$,^{15}N solution (CAS Number 10540-29-1) was purchased from Sigma-Aldrich Chemical (St. Quentin Fallavier, France). The stock solution of tamoxifen was prepared in methanol (1 mg/mL) and kept at −20 °C.

Exposure of algae to tamoxifen

An algal solution of 500 mL aged less than 10 days with a classical AFNOR medium (AFNOR 1980) and a density of 10^6 cells/mL (10 mg dry weight of algae) was exposed to 50 μg of ^{15}N-tamoxifen (100 μg/L). After exposure for 24 h, the algal solution was centrifuged at 3000 rpm for 20 min in glass tubes. A "control" solution was also centrifuged at the same time. The supernatant was removed, and the bottoms of the "control" and "exposed" tubes were stored in glass tubes at 5 °C throughout the experiment.

Exposure of daphnids to tamoxifen

Exposure via water

Daphnids were exposed to three concentrations of tamoxifen: 0, 5, and 50 μg/L, for 5 days. For each concentration, four beakers containing 1 L of rearing medium with ten daphnids aged 10 days were prepared. These beakers were then placed in a climatic room at 20 °C with a photoperiod of 16 h (500 lx). For each treatment (0, 5, and 50 μg/L), two beakers were reserved for the^{15}N-tamoxifen-contaminated diet. In order to limit the ingestion of dissolved contaminant by neonate daphnids, the latter were removed daily.

Exposure via feeding

Daphnids were fed for 2 h a day with contaminated or control algae by placing the daphnids in beakers containing 80 mL of medium with an algal density of 400,000 cells/mL. This discontinuous diet prevented the development of algae within the exposure medium and the dilution of tamoxifen in the algal biomass, thus feeding the daphnids with algae in isolation reduced the adsorption of tamoxifen on the algae.

Determination of ^{15}N-tamoxifen content in algae and daphnids

Sample preparation

A sample of algae (0.5 mL of concentrated) was extracted daily before feeding the daphnids. Algae were placed in 2-mL Eppendorf tubes frozen for 5 min in liquid nitrogen and freeze-dried before EA-IRMS analyses in order to quantify the tamoxifen content in the algae before each feeding step.

The daphnids were extracted daily before (3, 12, 24, 48, 72, and 96 h) and after (5, 27, 48, 51, 75, 96, and 99 h) feeding. They were then frozen for 5 min in liquid nitrogen and freeze-dried.

^{15}N measurements

After freeze-drying, the algae samples were weighed at 1 mg in tin capsules before analysis. Nitrogen concentration and $^{15}N/^{14}N$ ratio were measured using an isotope ratio mass spectrometer (Isoprime 100, Isoprime Ltd, Manchester, UK) coupled in continuous flow with an elemental analyser (FlashEA 1112 Thermo Electron, Milan, Italy). Aspartic acid was used to calibrate the N concentration measurements. For the isotope ratio measurements, two-point normalization of measured values was performed using international reference material IAEA-N1 and IAEA-305 according to IUPAC guidelines.$^{15}N/^{14}N$ ratio is expressed as $\delta^{15}N$ in ‰ relative to atmospheric N_2 (air):

$$\delta^{15}N = \left({}^{15}N/{}^{14}N_{sample}/{}^{15}N/{}^{14}N_{Air}-1\right) \times 10^3$$

and converted to atomic fraction (i.e., atomic %, Coplen 2011):

$$x\left({}^{15}N\right) = \left({}^{15}N/{}^{14}N_{sample}\right)/\left(1+{}^{15}N/{}^{14}N_{sample}\right)$$

In-house standards calibrated against IAEA-N1 and IAEA-N2 were interspersed within the samples, and the standard deviations for the replicate analyses were lower than 0.2‰.

Calculation of excess ^{15}N and tamoxifen content

Excess ^{15}N concentration, [^{15}N], was calculated from the difference between the ^{15}N fraction ($x(^{15}N)$) of algae exposed to ^{15}N-tamoxifen and the ^{15}N fraction ($x(^{15}N)_{control}$) measured in the control treatment without exposure as follows:

$$\left[^{15}\mathrm{N}\right] = \left(x\left(^{15}\mathrm{N}\right) - x\left(^{15}\mathrm{N}\right)_{\mathrm{control}}\right) \cdot [\mathrm{N}],$$

where [N] is the N concentration in the sample.

As ^{15}N-tamoxifen was the only ^{15}N tracer source in the experiment, and assuming no dissociation of tamoxifen by chemical reactions or metabolic pathways, the tamoxifen concentration was calculated from the ^{15}N concentration:

$$[\mathrm{Tamoxifen}]\left(\mu g \Big/ g\right) = \left[^{15}\mathrm{N}\right] \Big/ 15 \cdot M_{\mathrm{Tamoxifen}} \cdot 10^{6}.$$

with $M_{tamoxifen}$ (374.49 g/mol) being the molar mass of [^{13}C, ^{15}N]-tamoxifen.

Determination of dissolved tamoxifen concentration in water

At the end of the experiment, 150 mL of medium was collected in each beaker in which the daphnids were exposed. Because of the amount of water required for tamoxifen quantification, samples of each three replicates were pooled, resulting in a single tamoxifen concentration per treatment. Analytical measurements were performed by the Analytical Sciences Institute (CNRS, Lyon) using HPLC-MS/MS (Waters: UHPLC H-Class/Xevo TQ-S). Analytes were separated on a C18 column (Phenomenex Kinetex C18 Phényl Hexyl 100×2.1 mm, 2.6 μm) using as mobile phase a binary gradient formed by (A) Milli-Q quality water+0.01 % acetic acid, and (B) acetonitrile (ACN). The gradient started at 40 % B, rose to 100 % B in 2 min, leveled for 2 min at 100 % ACN, and was followed by column repackaging at 40 % ACN for 2 min. The flow was 0.5 mL/min, the furnace temperature was 40 °C, and the volume of injection was 2 μL. Eluted analyses were monitored by MS/MS equipped with an electrospray ion source operating in positive-ion mode, and the acquisition was performed in multi-reaction monitoring (MRM) mode. Detection and quantification limits were 0.15 μg/L (LOD) and 0.5 μg/L (LOQ), respectively.

Statistical analysis

The differences of δ^{15}N values of daphnids between the different treatments (water contamination; food contamination) were tested using three-way repeated measure analysis of variance (RM-ANOVA), with "time" being the repeated factor, and "water contamination level" and "algae contamination" the independent factors. Mauchly's test was used to test for sphericity and univariate test degrees of freedom were adjusted using the Huynh-Feldt epsilon correction factor when necessary. Post hoc tests (contrast method) were performed to determine whether significant pairwise differences occurred between treatments. Significance for statistical tests was accepted for $\alpha<0.05$. All the statistical analyses were performed using JMP 9 (SAS Institute Inc., Cary, NC) software.

Results

Evolution of δ^{15}N in daphnids

δ^{15}N values of daphnids fed with noncontaminated algae (δ^{15} N=−1.6±0.5‰) remained constant during the course of the experiment, with a mean value of +3±1‰. When exposed to algae artificially contaminated with ^{15}N-tamoxifen (δ^{15} N= 53±1‰), the δ^{15}N value of daphnids increased from +3 to +6 ‰ (Fig. 1). This significant increase of ^{15}N in the organisms when exposed to contaminated algae compared to noncontaminated algae demonstrated a transfer of ^{15}N-tamoxifen via the trophic route.

Daphnids exposed to ^{15}N-tamoxifen via surrounding water exhibited a significant increase of δ^{15}N values, up to +9‰ at 5 μg/L and up to +60‰ at 50 μg/L (Fig. 2). The ^{15}N enrichment showed that tamoxifen transfer from environmental water is a major contamination route.

When exposed to ^{15}N-tamoxifen via the trophic route and the surrounding medium, the δ^{15}N values of daphnids were close to the δ^{15}N values measured during exposure via surrounding water only (Fig. 3).

^{15}N tamoxifen quantification in algae and daphnids

Tamoxifen content in algae

The average tamoxifen content in algae measured before each feeding step was 390 μg/g (±SD 3.32 μg/g).

Daphnids exposed via the dietary route only (Fig. 4)

During the experiment, the tamoxifen concentration in daphnids increased from 15 to 20 μg/g after each feeding step, except at the fourth step (Fig. 4). This increase was due to the tamoxifen inside the algae grazed by daphnids during the 2-h feeding periods. This increase of content was systematically followed by a depuration phase, complete after the first feeding step (no more tamoxifen inside the daphnids), and then partial for the other feeding steps. This first step showed that 20 h was enough to purge the gut. However, the next steps showed that tamoxifen accumulated inside the daphnia even after the gut was purged. Consequently, considering the whole

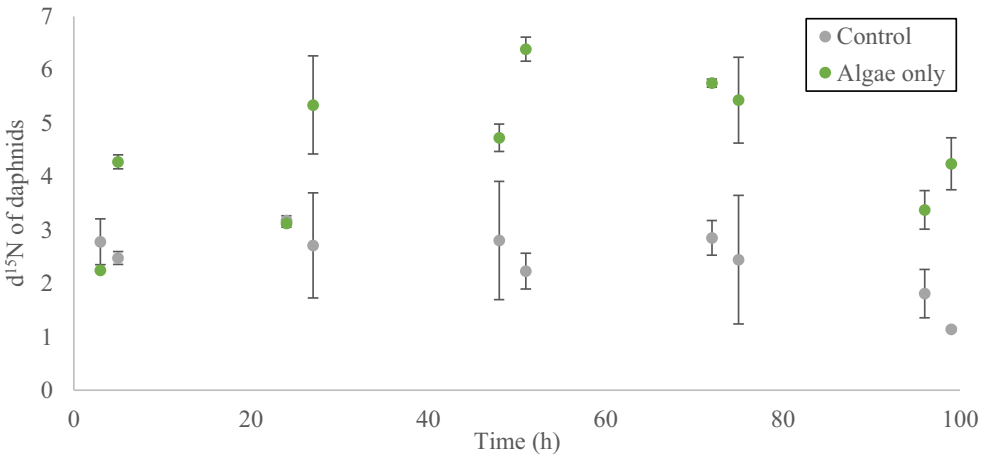

Fig. 1 Evolution of ratios $^{15}N/^{14}N$ ($\delta^{15}N$) in daphnids without toxicant (control: *gray circles*) and in daphnids exposed to ^{15}N-tamoxifen via the dietary route (algae only: *green circles*). *Error bars* show standard deviation

curve, we observed progressive bioaccumulation of tamoxifen in daphnids as the experiment progressed. This clearly showed that the dietary route was a significant route of contamination under our experimental conditions.

Daphnids exposed via medium only (Fig. 5a)

Figure 5a shows the tamoxifen content in daphnids exposed to tamoxifen via only the medium. We observed that daphnids had a very high tamoxifen content within the first minutes of exposure, about 10 μg/g and about a hundred micrograms per gram for daphnids exposed to 5 and 50 μg/L tamoxifen, respectively. We could also see that the content in daphnids decreased slightly during the feeding step. This can be explained by the fact that there was no tamoxifen in the feeding medium. The algae was not contaminated either so the tamoxifen content in the daphnids decreased via a depuration mechanism. Steady state was rapidly reached with a stable tamoxifen content in the daphnids. After the third feeding step, the tamoxifen content in the daphnids was stable in both treatments. At the end of this third feeding step, we reached a content in daphnids of up to 26 μg/g (SD±6) and 234 μg/g (SD±64) for 5 and 50 μg/L, respectively, in water. It is noteworthy that the same order of magnitude was obtained between the water of two treatments and the daphnids of two treatments (ten times more in each case). Thus, the bioconcentration factor of tamoxifen in daphnids was not linked to the initial concentration in water for the concentration range tested.

Daphnids exposed via medium and dietary routes (Fig. 5b)

Figure 5b shows the tamoxifen content in daphnids exposed via the medium and dietary routes. The dynamic bioaccumulation in these daphnids is close to that of daphnids exposed only via water: rapid bioaccumulation during the first few hours and at steady state reached around 48 h. Tamoxifen contents in daphnids were 46 μg/g (SD±13) and 299 μg/g (SD±95) for daphnids exposed to 5 and 50 μg/L in water and to contaminated algae, respectively.

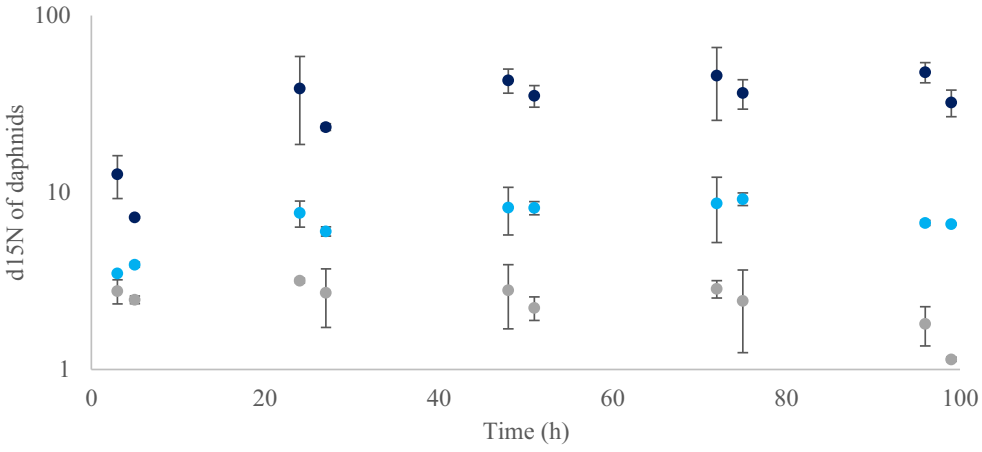

Fig. 2 Evolution of ratios $^{15}N/^{14}N$ ($\delta^{15}N$) in daphnids without toxicant (control: *gray circles*) and in daphnids exposed to ^{15}N-tamoxifen via medium only (5 μ/L, *light blue circles*, and 50 μg/L, dark *blue circles*). *Error bars* show standard deviations

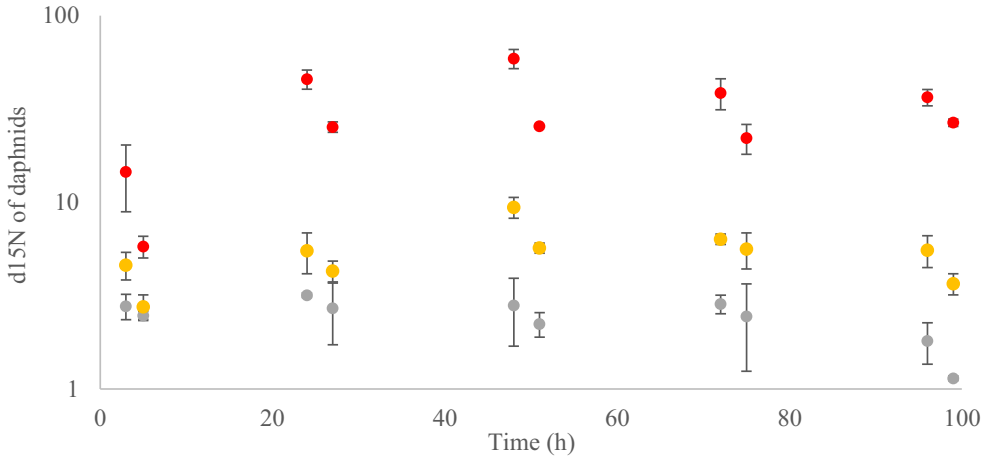

Fig. 3 Evolution of ratios $^{15}N/^{14}N$ ($\delta^{15}N$) in daphnids without toxicant (control: *gay circles*) and in daphnids exposed to ^{15}N tamoxifen via medium (5 μ/L, *yellow circles*, and 50 μg/L, *red circles*) and the dietary route. *Error bars* show standard deviations

Discussion

Bioconcentration and bioaccumulation factors

For better comprehension of the respective contributions of medium and dietary routes to tamoxifen contamination, we compared tamoxifen concentrations dissolved in water and its content in daphnids at the end of the experiment (at steady state) (Table 1). Indeed, we could calculate BCF and BAF only when the concentrations were at steady state between medium and the organism.

We observed that the concentrations measured in water at the 99th hour were nearly half the initial concentration, most likely because of the adsorption of the tamoxifen onto the walls of the beakers (data not shown).

First, the results of these chemical analyses allowed calculating the bioconcentration factor (BCF) (for daphnids exposed only via water) and bioaccumulation factors (BAF) (for daphnids exposed via medium and the dietary route). These values (Table 1) show the high bioaccumulability of tamoxifen in daphnids.

Then, by comparing the BCFs and BAFs calculated for all the treatments, we saw that the dietary route contributed more to bioaccumulation in daphnids exposed to 5 μg/L in water than in daphnids exposed to 50 μg/L.

Finally, we observed that the tamoxifen content in daphnids at the end of the experiment was always lower than that in algae used to feed them (390 μg/g). Thus, under these experimental conditions, we did not observe biomagnification of tamoxifen. This can be explained by the fact that, in our experiment, the tamoxifen concentration to which the algae were exposed (100 μg/L) was always higher than that to which the daphnids were exposed (5 and 50 μg/L).

To our knowledge, tamoxifen is the most bioaccumulative PC ever measured experimentally in an invertebrate. However, other organic compounds have already been measured as more bioaccumulative, notably inside *Daphnia magna* (Geyer et al. 1991).

However, it is noteworthy that our method is an indirect analytic method. In our work, we consider that a nitrogen atom corresponds to a molecule of tamoxifen. However, it is possible that this nitrogen atom is not an unchanged

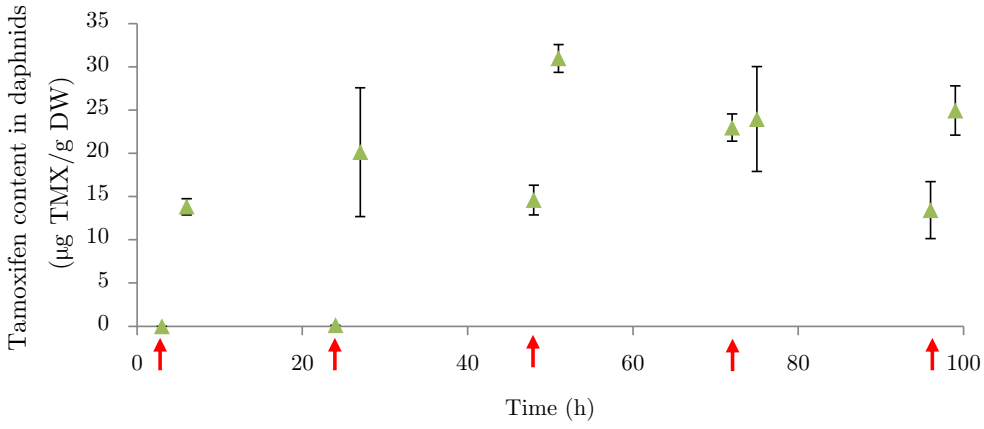

Fig. 4 Tamoxifen content in daphnids (μg tamoxifen/g dry weight) exposed to tamoxifen via only the dietary route for 5 days. *Error bars* shows standard deviations. *Red arrows* show feeding steps

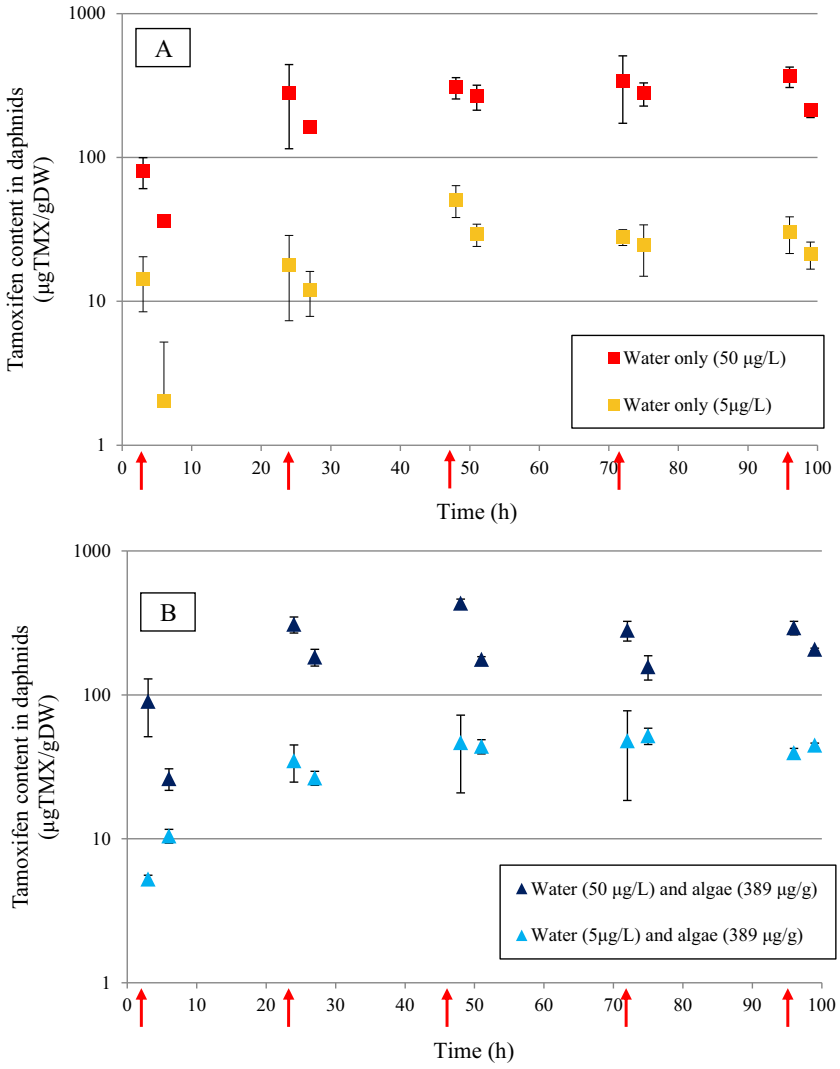

Fig. 5 **a**, **b** Evolution of tamoxifen content in daphnids (µg tamoxifen/g dry weight) exposed to tamoxifen via only water (5 µg/L, *yellow square*, and 50 µg/L, *red square*) and via water and food (5 µg/L, *dark blue triangle*, and 50 µg/L, *light blue triangle*). *Error bars* show standard deviations. *Red arrows* show feeding steps

molecule of tamoxifen but a metabolite of it. Indeed, it has already been demonstrated that human pharmaceuticals can be metabolized by freshwater crustaceans (Jeon et al. 2013). We note that the pharmaceuticals studied in the latter work were not bioaccumulative compounds.

Bioconcentration and bioaccumulation dynamics

When observing our tamoxifen bioconcentration dynamics inside daphnids, we saw that it was consistent with other works on the bioconcentration of pharmaceuticals inside

Table 1 Tamoxifen ^{15}N concentration in water and tamoxifen ^{15}N content in daphnids exposed to tamoxifen via only the dietary route for 5 days

	Treatments			
	Water 5 µg/L	Water 5 µg/L and algae 390 µg/g	Water 50 µg/L	Water 50 µg/L and algae 390 µg/g
Measured TMX concentration in water at the end of the experiment (µg/L)	2	1.9	29.5	25.9
Mean (n=4) TMX content (± SD) in daphnids at the end of the experiment (µg/g)	25.69 (±7.6)	42.13 (±3.5)	250.3 (±53)	289.2 (±95)
Bioconcentration factor (± SD)	12844 (±3791)		8483 (±1794)	
Bioaccumulation factor (± SD)		22172 (±1844)		11167 (±3689)

invertebrates (Meredith-Williams et al. 2012; Anskjær et al. 2013). In these studies, the authors observed a very high bioconcentration at the beginning of exposure and then a stabilization of the content inside organisms after 24 to 48 h. Nevertheless, the PC contents in these studies were lower than those observed in our works concerning tamoxifen.

There is an increasing number of works on PC bioconcentration in invertebrates in the literature, but studies on the bioaccumulation of these contaminants are still rare. These studies are more common for other organic contaminants (e.g., perfluoroalkyl, polychlorobiphnyles, and orhexachlorobenzene). However, all these studies demonstrated that for pelagic organisms, aqueous medium is the predominant route of contamination compared to the dietary route (Muñoz et al. 1996; Dai et al. 2013; Kim et al. 2014). However, for benthic organisms, the relative contribution to the bioaccumulation of the dietary route and water can be modified (Dussault et al. 2009; Lopes et al. 2012; Bertin et al. 2014).

Ecosystemic consequences

We can describe at least two of the consequences of these bioaccumulations at ecosystem scale: biomagnification and decreasing fitness. Indeed, many works have already shown that organic contaminant transfer, notably of PCs, can occur along the trophic web and have harmful effects on the organisms exposed (Correa-Reyes et al. 2007; Lajeunesse et al. 2011; Dai et al. 2013). Moreover, it has been demonstrated that PCs can be transferred after several generations (Kim et al. 2014). This "multigenerational effect" is consistent with the work of Chiaia-Hernandez et al. (2013), who showed that several PCs could be significantly accumulated in daphnia eggs. Lastly, Montiglio and Royauté (2014) highlighted the need to take into account anthropogenic contaminants in the behavioral disturbances of several organisms (e.g., fish, crustaceans, insects, birds, etc.).

Conclusion and perspectives

This work, as well as previous experiments on bioconcentration in microalgae (Orias et al. 2015), showed that tamoxifen has strong bioaccumulative potential in the first levels of the trophic web considered. The experiment described in this paper focused on the transfer of tamoxifen in daphnids, the second level of this experimental trophic web, on the one hand due to immersion in the contaminated medium and, on the other hand, from the dietary route via the consumption of contaminated algae. Under these experimental conditions, we observed that this consumption plays a key role, especially when the concentration of medium is lower. In other words, we can say that the lower the concentration in medium, the greater the role played by the dietary route in contamination by tamoxifen. In the future, it would be interesting to test lower levels of food and medium contamination in order to get closer to environmental conditions. Moreover, assays integrating a secondary consumer (i.e., a fish species) could enrich knowledge on the bioaccumulation of pharmaceuticals such as tamoxifen along a longer trophic web.

In addition, in this study, we used stable isotopes for the first time in order to monitor an organic contaminant in an invertebrate. Our results highlighted the advantages of this method for studying the bioaccumulation of contaminants in aquatic invertebrates (especially the possibility of analyzing the material directly without prior extraction, and the small quantity of living material required for the analysis). However, stable isotope analysis does not allow identifying the potential metabolites of the molecule studied. Therefore, it is now necessary to validate our method using a direct analysis method such as chromatography.

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References

AFNOR (1980) Détermination de l'inhibition de la croissance de Scenedesmus subspicatus par une substance

Anskjær G, Rendal C, Kusk K (2013) Effect of pH on the toxicity and bioconcentration of sulfadiazine on Daphnia magna. Chemosphere 91(8):1183–1188

Barnes KK, Kolpin DW, Furlong ET, Zaugg SD, Meyer MT, Barber LB (2008) A national reconnaissance of pharmaceuticals and other organic wastewater contaminants in the United States—I) Groundwater. Sci Total Environ 402:192–200. doi:10.1016/j.scitotenv.2008.04.028

Bertin D, Ferrari BJD, Labadie P, Sapin A, Garric J, Budzinski H, Houde M, Babut M (2014) Bioaccumulation of perfluoroalkyl compounds in midge (Chironomus riparius) larvae exposed to sediment. Environ Pollut 189:27–34. doi:10.1016/j.envpol.2014.02.018

Blair BD, Crago JP, Hedman CJ, Klaper RD (2013) Pharmaceuticals and personal care products found in the Great Lakes above concentrations of environmental concern. Chemosphere 93:2116–2123. doi:10.1016/j.chemosphere.2013.07.057

Chiaia-Hernandez AC, Ashauer R, Moest M, Hollingshaus T, Jeon J, Spaak P, Hollender J (2013) Bioconcentration of organic contaminants in daphnia resting eggs. Environ Sci Technol 47:10667–10675. doi:10.1021/es401763d

Claessens M, Vanhaecke L, Wille K, Janssen CR (2013) Emerging contaminants in Belgian marine waters: single toxicant and mixture risks of pharmaceuticals. Mar Pollut Bull 71:41–50. doi:10.1016/j.marpolbul.2013.03.039

Clément B, Zaid S (2004) A new protocol to measure the effects of toxicants on daphnid–algae interactions. Chemosphere 55(10):1429–1438

Coetsier CM, Spinelli S, Lin L, Roig B, Touraud E (2009) Discharge of pharmaceutical products (PPs) through a conventional biological

sewage treatment plant: MECs vs PECs? Environ Int 35:787–792. doi:10.1016/j.envint.2009.01.008

Coplen TB (2011) Guidelines and recommended terms for expression of stableisotope-ratio and gas-ratiom easurement results. Rapid Commun Mass Spectrom 25:2538–2560

Correa-Reyes G, Viana MT, Marquez-Rocha FJ, Licea AF, Ponce E, Vazquez-Duhalt R (2007) Nonylphenol algal bioaccumulation and its effect through the trophic chain. Chemosphere 68:662–670. doi:10.1016/j.chemosphere.2007.02.030

Dai Z, Xia X, Guo J, Jiang X (2013) Bioaccumulation and uptake routes of perfluoroalkyl acids in daphnia magna. Chemosphere 90:1589–1596. doi:10.1016/j.chemosphere.2012.08.026

Dussault ÈB, Balakrishnan VK, Borgmann U, Solomon KR, Sibley PK (2009) Bioaccumulation of the synthetic hormone 17α-ethinylestradiol in the benthic invertebrates Chironomus tentans and Hyalella azteca. Ecotoxicol Environ Saf 72:1635–1641. doi:10.1016/j.ecoenv.2009.04.019

Fick J, Söderström H, Lindberg RH, Phan C, Tysklind M, Larsson DGJ (2009) Contamination of surface, ground, and drinking water from pharmaceutical production. Environ Toxicol Chem 28:2522–2527. doi:10.1897/09-073.1

Geyer HJ, Scheunert I, Brüggemann R, Steinberg C, Korte F, Kettrup A (1991) QSAR for organic chemical bioconcentration in Daphnia, algae, and mussels. QSAR Environ Toxicol 109–110:387–394. doi:10.1016/0048-9697(91)90193-I

Jean J, Perrodin Y, Pivot C, Trepo D, Perraud M, Droguet J, Tissot-Guerraz F, Locher F (2012) Identification and prioritization of bioaccumulable pharmaceutical substances discharged in hospital effluents. J Environ Manage 103:113–121. doi:10.1016/j.jenvman.2012.03.005

Jeon J, Kurth D, Hollender J (2013) Biotransformation pathways of biocides and pharmaceuticals in freshwater crustaceans based on structure elucidation of metabolites using high resolution mass spectrometry. Chem Res Toxicol 26:313–324. doi:10.1021/tx300457f

Kim HY, Jeon J, Hollender J, Yu S, Kim SD (2014) Aqueous and dietary bioaccumulation of antibiotic tetracycline in D. magna and its multigenerational transfer. J Hazard Mater 279:428–435. doi:10.1016/j.jhazmat.2014.07.031

Kolpin DW, Furlong ET, Meyer MT, Thurman EM, Zaugg SD, Barber LB, Buxton HT (2002) Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999−2000: a national reconnaissance. Environ Sci Technol 36:1202–1211. doi:10.1021/es011055j

Lajeunesse A, Gagnon C, Gagné F, Louis S, Čejka P, Sauvé S (2011) Distribution of antidepressants and their metabolites in brook trout exposed to municipal wastewaters before and after ozone treatment—evidence of biological effects. Chemosphere 83:564–571. doi:10.1016/j.chemosphere.2010.12.026

Loos R, Gawlik BM, Locoro G, Rimaviciute E, Contini S, Bidoglio G (2009) EU-wide survey of polar organic persistent pollutants in European river waters. Environ Pollut 157:561–568. doi:10.1016/j.envpol.2008.09.020

Loos R, Locoro G, Comero S, Contini S, Schwesig D, Werres F, Balsaa P, Gans O, Weiss S, Blaha L, Bolchi M, Gawlik BM (2010) Pan-European survey on the occurrence of selected polar organic persistent pollutants in ground water. Water Res 44:4115–4126. doi:10.1016/j.watres.2010.05.032

Lopes C, Persat H, Babut M (2012) Transfer of PCBs from bottom sediment to freshwater river fish: a food-web modelling approach in the Rhône River (France) in support of sediment management. Ecotoxicol Environ Saf 81:17–26. doi:10.1016/j.ecoenv.2012.04.007

López-Serna R, Petrović M, Barceló D (2012) Occurrence and distribution of multi-class pharmaceuticals and their active metabolites and transformation products in the Ebro River basin (NE Spain). Sci Total Environ. doi:10.1016/j.scitotenv.2012.06.027

Meredith-Williams M, Carter LJ, Fussell R, Raffaelli D, Ashauer R, Boxall ABA (2012) Uptake and depuration of pharmaceuticals in aquatic invertebrates. Environ Pollut 165:250–258

Montiglio P-O, Royauté R (2014) Contaminants as a neglected source of behavioural variation. Anim Behav 88:29–35. doi:10.1016/j.anbehav.2013.11.018

Muñoz MJ, Ramos C, Tarazona J (1996) Bioaccumulation and toxicity of hexachlorobenzene in chlorella vulgaris and daphnia magna. Aquat Toxicol 35:211–220. doi:10.1016/0166-445X(96)00010-0

Orias F, Simon L, Perrodin Y (2015) Experimental assessment of the bioconcentration of 15N-tamoxifen in Pseudokirchneriella subcapitata. Chemosphere 122:251–256. doi:10.1016/j.chemosphere.2014.11.070

Roberts PH, Thomas KV (2006) The occurrence of selected pharmaceuticals in wastewater effluent and surface waters of the lower Tyne catchment. Sci Total Environ 356:143–153. doi:10.1016/j.scitotenv.2005.04.031

Schultz MM, Furlong ET, Kolpin DW, Werner SL, Schoenfuss HL, Barber LB, Blazer VS, Norris DO, Vajda AM (2010) Antidepressant pharmaceuticals in two U.S. Effluent-impacted streams: occurrence and fate in water and sediment, and selective uptake in fish neural tissue. Environ Sci Technol 44:1918–1925. doi:10.1021/es9022706

Thomas KV, Hilton MJ (2004) The occurrence of selected human pharmaceutical compounds in UK estuaries. Mar Pollut Bull 49:436–444. doi:10.1016/j.marpolbul.2004.02.028

Vazquez-Roig P, Andreu V, Blasco C, Picó Y (2012) Risk assessment on the presence of pharmaceuticals in sediments, soils and waters of the Pego–Oliva Marshlands (Valencia, eastern Spain). Sci Total Environ 440:24–32. doi:10.1016/j.scitotenv.2012.08.036

Yang Y, Fu J, Peng H, Hou L, Liu M, Zhou JL (2011) Occurrence and phase distribution of selected pharmaceuticals in the Yangtze Estuary and its coastal zone. J Hazard Mater 190:588–596. doi:10.1016/j.jhazmat.2011.03.092

Zenker A, Cicero MR, Prestinaci F, Bottoni P, Carere M (2014) Bioaccumulation and biomagnification potential of pharmaceuticals with a focus to the aquatic environment. J Environ Manage 133:378–387. doi:10.1016/j.jenvman.2013.12.017

8.3 Synthèse

Pour la première fois à notre connaissance, la bioaccumulation et la bioconcentration d'un contaminant organique ont été mesurées chez un invertébré. Les résultats de ces expérimentations montrent que le tamoxifen est très rapidement bioconcentrable dans les daphnies et en forte proportion. Ces travaux montrent également que le régime alimentaire à un rôle non négligeable dans la contamination par le RP étudié, et que plus la concentration dans le milieu est faible, plus cet impact est important. Il est maintenant nécessaire de remonter encore le long de la chaine alimentaire en étudiant la dynamique du tamoxifen chez un consommateur secondaire.

Chapitre 9

Bioconcentration du ^{15}N tamoxifen chez *Danio rerio*

9.1 Enjeux et démarche

A ce stade, nous avons réussi à mesurer un transfert important du tamoxifen dans les deux premiers maillons de la chaine alimentaire. Ainsi, nous arrivons maintenant au troisième niveau : le consommateur secondaire. Chez les daphnies, il a été techniquement possible d'évaluer à la fois la bioaccumulation et la bioconcentration. Chez le poisson, nous n'avons toutefois eu seulement la possibilité d'évaluer la bioconcentration. De même que pour les deux premiers niveaux trophiques étudiés, l'objectif de ces expérimentations était double : (i) mesurer si nous observons un enrichissement en ^{15}N significatif pour quantifier une accumulation de tamoxifen dans notre organisme, ici le poisson (ii) déterminer la dynamique du tamoxifen dans le temps et dans différents organes. Nous avons également mesuré l'évolution du tamoxifen dissout afin de déterminer à quelles concentrations les poissons étaient réellement exposés. Les poissons ont été exposés 21 jours à 3 concentrations de tamoxifen : 0,1 μg/L, 1 μg/L et 10 μg/L. A chaque prélèvement, foie, gonades et muscles ont été récupérés et l'enrichissement en ^{15}N y est mesuré. Nous nous sommes intéressés à ces trois organes à cause de leur nature différente, présentant toutes un intérêt spécifique : (i) Le foie est l'organe responsable de la détoxification des contaminants et est très riche en lipide. (ii) Les gonades sont très riches en lipides. Ainsi, une molécule très hydrophobe comme le tamoxifen risque d'être présente dans cet organe. (iii) Les muscles sont pauvres en lipides est souvent témoins d'une contamination chronique. La présence de tamoxifen dans cet organe après 21 jours pourrait présenter un risque supplémentaire pour les organismes exposés.

9.2 Article 7 : *Bioconcentration of ^{15}N-tamoxifen at environmental concentration in liver, gonad and muscle of* Danio rerio

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Bioconcentration of ^{15}N-tamoxifen at environmental concentration in liver, gonad and muscle of *Danio rerio*

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ABSTRACT

Pharmaceutical compounds (PCs) are ubiquitous in aquatic ecosystems. In addition to the direct ecotoxicological risk presented by certain PCs, others can accumulate inside organisms and along trophic webs, subsequently contaminating whole ecosystems. We studied the bioconcentration of a bioaccumulative PC already found several times in the environment: tamoxifen. To this end, we exposed *Danio rerio* for 21 d to ^{15}N-tamoxifen concentrations ranging from 0.1 to 10 μg/L and used an analytic method based on stable isotopes to evaluate the tamoxifen content in these organisms. The evolution of the ^{15}N/^{14}N ratio was thus measured in liver, muscle and gonads of exposed fish compared to control fish. We succeeded in quantifying ^{15}N-tamoxifen bioconcentrations at all the exposure concentrations tested. The highest bioconcentration factors of tamoxifen measured were 14,920 in muscle, 73,800 in liver and 85,600 in gonads of fish after 21 d exposure at a nominal concentration of 10 μg/L. However, these bioconcentration factors have to be considered as maximal values (BCF_{MAX}). Indeed, despite its proven stability, tamoxifen can be potentially partially degraded during experiments. We now need to refine these results by using a direct analytic method (i.e. LC–MS/MS).

1. Introduction

Nowadays, pharmaceutical compounds (PCs) are found in all parts of aquatic ecosystems: water, sediment and organisms. Two main sources are responsible for the emission of PCs: (i) wastewater from pharmaceutical factories (Collado et al., 2014; Larsson et al., 2007), (ii) urban wastewater (domestic and hospital) (Kümmerer, 2009; Verlicchi et al., 2010). It has already been demonstrated that these PCs could have harmful effects on organisms or at the ecosystem scale, notably on fish, even at very low concentrations. Indeed, Brodin et al. (2013) have already shown that low concentrations of psychiatric drugs can modify the behavior of natural fishes. Moreover, Kidd et al. (2007) illustrated that only 5 ng/L of ethinylestradiol can lead to the durable collapse of the fish population of a whole lake. In addition to direct ecotoxicity, some of these PCs are sometimes found at concentrations many times higher inside organisms than in water (Zenker et al., 2014): in this case we use the term bioaccumulative compounds.

Here, we focus our work on a particularly bioaccumulative pharmaceutical: tamoxifen (Jean et al., 2012). This molecule is an anticancer drug available as tamoxifen citrate used to treat breast cancer. It has already been detected not only in sediment (Yang et al., 2011) but also in water (Ashton et al., 2004) at significant concentrations (several hundred ng/kg and several hundred ng/L, respectively). Theoretical bioconcentration factors (BCF) of tamoxifen have been estimated at around 7000 (Jean et al., 2012), using a quantitative structure activity relation (QSAR).

In this study, we measured how tamoxifen bioconcentrates inside a fish classically used in ecotoxicology, *Danio rerio*, at several concentrations, including environmental concentrations. Therefore we exposed this organism for 21 d at concentrations from 0.1 μg/L to 10 μg/L. After fish sampling at several time steps, we removed liver, gonads and muscle. These organs were chosen because: 1) tamoxifen is expected to be metabolized in liver (by cytochrome P450), 2) gonads are very rich in lipids, in which it is expected to accumulate (high K_{ow}), and 3) muscle, which is an indicator of chronic contamination.

Due to difficulties linked to the measurement of this kind of compound inside a biological matrix, we performed the measurements with a stable isotope labelled molecule (^{15}N tamoxifen) using an elemental analyzer coupled with a mass spectrometer (EA-IRMS). Therefore the evolution of the ^{15}N/^{14}N ratio was first measured in each organ, after which tamoxifen (and/or

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metabolites) enrichment was quantified. This original analytical approach had been developed during previous works (Orias et al., 2015).

2. Materials and methods

2.1. Tamoxifen ^{15}N

Tamoxifen $^{15}N^{13}C_2$ was purchased from Sigma-Aldrich chemical (St Quentin Falavier, France), prepared in methanol (1 mg/mL) and kept in a glass ampoule at −20 °C.

2.2. Danio rerio

360 female fishes (*D. rerio*) aged 3 months, taken from a fish farm certified for animal experimentation (Elevage de la Grande rivière, La fond Garel, 69490 Saint Forgeux), were used in these experiments. We chose female fishes because their gonads are larger than male fish gonads and have a higher lipid content, thereby increasing the potential bioconcentration of the contaminant studied.

2.3. Exposure conditions

The aquariums used were one piece glass tanks without seals, with a capacity of 12 L. They were filled with 10 L of synthetic medium composed of osmosed water and trace elements ($MgSO_4$ 180 mg/L, $CaCl_2$ 200 mg/L, $NaHCO_3$ 100 mg/L, $KHCO_3$ 20 mg/L). In order to limit contaminant adsorption, we eliminated the use of plastic equipment inside the aquariums. Oxygen was provided by a glass pipette and the exposure room was thermostated at 26 ± 1 °C in order to prevent the use of devices inside the aquariums. The latter were then filled with medium without contaminant and left for one week to ensure the stabilization of physicochemical parameters. Before the experiment started, the fish were distributed in all 12 aquariums (33 fishes per aquarium) for ten days for acclimation. The aquariums were covered with a plastic film to limit water evaporation. Lighting was provided by neon tubes imitating sunlight with a photoperiod of 16 h. pH, conductivity, nitrates, nitrites and the temperature of the water and air were measured daily. The fish were fed until satiety 6 times a week with Tetramin® flakes. Three times a week, feces were flushed out of the aquariums with a hand pump to limit tamoxifen adsorption on them. The fish were exposed to 3 tamoxifen concentrations (0.1 μg/L, 1 μg/L and 10μg/L) for 21 d (triplicates). Mediums were half renewed at 7 d and 14 d. The quantity of tamoxifen added at each renewal was the same as than on 0 d. Before and after each renewal, 200 mL of medium was sampled for chemical analysis (cf. Section 2.7).

Finally, these experiments conformed to European regulations concerning animal experiments and were authorized by the French Ministry of Higher Education and Research (Project no. DR2013-33, approved since 07/04/14).

2.4. Determination of dissolved tamoxifen concentration in water

Water samples were analyzed at the beginning of the experiment (0 d), before (7 d and 14 d), after each renewal (7 d+ and 14 d+) and at the end of exposure (21 d) in all treatments. The water samples were pooled by exposure concentration, by time and concentration (6 all in all), in order to obtain a sufficient amount of tamoxifen, and then filtered. Analytical measurements were performed by the Analytical Sciences Institute (CNRS, Lyon) using HPLC–MS/MS (Waters: UHPLC H-Class/Xevo TQ-S) with the following analytical parameters: Column: Phenomenex Kinetex C18 Phenyl Hexyl 100 × 2.1 mm^2, 2.6 μm; Mobile phases (A)= MilliQ quality water+0.01% acetic acid (B)=Acetonitrile (ACN); Gradient: Departure 40% B, rising to 100% B in two minutes, level for two minutes at 100% ACN, column repackaging at 40% ACN for two minutes; Flow: 0.5 mL/min; Furnace temperature: 40 °C; Injection: 2 μL; Ionization source: Positive mode electrospray; Acquisition parameters: Multi-Reaction Monitoring (MRM) mode. Detection and quantification limits were 0.15 μg/L (LOD) and 0.5 μg/L (LOQ), respectively.

2.5. Fish sampling and dissection

In order to assess tamoxifen bioconcentration dynamics, fish were sampled at: 0 d (2 h exposure), 1 d, 2 d, 3 d, 4 d, 7 d (before renewal), 10 d, 17 d, 14 d (before renewal) and 21 d. Each time, 3 fish were sampled for each concentration and each system (all in all 36 fishes at each time). Immediately afterwards, the fish were plunged in cold water (3 °C) to euthanize them. Each fish was then slightly dried on absorbent paper, weighed and measured (Fork length). Livers, gonads and muscles were then removed and pulled by triplicate. So, each sample is a combination of organs from three fishes and there is three sample by treatment. Finally, samples were plunged in liquid nitrogen and lyophilized for 7 d.

2.6. ^{15}N measurements

After freeze-drying, fish samples were weighed at 1 mg in tin capsules before analysis. Nitrogen concentration and $^{15}N/^{14}N$ ratio were measured using an isotope ratio mass spectrometer (Isoprime 100, Isoprime Ltd., Manchester, UK) coupled in continuous flow with an elemental analyzer (Flash EA 1112 Thermo Electron, Milan, Italy). Aspartic acid was used to calibrate the N concentration measurements. For isotope ratio measurements, two-point normalization of measured values was performed using international reference material IAEA-N1 and IAEA-305 according to IUPAC guidelines. $^{15}N/^{14}N$ ratios were expressed as $\delta^{15}N$ in‰ relative to atmospheric N_2 (Air):

$$\delta^{15}N = \left(^{15}N/^{14}N_{sample}/^{15}N/^{14}N_{Air}-1\right) \times 10^3$$

and converted to atom fraction (i.e. atom %; Coplen, 2011):

$$x\left(^{15}N\right) = \left(^{15}N/^{14}N_{sample}\right)/\left(1 + {}^{15}N/^{14}N_{sample}\right)$$

In-house standards calibrated against IAEA-N1 and IAEA-N2 were interspersed within the samples and the standard deviations for replicate analyses were lower than 0.2‰.

2.7. Calculation of excess ^{15}N and tamoxifen concentration

Excess ^{15}N concentration, [^{15}N], was calculated from the difference between the ^{15}N fraction ($x(^{15}N)$) of fish exposed to ^{15}N-tamoxifen and the ^{15}N fraction ($x(^{15}N)_{control}$) measured in the control treatment without exposure as follows:

$$[^{15}N] = (x(^{15}N) - x(^{15}N)_{control}) \cdot [N],$$

where [N] is the N concentration in the sample.

As ^{15}N-tamoxifen was the only ^{15}N tracer source in the experiment, and by considering that no dissociation of tamoxifen had occurred through chemical reactions or metabolic pathways (DellaGreca et al., 2007; Li et al., 2001; Negreira et al., 2014; Zhou et al., 2009; ECP, 2014), the concentration of tamoxifen was calculated from the ^{15}N concentration:

$$[\text{Tamoxifen}]\ \left(\mu g\ g^{-1}\right) = \left[^{15}N\right]/15 \cdot M_{\text{Tamoxifen}} \cdot 10^6$$

with $M_{\text{tamoxifen}}$ (374.49 g/mol) the molar mass of [^{13}C,

^{15}N]-tamoxifen.

These tamoxifen concentrations should be considered as maximal concentrations. Indeed, despite the stability of tamoxifen, partial degradation is always possible during the experiment.

A detection limit of tamoxifen in the fish samples of 1.5 μg g^{-1} was calculated using this equation from the detection limit of the difference between the ^{15}N/^{14}N ratio, less than 0.2‰, resulting in an estimation of the quantification limit of 4.6 μg g^{-1}.

2.8. Bioconcentration factor of the tamoxifen calculation

To calculate the BCF of a contaminant, it is necessary to reach equilibrium between excretion and assimilation constants in organisms. Here, considering the bioconcentration dynamics in the fish studied, we estimated that equilibrium was reached at 21 d.

The bioconcentration factors of tamoxifen were then calculated according to the following equation:

$$\text{BCF} = \frac{\text{Tamoxifen content quantified in organ (g/kg) at equilibrium}}{\text{Tamoxifen concentration measured in water (g/kg) at equilibrium}}$$

As in Section 2.7, these BCF should be considered as maximal BCFs, considering the potential partial degradation of tamoxifen. That is why from now in this paper, we will refer to BCF_{MAX}.

2.9. Statistical analysis

δ^{15}N values were compared between treatments with a repeated measures analysis of variance (ANOVA) with the tamoxifen concentration ("treatment") and time as independent factors, followed by a Tukey HSD posthoc test to determine which treatments differed. Statistical analyses were performed using JMP8 software (SAS Institute) and statistical significance was accepted at $\alpha < 0.05$.

3. Results

3.1. Physicochemical and biological parameters

During the 21 d of exposure, the temperatures of air and water fluctuated between 25 and 27 °C. Conductivity oscillated between 650 and 750 μS/cm and pH between 6.8 and 7.6. Gradually, during the experiment, we observed an increase of nitrate and nitrite concentrations reaching up to 250 mg/L and 10 mg/L, respectively, by the end of the experiment. Nevertheless, it is noteworthy that we did not observe any mortality and *D. rerio* is tolerant to these amounts of nitrite and nitrate concentrations (Macova et al., 2008). Otherwise, the weight and size measurements did not allow us to highlight differences in weight or size between treatments (423 ± 129 mg and 27 ± 2.4 mm).

Tamoxifen concentrations measured in water by HPLC for the highest exposure concentration (10 μg/L) at the beginning and at the end (0 d and 21 d) of the experiment, and also before (7 d and 14 d) and after (7 d+ and 14 d+) each renewal, are presented in Table 1. From the beginning of the experiment, we observed that the real concentration was under 50% of the nominal concentration (10 μg/L). At 7 d, the concentration of tamoxifen in water was under 150 ng/L (LOD). After both renewals, the dissolved tamoxifen concentration did not reach more than 33% of the nominal concentration and fell to around 500 ng/L after 7 d.

Table 1
Tamoxifen (TMX) concentration in water during the experiment for the "10 μg/L" treatment. "+" means after renewal of half of the medium.

Time (d)	0	7	7+	14	14+	21
TMX concentration in water (μg TMX/L)	4.8	< LOD	3.3	0.6	3.2	0.5

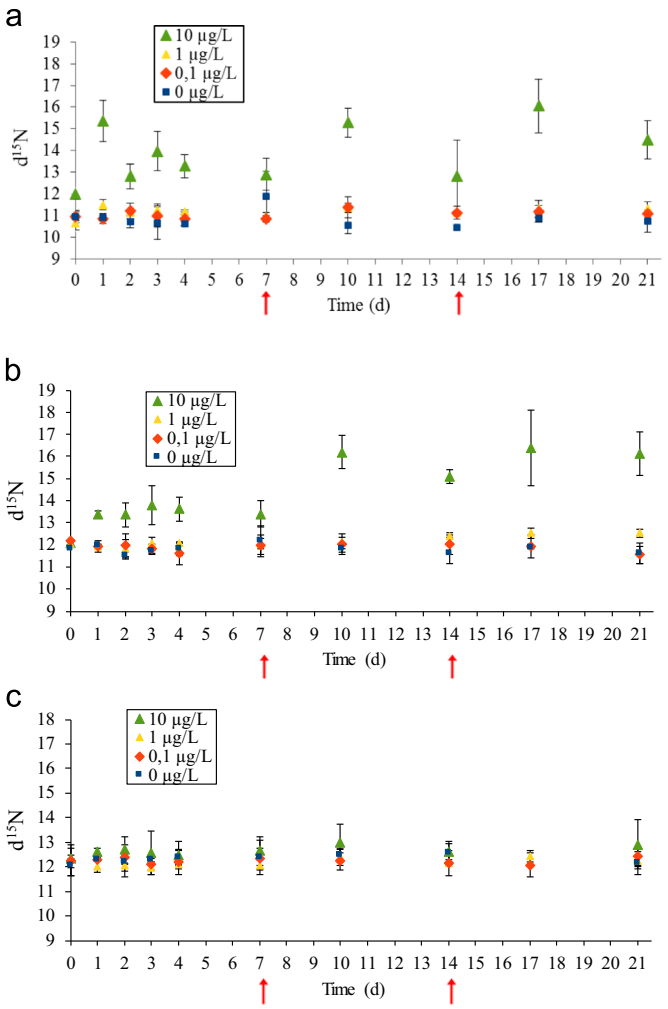

Fig. 1. (a) Evolution of the ^{15}N/^{14}N ratio (d^{15}N) in *Danio rerio* livers exposed to ^{15}N tamoxifen for 21 d. Red arrows mean half-renewal and error bars show standard deviations (2SE). (b) Evolution of the ^{15}N/^{14}N ratio (d^{15}N) in *Danio rerio* gonads exposed to ^{15}N tamoxifen 21 d. Red arrows mean half-renewal and error bars show standard deviations (2SE). (c) Evolution of the ratio ^{15}N/^{14}N (d^{15}N) in *Danio rerio* muscles exposed to ^{15}N tamoxifen for 21 d. Red arrows mean half-renewal and error bars show standard deviations (2SE). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. Evolution of ratio ^{15}N/^{14}N in organs

Without exposure to tamoxifen, the δ^{15}N of liver, gonads and muscles remained constant throughout the experiment (Fig. 1a; δ^{15}N = 11‰ for liver, 12‰ for gonads and muscles). At the exposure concentration of 10 μg/L tamoxifen, liver δ^{15}N (Fig. 1a) exhibited a significant increase from 11‰ to 15‰ (repeated measures ANOVA, "Concentration" effect, and Tukey HSD posthoc, $p < 0.0001$). As ^{15}N-tamoxifen was the only supply of ^{15}N in the experiment compared to the control, this significant ^{15}N enrichment indicates that tamoxifen was incorporated in liver tissues. ^{15}N enrichment (and therefore tamoxifen incorporation) occurred from the first hours of exposure, followed by a slow decrease to a δ^{15}N value of 13‰ at 7 d. ^{15}N enrichment increased up to 16‰ after each renewal of the medium (i.e. 7 d and 14 d). A slight increase of liver δ^{15}N (from 11‰ to 12‰) was measured for an exposure concentration of 0.1 and 1 μg/L of tamoxifen (Fig. 1a), but

this ^{15}N enrichment was not significant (Tukey HSD posthoc, respectively $p=0.743$ and $p=0.158$ for 0.1 and 1 μg/L).

In fish exposed to a tamoxifen concentration of 10 μg/L, $\delta^{15}N$ values of gonads rapidly increased from 12‰ to 13.5‰ at 7 d, and to 16.5‰ after the first medium renewal (Fig. 1b). ^{15}N enrichment significantly increased with the concentration of tamoxifen (repeated measures ANOVA, "Concentration" effect, $p<0.0001$), and ^{15}N enrichment was significantly higher at exposure to 10 μg/L than to 0.1 and 1 μg/L (Tukey HSD posthoc, $p<0.0001$). A slight increase of gonad $\delta^{15}N$ (from 12‰ to 13‰) was measured for an exposure concentration of 0.1 and 1 μg/L of tamoxifen (Fig. 1b), but this ^{15}N enrichment was not significant at 0.1 μg/L (Tukey HSD posthoc, $p=0.83$ and $p<0.01$ for 0.1 and 1 μg/L, respectively).

In muscles, $\delta^{15}N$ values exhibited a slight increase from 12‰ to 12.6‰ in the treatment with a tamoxifen concentration of 10 μg/L (Fig. 1c), significantly higher than in the control experiment and a lower tamoxifen concentration (repeated measure ANOVA, "Concentration" effect, and Tukey HSD posthoc, $p<0.01$). No significant ^{15}N enrichment was measured in the treatment with a tamoxifen concentration of 0.1 and 1 μg/L (Tukey HSD posthoc, $p>0.19$).

3.3. Estimation of tamoxifen content in fish from ^{15}N concentration

In these experiments, ^{15}N tamoxifen was the only source of ^{15}N compared to the control treatment without exposure to tamoxifen. We therefore attempted to estimate the concentration of tamoxifen incorporated in the different fish tissues on the basis of ^{15}N enrichment (cf. Section 2.6). As the increase of $\delta^{15}N$ during the experiments remained low, we calculated a tamoxifen concentration only when significant differences of $\delta^{15}N$ were measured in comparison to the control (i.e. for the treatment at 10 μg/L for the 3 organs, and the treatment at 1 μg/L for gonads). Moreover, the concentrations calculated are shown only when average values are higher than the quantification limit of 4600 ng/g.

3.3.1. Exposition at 10 μg/L

At the highest concentration tested, 10 μg/L, all the organs were contaminated rapidly and durably. Indeed, in the three organs observed, average tamoxifen contents were higher than the quantification limit from the beginning to the end of the experiment. The least contaminated of the three organs was the muscle, which presented average contents of 5100 (±4700) ng/g at 2 d and 7460 (±2600) ng/g at the end of the experiment. Gonad and liver presented much higher contents. Indeed, the highest average contents in the liver (17 d) and in the gonad (21 d) were respectively 45,400 (±6900) and 42,800 (±10,200) ng/g. Bioconcentration dynamics inside these organs were quite similar. During the first days of the experiment, the average contents increased slightly until 3 d (liver: 29,900 (±7000) ng/g; gonad: 21,700 (±9700) ng/g) and then decreased before the first renewal. Three days later (10 d), we observed a strong increase in all the organs, particularly in liver (44,200 ± 8000 ng/g) and gonads (40,200 ± 9100 ng/g), before decreasing again until the second renewal, with respective contents of 19,200 (±15,400) ng/g and 32,000 ± (3000) ng/g. Finally, after the second renewal, we found similar contents in liver and gonad, between 35,000 and 45,000 ng/g (Fig. 2a).

3.3.2. Exposure at 1 μg/L

With our analytic method based on the $^{15}N/^{14}N$ measurement, we did not succeed in highlighting any significant muscle bioconcentration of tamoxifen in fish exposed to 1 μg/L. In liver, only four significant contents were measured from 4700 (±2510) ng/g at 3 d to 7900 (±1920) ng/g at 10 d. The final average tamoxifen content measured in liver was 5570 (±1800) ng/g. In gonad, three average contents were quantified at 14 d, 17 d and 21 d with 7370 (±610), 5860 (±2210) and 8580 ± 1750 ng/g, respectively (Fig. 2b).

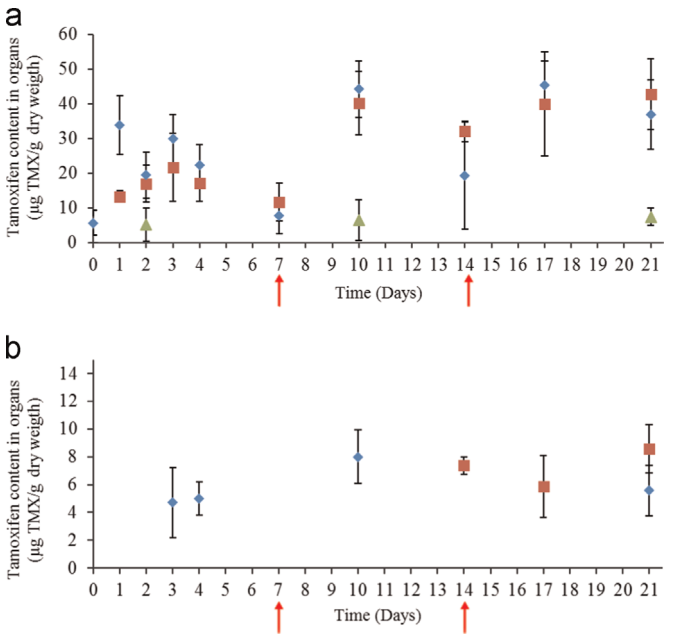

Fig. 2. (a) Tamoxifen content in liver (blue diamond), gonads (red square) and muscle (green triangle) as a function of time of fish exposed to 10 μg/L tamoxifen ^{15}N. Red arrows show renewal of half of the medium. Error bars show standard errors (2SE). (b) Tamoxifen content in liver (blue diamond), gonads (red square) and muscle (green triangle) as a function of time of fish exposed to 1 μg/L tamoxifen ^{15}N. Red arrows show renewal of half of the medium. Error bars show standard errors (2SE). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3.3. Exposureat 0.1 μg/L

Only one average content was quantified at this concentration. At 10 d, we found 7140 (±4540) ng/g in fish liver.

3.4. Bioconcentration factors of tamoxifen (BCF_{MAX})

The maximum bioconcentration factors calculated for each organ are gathered in Table 2. It shows that gonads and liver are the organs (of the three studied here) that bioconcentrate tamoxifen most, with an average BCF_{MAX} reaching 85,600 and 73,800, respectively. Muscle bioconcentrates tamoxifen but with a lower BCF_{MAX} of around 15,000.

4. Discussion

4.1. ^{15}N enrichment and bioconcentration

^{15}N labeled tamoxifen was used to trace the incorporation of tamoxifen in *D. rerio* organs (liver, gonads and muscles).

Table 2
Tamoxifen contents (μg/g) and bioconcentration factors in muscles, gonads and livers of *Danio rerio* after 21 d exposure to 10 μg/L tamoxifen.

	TMX content in organs at day 21 (ng TMX/g dry weight)		BCF_{MAX} (after 21 d) at 10 μg/L	
	Mean	Standard error	Mean	Standard error
Muscles	7460	2600	14,920	5200
Gonads	42,800	10,200	85,600	20,400
Livers	36,900	10,000	73,800	20,000

Significant ^{15}N enrichment at 10 μg/L exposure demonstrated that tamoxifen was transferred from water to these three organs. Environmental water is therefore a major contamination pathway for tamoxifen. Higher ^{15}N enrichments in liver and gonads than in muscles were measured. These results indicate higher tamoxifen accumulation in these organs, as was expected, as liver and gonads exhibit higher metabolic turnover rates and lipid contents than muscles. At lower exposure concentrations, ^{15}N enrichment was much lower, but nevertheless present in gonads and liver. Tamoxifen therefore accumulated in these organs even at low concentration in water, close to concentrations reported for tamoxifen in river water (Ashton et al., 2004; Yang et al., 2011).

Several studies have already shown the bioaccumulative potential of PCs in fish. Pharmaceuticals are found in all fish compartments (i.e. brain, plasma, gills, liver, muscle, etc.) even in fish exposed at environmental concentrations (Al-Ansari et al., 2010; Chair et al., 1996; Garcia et al., 2012; Lajeunesse et al., 2011; Mimeault et al., 2005). However, in the literature, information on the mechanisms of this bioconcentration is still scarce.

In our experiments, the liver is the only organ in which we could quantify tamoxifen at all the concentrations tested (0.1, 1 and 10 μg/L). These results show that liver is the organ most concerned by the bioconcentration of tamoxifen. The other organs were also contaminated when we increased the exposure concentration. We were able to quantify a significant tamoxifen content in gonads immediately on reaching 1 μg/L, but only at 10 μg/L for muscle. This could the consequence of progressive liver "saturation" when exposure to the concentration increased. Above 1 μg/L, the metabolization capacities of liver were exceeded and tamoxifen diffused into other organs. As gonads have a high lipid content, tamoxifen bioconcentration was privileged in this organ. These results are consistent with those of Liu et al. (2014), who observed a change in the distribution of roxythromycin, another PC, in the organs of *Carassius auratus* according to exposure concentration. However, we need more data for the lowest concentration (i.e. 0.1 μg/L) to fully validate this hypothesis.

At the highest concentrations (1 and 10 μg/L), we could see that the average contents inside liver and gonads were similar (around 40,000 ng/g) at the end of exposure. However, if the respective masses of these organs in *D. rerio* are considered, it can be seen that the highest contaminant load was present in gonads. Indeed, liver and gonads are approximately and respectively 1% and 10% of the organism's dry weight (Naderi et al., 2014). These observations lead to reflection on the consequences of this bioconcentration on future fish generations.

The maximal bioconcentration factors obtained for tamoxifen in our experiments are the highest ever measured. Some authors have identified several bioaccumulative PCs such as diclofenac (BCF between 69 and 2730) (Schwaiger et al., 2004), carbamazepine (BCF from 1.9 to 4.6) (Garcia et al., 2012) and a sertraline metabolite (i.e. desmethylsertraline), with a BCF in *Salvelinus fontinalis* liver (i.e. brook trout) of 12,250 (Lajeunesse et al., 2011).

4.2. Nominal, measured and real exposure concentrations in water

Great care had to be taken to limit the adsorption of tamoxifen and thus maximize tamoxifen dissolution during the experiment: air heating rather than water in order to prevent the use of plastic devices inside the aquariums, use of a glass pipette for oxygen, use of single piece tanks without plastic seals and the drainage of feces. Nevertheless, when we observed the values measured for dissolved tamoxifen, we realized that they were insufficient because the nominal concentration had not been reached even at the beginning of the experiment. This gap between real and nominal values at the beginning of the experiment (52%) was certainly due to the adsorption of tamoxifen, a highly hydrophobic compound, on the wall of the aquarium. Before the first renewal (7 d), the dissolved tamoxifen concentration was under the LOD (150 ng/L), probably because of the continuous adsorption on the wall of the aquariums but also on (and inside) the fish, and on the suspended matter accumulating in the aquariums during this period. Indeed, accumulated feces can trap tamoxifen and thus remove a large amount of tamoxifen from the aquarium. After the following renewals (day 7+ and day 14+), we saw a strong decrease of the dissolved concentration but with similar concentrations at day 14 and day 21 (0.6 and 0.5 μg/L, respectively). These analytic results show that tamoxifen remained in the medium even after several days and despite a high concentration of suspended matter.

4.3. Tamoxifen and metabolites

The analytical method used to measure the tamoxifen content in a biological matrix is an indirect method. In our study, initially, we considered that each supplementary ^{15}N atom inside the organism corresponded to one molecule of tamoxifen. Indeed, tamoxifen is known to be a relatively stable molecule. This state has been shown under different conditions: in the aquatic environment (ECP, 2014), in wastewater treatment plant effluent (Negreira et al., 2014; Zhou et al., 2009); under gastric conditions (Li et al., 2001), and under sunlight conditions (DellaGreca et al., 2007).

However, despite this stability, partial degradation could nonetheless have occurred under the specific conditions of our experiments. That is why we decided to consider that the tamoxifen bioconcentration measured corresponded to the maximum, and that the calculated BCF corresponded to BCF_{MAX}.

Moreover, if the hypothesis according to which "no biodegradation of tamoxifen is possible" is correct for organisms such as algal cells, this is probably not quite true for organisms such as fish. Indeed, fish such as *D. rerio* share a large proportion of biocellular characteristics with human beings (Gunnarsson et al., 2008). In addition, several authors have demonstrate that metabolites could be accumulate inside fish organs (Brooks et al., 2005; Lajeunesse et al., 2011).

5. Conclusion

In this work, we measured for the first time the bioconcentration dynamics of an anticancer drug detected several times in the environment in three organs (liver, muscle and gonads) of a vertebrate organism (*D. rerio*). Our results show that tamoxifen accumulates considerably in fish, especially in fish gonad and liver, with a BCF_{MAX} up to 85,600. We also showed that even at environmental concentrations (i.e. several hundred ng/L), tamoxifen bioconcentrates significantly. In our experiments, we confirm that bioconcentration differs according to the organ considered (Lajeunesse et al., 2011): tamoxifen content is higher in organs with high lipid contents. Bioconcentration also depends on the exposure concentration. Indeed, we obtained higher contents inside organisms exposed to higher nominal tamoxifen concentrations.

Additionally, in these experiments we used isotopic chemistry for the first time to measure the bioconcentration of a PC in a vertebrate. This method has several advantages: all matrixes can be analyzed (e.g. sediments, plants, invertebrates, etc.), a few hundred μg are enough for a sample, and all the extraction processes leading to the loss of contaminant can be avoided. However, this method has a major disadvantage as it measures every ^{15}N labelled molecule, without distinguishing between the original molecule and potential metabolites. However, in our work, this limit was offset by the fact that tamoxifen is a persistent molecule (ECP, 2014; DellaGreca et al., 2007; Li et al., 2001; Negreira et al., 2014; Zhou et al., 2009) that metabolizes little. It is noteworthy

that fish are nevertheless probably capable of metabolizing tamoxifen (Gunnarsson et al., 2008). Thus in future research it will be necessary to perform analytic experiments with LC–MS/MS analysis, for example, to determine the precise rates of original tamoxifen and metabolites in the matrixes analyzed.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2015.06.033.

References

Al-Ansari, A.M., Saleem, A., Kimpe, L.E., Sherry, J.P., McMaster, M.E., Trudeau, V.L., Blais, J.M., 2010. Bioaccumulation of the pharmaceutical 17α-ethinylestradiol in shorthead redhorse suckers (*Moxostoma macrolepidotum*) from the St. Clair River. Can. Environ. Pollut. 158, 2566–2571. http://dx.doi.org/10.1016/j.envpol.2010.05.020.

Ashton, D., Hilton, M., Thomas, K.V., 2004. Investigating the environmental transport of human pharmaceuticals to streams in the United Kingdom. Sci. Total Environ. 333, 167–184. http://dx.doi.org/10.1016/j.scitotenv.2004.04.062.

Brodin, T., Fick, J., Jonsson, M., Klaminder, J., 2013. Dilute concentrations of a psychiatric drug alter behavior of fish from natural populations. Science 339, 814–815.

Brooks, B.W., Chambliss, C.K., Stanley, J.K., Ramirez, A., Banks, K.E., Johnson, R.D., Lewis, R.J., 2005. Determination of select antidepressants in fish from an effluent-dominated stream. Environ. Toxicol. Chem. 24, 464–469. http://dx.doi.org/10.1897/04-081R.1.

Chair, M., Nelis, H.J., Leger, P., Sorgeloos, P., de Leenheer, A.P., 1996. Accumulation of trimethoprim, sulfamethoxazole, and N-acetylsulfamethoxazole in fish and shrimp fed medicated *Artemia franciscana*. Antimicrob. Agents Chemother. 40, 1649–1652.

Collado, N., Rodriguez-Mozaz, S., Gros, M., Rubirola, A., Barceló, D., Comas, J., Rodriguez-Roda, I., Buttiglieri, G., 2014. Pharmaceuticals occurrence in a WWTP with significant industrial contribution and its input into the river system. Environ. Pollut. 185, 202–212. http://dx.doi.org/10.1016/j.envpol.2013.10.040.

Coplen, T.B., 2011. Guidelines and recommended terms for expression of stable-isotope-ratio and gas-ratio measurement results. Rapid Commun. Mass Spectrom. 25, 2538–2560. http://dx.doi.org/10.1002/rcm.5129.

DellaGreca, M., Iesce, M.R., Isidori, M., Nardelli, A., Previtera, L., Rubino, M., 2007. Phototransformation products of tamoxifen by sunlight in water. Toxicity of the drug and its derivatives on aquatic organisms. Chemosphere 67, 1933–1939. http://dx.doi.org/10.1016/j.chemosphere.2006.12.001.

ECP, Environmentally Classified Pharmaceuticals, 2014–2015, ⟨www.janusinfo.se/environement⟩.

Garcia, S.N., Foster, M., Constantine, L.A., Huggett, D.B., 2012. Field and laboratory fish tissue accumulation of the anti-convulsant drug carbamazepine. Ecotoxicol. Environ. Saf. 84, 207–211. http://dx.doi.org/10.1016/j.ecoenv.2012.07.013.

Gunnarsson, L., Jauhiainen, A., Kristiansson, E., Nerman, O., Larsson, D.G.J., 2008. Evolutionary conservation of human drug targets in organisms used for environmental risk assessments. Environ. Sci. Technol. 42, 5807–5813. http://dx.doi.org/10.1021/es8005173.

Jean, J., Perrodin, Y., Pivot, C., Trepo, D., Perraud, M., Droguet, J., Tissot-Guerraz, F., Locher, F., 2012. Identification and prioritization of bioaccumulable pharmaceutical substances discharged in hospital effluents. J. Environ. Manag. 103, 113–121. http://dx.doi.org/10.1016/j.jenvman.2012.03.005.

Kidd, K.A., Blanchfield, P.J., Mills, K.H., Palace, V.P., Evans, R.E., Lazorchak, J.M., Flick, R.W., 2007. Collapse of a fish population after exposure to a synthetic estrogen. Proc. Natl. Acad. Sci. USA 104, 8897–8901. http://dx.doi.org/10.1073/pnas.0609568104.

Kümmerer, K., 2009. The presence of pharmaceuticals in the environment due to human use – present knowledge and future challenges. J. Environ. Manag. 90, 2354–2366. http://dx.doi.org/10.1016/j.jenvman.2009.01.023.

Lajeunesse, A., Gagnon, C., Gagné, F., Louis, S., Čejka, P., Sauvé, S., 2011. Distribution of antidepressants and their metabolites in brook trout exposed to municipal wastewaters before and after ozone treatment – evidence of biological effects. Chemosphere 83, 564–571. http://dx.doi.org/10.1016/j.chemosphere.2010.12.026.

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

Liu, J., Lu, G., Wang, Y., Yan, Z., Yang, X., Ding, J., Jiang, Z., 2014. Bioconcentration, metabolism, and biomarker responses in freshwater fish *Carassius auratus* exposed to roxithromycin. Chemosphere 99, 102–108. http://dx.doi.org/10.1016/j.chemosphere.2013.10.036.

Li, X.-F., Carter, S., Dovichi, N.J., Zhao, J.Y., Kovarik, P., Sakuma, T., 2001. Analysis of tamoxifen and its metabolites in synthetic gastric fluid digests and urine samples using high-performance liquid chromatography with electrospray mass spectrometry. J. Chromatogr. A 914, 5–12. http://dx.doi.org/10.1016/S0021-9673(01)00538-6, Int. Symp. High Perform. Liq. Phase Sep. Relat. Technol..

Macova, S., Pistekova, V., Svobodova, Z., Votavova, P., Voslarova, E., Bedanova, I., 2008. Comparison of acute nitrite toxicity for various stages of *Danio rerio*. Toxicol. Lett. 180 (Supplement), S190. http://dx.doi.org/10.1016/j.toxlet.2008.06.195, Abstr. 45th Congr. Eur. Soc. Toxicol..

Mimeault, C., Woodhouse, A.J., Miao, X.-S., Metcalfe, C.D., Moon, T.W., Trudeau, V.L., 2005. The human lipid regulator, gemfibrozil bioconcentrates and reduces testosterone in the goldfish, *Carassius auratus*. Aquat. Toxicol. 73, 44–54. http://dx.doi.org/10.1016/j.aquatox.2005.01.009.

Naderi, M., Wong, M.Y.L., Gholami, F., 2014. Developmental exposure of zebrafish (*Danio rerio*) to bisphenol-S impairs subsequent reproduction potential and hormonal balance in adults. Aquat. Toxicol. 148, 195–203. http://dx.doi.org/10.1016/j.aquatox.2014.01.009.

Negreira, N., de Alda, M.L., Barceló, D., 2014. Cytostatic drugs and metabolites in municipal and hospital wastewaters in Spain: filtration, occurrence, and environmental risk. Sci. Total Environ. 497–498, 68–77. http://dx.doi.org/10.1016/j.scitotenv.2014.07.101.

Orias, F., Simon, L., Perrodin, Y., 2015. Experimental assessment of the bioconcentration of 15N-tamoxifen in *Pseudokirchneriella subcapitata*. Chemosphere 122, 251–256. http://dx.doi.org/10.1016/j.chemosphere.2014.11.070.

Schwaiger, J., Ferling, H., Mallow, U., Wintermayr, H., Negele, R.D., 2004. Toxic effects of the non-steroidal anti-inflammatory drug diclofenac: Part I: histopathological alterations and bioaccumulation in rainbow trout. Aquat. Toxicol. 68, 141–150. http://dx.doi.org/10.1016/j.aquatox.2004.03.014.

Verlicchi, P., Galletti, A., Petrovic, M., Barceló, D., 2010. Hospital effluents as a source of emerging pollutants: an overview of micropollutants and sustainable treatment options. J. Hydrol. 389, 416–428. http://dx.doi.org/10.1016/j.jhydrol.2010.06.005.

Yang, Y., Fu, J., Peng, H., Hou, L., Liu, M., Zhou, J.L., 2011. Occurrence and phase distribution of selected pharmaceuticals in the Yangtze Estuary and its coastal zone. J. Hazard. Mater. 190, 588–596. http://dx.doi.org/10.1016/j.jhazmat.2011.03.092.

Zenker, A., Cicero, M.R., Prestinaci, F., Bottoni, P., Carere, M., 2014. Bioaccumulation and biomagnification potential of pharmaceuticals with a focus to the aquatic environment. J. Environ. Manag. 133, 378–387. http://dx.doi.org/10.1016/j.jenvman.2013.12.017.

Zhou, J.L., Zhang, Z.L., Banks, E., Grover, D., Jiang, J.Q., 2009. Pharmaceutical residues in wastewater treatment works effluents and their impact on receiving river water. J. Hazard. Mater. 166, 655–661. http://dx.doi.org/10.1016/j.jhazmat.2008.11.070.

9.3 Synthèse

A la plus forte concentration d'exposition testée (10 μg/L), nous sommes parvenus à mettre en évidence une bioconcentration significative dans tous les organes du poisson après seulement 21 jours d'exposition. A la concentration de 0,1 μg/L, inférieure à des concentrations déjà mesurées dans l'Environnement, seul le foie présente une bioconcentration quantifiable. Cependant, au niveau individuel, les gonades représentent le réservoir de contaminant le plus important. Les conséquences intergénérationnelles de cette bioconcentration représentent un risque supplémentaire à celui de l'écotoxicité directe du contaminant.

CONCLUSION ET PERSPECTIVES GENERALES

Conclusion générale

L'étude bibliographique approfondie concernant les effluents hospitaliers, nous a permis de développer plusieurs points :

- Tout d'abord, nous avons publié la liste exhaustive des substances déjà recherchées dans ces effluents à travers le monde. Nous avons identifié que près de 300 substances comprenant des détergents, des métaux ou encore des résidus pharmaceutiques avaient déjà été recherchées dans les effluents hospitaliers.
- Ensuite, nous avons réuni toutes les données d'écotoxicité disponibles pour ces substances afin d'établir des concentrations sans effet (PNEC). Nous avons calculé deux types de PNEC : celles basée sur des données de modélisation (i.e. ECOSAR (Sanderson et al., 2003)) au nombre de 61, et celles basées sur des données expérimentales, au nombre de 155. Parmi ces 155 substances, on trouve 12 métaux et 143 autres substances. Pour 81 des substances recherchées, nous n'avons pas été en mesure de calculer de PNEC en raison du manque de données écotoxicologiques.
- Enfin, nous avons focalisé notre travail sur les 197 résidus pharmaceutiques déjà recherchés dans les effluents hospitaliers. Nous avons caractérisé leur contribution au danger écotoxicologique global des effluents hospitaliers en comparant les concentrations maximum auxquelles on les avait déjà mesurées dans les effluents à leur PNEC. Ainsi, nous avons calculé 127 quotients de danger écotoxicologique. Parmi ceux-ci, nous avons identifié les 15 RP les plus dangereux, conduisant à des quotients de danger supérieurs à 1000.

Nous nous sommes ensuite intéressés à l'écotoxicité directe de 3 molécules modèles très bioaccumulables : le ritonavir, l'amiodarone et le tamoxifen. Parmi elles, nous avons montré, suite à la mise en œuvre d'une batterie de bioessais complémentaires, que c'était le tamoxifen qui était la molécule la plus écotoxique.

Puis nous avons étudié le rôle que pouvait jouer les excipients sur l'écotoxicité des principes actifs des médicaments. Nous avons alors observé que la nature de l'excipient était à prendre en compte lorsque des tests d'écotoxicité étaient effectués sur un médicament (principe actif + excipient). En effet, si nous n'avons pas observé d'impact significatif de l'hydrochlorate sur l'écotoxicité de l'amiodarone, le citrate a significativement augmenté l'écotoxicité du tamoxifen dans tous les tests d'écotoxicité mis en œuvre.

Enfin, étant donné que le tamoxifen était la molécule la plus écotoxique parmi nos molécules modèles, nous avons choisi de caractériser le risque écotoxicologique qu'il pouvait représenter pour les écosystèmes aquatiques de quelques cours d'eau étudié par d'autres sur le plan international. Nous avons ainsi mis en évidence que le tamoxifen présentait un risque écotoxicologique significatif pour deux des cours d'eau où il avait été mesuré.

Une fois caractérisée l'écotoxicité directe du tamoxifen, nous nous nous sommes intéessé à son écotoxicité potentielle indirecte liée à sa bioconcentration dans les organismes. Ainsi, nous avons évalué sa bioaccumulabilité chez *P. subcapitata*, *D. magna* et *D. rerio*.

- Chez les algues, nous avons mesuré une bioconcentration très rapide. En effet, dès les premières minutes d'exposition, nous avons mesuré des teneurs en tamoxifen 10000 fois supérieures dans les algues que dans l'eau. Le facteur de bioconcentration le plus important que nous avons mesuré est de 26700.
- Dans le niveau trophique supérieur, les daphnies, nous avons également mesuré une bioconcentration forte et rapide du tamoxifen avec des teneurs près de 1000 fois supérieures dans les daphnies que dans l'eau, ceci après seulement 2 heures d'exposition. Chez les daphnies, nous avons également pu tester l'impact d'une alimentation contaminée sur la bioaccumulation du tamoxifen. Nous avons mis en évidence que plus la concentration du milieu était basse, plus l'impact de l'alimentation dans la contamination était important. Autrement dit, plus la concentration dans le milieu sera faible, plus l'alimentation contribuera de manière importante à la contamination de l'organisme.
- Chez le dernier organisme que nous avons testé, le poisson zèbre, nous avons mesuré la bioconcentration du tamoxifen dans trois organes : le foie, les gonades et le muscle. Nous avons déterminé que le foie et les gonades étaient les organes privilégiés de bioconcentration du tamoxifen avec de fortes teneurs dès les premiers jours d'exposition. Au regard de la masse de gonades présente chez ces poissons, nous avons également déterminé que cet organe accumulait la grande majorité du tamoxifen bioconcentré à l'échelle de l'organisme. Les facteurs de bioconcentration maximaux mesurés dans les foies et les gonades de ces organismes sont respectivement de l'ordre de 73800 et 85600 après 21 jours d'exposition.

Enfin, au cours de ces travaux, nous avons développé une méthode nous permettant d'étudier le transfert dans les organismes de molécules bioaccumulables. Cette méthode d'analyse qui s'appuie sur la mesure du ratio isotopique d'un élément donné, présente de nombreux avantages :

- Le premier d'entre eux est la possibilité de mesurer une accumulation de contaminant dans n'importe quelle matrice : organisme végétal, animal ou sédiment. Cet avantage serait particulièrement utile dans une approche de type micro ou mésocosme. En effet, il serait possible de suivre dans tous les compartiments du système le devenir du contaminant considéré.

– Grâce à cette technique d'analyse, nous n'avons pas besoin d'effectuer d'étape d'extraction sur nos échantillons. Cette étape est cruciale car c'est à ce moment qu'il est possible de perdre une quantité non négligeable de contaminant. Ces pertes peuvent être compensées par l'introduction de standards interne et/ou externe mais sans être certain que ces standards se comportent exactement comme le contaminant. De plus, les protocoles d'extraction ou les standards n'existent pas toujours. Ainsi, il n'est pas possible de travailler avec n'importe quelle substance.
– Cette technique présente également l'avantage de ne nécessiter qu'une très faible masse d'échantillon. En effet, dans nos travaux, le seuil minimal était de 150 μg de matériel. Il est assez facile d'imaginer l'avantage que cela représente quand on sait que les techniques d'analyse par chromatographie par exemple nécessitent plusieurs dizaine de mg de matériel.
– Une autre méthode d'analyse indirecte est utilisée dans les études de transfert de substance dans les organismes, et repose sur l'utilisation d'isotopes radioactifs. Avec la méthode que nous avons développé, les risques pratiques liés à l'utilisation de ce type de substances (i.e. radioactivité) sont inexistants. Par ailleurs, l'utilisation d'isotope stable ne peut pas perturber le développement de l'organisme contrairement une la radioactivité.

Cependant, si nous avons réussi à nous affranchir des contraintes liées aux techniques classiques d'analyse dans ce type d'étude (i.e. chromatographie ou radioactivité), nous avons été confrontés à certaines limites :

– La première limite de notre technique d'analyse concerne la possible surévaluation de la concentration en tamoxifen, pouvant être liée au fait que chaque atome d'azote présent dans les tissus est assimilé à une molécule de tamoxifen, alors qu'il pourrait s'agir partiellement de métabolites du tamoxifen, voire d'une produit de dégradation final. La dégradation jusqu'à un produit final, nous parait exclue ou minoritaire, à l'échelle de temps à laquelle on a travaillé, compte-tenu du caractère très stable de la molécule (DellaGreca et al., 2007). A l'inverse, les métabolites du tamoxifen peuvent être pris en compte dans les mesures basées sur l'analyse élémentaire. Dans ces conditions, on pourrait plutôt considérer que « tamoxifen » signifie « tamoxifen + métabolites » dans les résultats de nos expérimentions, ce qui reste très intéressant, voire un « plus », en évitant d'exclure du raisonnement des molécules qui peuvent contribuer fortement à l'impact environnemental de cette substance. Nous avons attiré l'attention sur ce point dans la discussion et les conclusions de nos différentes publications. De plus, nous avons réalisé en fin de thèse une étude exploratoire de comparaison de l'analyse isotopique et de l'analyse « classique » (i.e. HPLC-MS), désormais possible pour le tamoxifen (hors métabolites), sur un des tissus échantillonnés : les gonades de poisson. Cette étude exploratoire, décrite en annexe X, va dans le sens de la présence à la fois du tamoxifen et de ses métabolites dans les tissus exposés, et conclue à la possibilité et l'intérêt, via

la conduite en parallèle de ces deux types d'analyses, d'exprimer désormais deux types de BCF, en vue de leur usage dans l'évaluation des risques écotoxicologiques : le BCF du tamoxifen « strict » et le BDC du « tamoxifen + métabolites ».

– Une autre contrainte de notre méthode est la limite de quantification qui, dans les conditions où nous avons travaillé, est très haute. En effet, quand les méthodes d'analyse par chromatographie nous proposent des limites de quantification de l'ordre du ng/g, nous ne sommes pas capables de quantifier précisément au-dessus du μg/g avec l'analyse isotopique. Cependant, cette limite doit pouvoir être baissée en augmentant les prises d'essai qui dans nos travaux étaient très basses.
– La dernière contrainte majeure liée à notre méthode d'analyse est l'impossibilité de l'appliquer in situ. En effet, il est indispensable d'utiliser des contaminants marqués pour observer une variation du ratio isotopique. L'utilisation de notre méthode d'analyse isotopique est strictement limitée aux études *in vitro*.

Perspectives générales

Les résultats obtenus lors de nos expérimentations présentent de nombreuses perspectives.

Chez les algues nous avons observé des facteurs de bioconcentration (BCF) très importants (i.e. jusqu'à 26700) pouvant être liés au fait que cet organisme est incapable d'excréter et/ou de dégrader le tamoxifen comme peuvent le faire les autres organismes testés. Par ailleurs, il est impossible de déterminer si ce contaminant s'adsorbe simplement à la surface des algues ou s'il est internalisé dans la cellule. Des techniques de microscopie couplées à un marquage des molécules de tamoxifen pourraient nous indiquer à quel endroit se positionne les contaminants. Par ailleurs, dans les conditions expérimentales que nous avons testées, tout le tamoxifen en solution a été fixé par les algues en suspension. Ainsi, nous ne connaissons pas le BCF maximal du couple algue/tamoxifen. La modélisation pourrait venir en aide si nous arrivons à déterminer le taux de saturation des cellules algales, par exemple en exposant des algues au tamoxifen sans lumière, rendant la croissance nulle et donc la dilution dans la biomasse nulle.

L'objectif de l'expérience sur les daphnies était notamment de déterminer quel pouvait être l'impact d'un régime alimentaire contaminé sur l'accumulation du contaminant à l'échelle de l'organisme. Nous avons mis en évidence que cet apport alimentaire était non négligeable car en n'exposant les daphnies que par la voie alimentaire, on observait une accumulation du tamoxifen dans l'organisme (teneur en tamoxifen dans les daphnies jusqu'à 30 μg/g). En revanche, en comparaison de l'apport lié au milieu d'exposition, la contamination liée régime alimentaire est beaucoup plus faible. Notons cependant que plus la concentration dans le milieu est faible plus la fraction de contaminant apportée par le régime alimentaire est importante. Pour vérifier si cela est lié à la durée d'exposition aux deux voies de contamination, il faudrait effectuer la même expérience mais en augmentant la fréquence des étapes d'alimentation. Par ailleurs, dans nos expérimentations, nous avons choisi de contaminer très fortement l'alimentation des daphnies, en l'occurrence les algues, en exposant ces cellules à une forte concentration en tamoxifen (i.e. 100 μg/L), supérieure à celle du milieu d'exposition des daphnies (i.e.5 et 50 μg/L). Ainsi, nous n'avons pas pu évaluer la bioamplification du tamoxifen entre ces deux maillons de la chaîne alimentaire. En effet, pour évaluer ce phénomène, il est nécessaire que les deux organismes soient exposés aux mêmes concentrations de contaminant. Récemment, une nouvelle perspective s'est ouverte dans ce domaine. Les proto-

coles analytiques sont désormais disponibles pour analyser le tamoxifen dans les organismes invertébrés par chromatographie. Il serait donc intéressant de dimensionner de nouvelles expériences de sorte à pouvoir comparer les teneurs en tamoxifen mesurées dans les daphnies par les deux méthodes (i.e. HPLC-MS-MS et EA-IRMS) afin d'étudier la capacité excrétrice des invertébrés.

Le poisson zèbre, chez qui nous avons étudié la bioconcentration du tamoxifen, a également montré des résultats positifs. Les trois organes de Danio rerio étudiés ont montré des modifications significatives du ratio $^{15}N/^{14}N$, mais dans des proportions plus ou moins importantes. C'est dans le foie et les gonades que nous retrouvons les plus fortes teneurs en tamoxifen après 21 jours d'exposition, dans des proportions similaires (i.e. BCF entre 13000 et 85600). Ainsi, en considérant la masse des différents organes, nous avons mis en évidence que les gonades étaient le réservoir de contaminant prédominant à l'échelle de l'organisme. Ces résultats nous encouragent à étudier ces phénomènes à plus long terme. D'une part, pour observer ce que ces phénomènes d'accumulation dans les gonades pouvaient engendrer sur la descendance. D'autre part, pour observer si avec une exposition plus longue les muscles pouvaient être également contaminés via ces phénomènes d'accumulation avec les conséquences sanitaires imaginables. Au niveau expérimental, quelques améliorations peuvent être apportées au protocole mis en place. Dans cette expérience sur la bioconcentration du tamoxifen dan le poisson, nous avons régulièrement éliminé les fèces du système. Or, que ce soit par l'excrétion ou par l'adsorption sur ces dernières, le tamoxifen sort du système. Grâce à notre méthode nous permettant de mesurer la teneur en tamoxifen sur toutes les matrices, nous aurions dû mesurer ce ratio dans les fèces pour évaluer la quantité de tamoxifen qu'elles contenaient. Par ailleurs, pour limiter les variations dans le ratio $^{15}N/^{14}N$ du témoin, il serait utile de standardiser l'alimentation. En effet, dans nos expériences, nous avons nourri les poissons avec du Tetraminő en paillette qui comprend différents type d'aliments broyés (i.e. algues, invertébrés,...) avec des ratios $^{15}N/^{14}N$ différents qui peuvent ainsi perturber la teneur de base en ^{15}N du témoin. A terme, afin d'approfondir nos connaissances sur le transfert des contaminants dans les chaînes alimentaires, l'idéal serait d'alimenter nos poissons avec des invertébrés élevés dans un milieu contaminé et non contaminé ayant reçu une alimentation en algues contaminées et non contaminées. En somme, l'objectif ultime est de réaliser tous les traitements présentés dans la chaîne alimentaire artificielle présentée au début de la partie 3 de ce manuscrit (figure 6.1).

En complément des travaux réalisés sur ces trois organismes, des études sur d'autres organismes modèles seraient utiles pour mieux évaluer les risques liés à la bioaccumulation dans les écosystèmes. Par exemple, pour les algues, nous pourrions évaluer la bioconcentration du tamoxifen chez des algues mobiles comme les *Chlamydomonas reinhardtii*. Pour les invertébrés, nous pourrions travailler avec des mollusques (e.g. *Potamopyrgus antipodarum*) qui sont des organismes filtreurs et très riches en lipides. Ainsi, on peut s'attendre à des fac-

teurs de bioconcentration et de bioaccumulation encore plus élevés que chez la daphnie. Le poisson modèle que nous avons choisi est un poisson tropical qui vit dans eaux relativement chaudes (i.e. 26-28 $^{\circ}$ C) assez éloignées des conditions environnementales de nos écosystèmes européens. Ainsi, travailler sur une espèce d'eau plus froide pourrait nous fournir des informations intéressantes sur la bioconcentration sous nos latitudes.

Lorsque les données d'écotoxicité d'une substance sont disponibles, il est à noter qu'il s'agit le plus souvent de données d'écotoxicité aiguë. Or, il est indispensable de connaître l'effet à long terme d'une substance pour évaluer au mieux les risques qu'elle représente pour les écosystèmes. Dans l'idéal, pour évaluer au mieux le risque écotoxicologique vis-à-vis d'un organisme, il est même nécessaire d'évaluer l'impact d'une gamme de concentration à tous les stades de développement de l'organisme considéré. Pour mieux évaluer le comportement des RP à l'échelle de l'écosystème, certaines équipes de recherche ont effectuée des tests d'écotoxicité à l'échelle du microcosme voire du mésocosme. Cette approche permet non seulement d'évaluer l'impact du contaminant sur l'organisme mais également sur les relations qu'il peut avoir avec les autres organismes avec lesquels il est susceptible d'interagir. Concernant la méthode d'analyse isotopique que nous avons développée, elle pourrait être appliquée sur d'autres molécules pour répondre à de problématiques différentes. D'une part, travailler avec des molécules faciles à analyser dans les différentes matrices biologiques (e.g. diclofenac, paracétamol). En utilisant de manière complémentaire les analyses par chromatographie et par isotopie, nous pourrions mieux comprendre les mécanismes de dégradation de ces molécules dans des organismes non cibles des RP (i.e. organismes vivants autres que l'Homme). D'autre part, nous pourrions approfondir les expérimentations sur les molécules prioritaires bioaccumulables. Le meilleur candidat pour cela semble être l'amiodarone. En effet, cette molécule présente un seul azote dans sa structure moléculaire. Elle est analysable par chromatographie dans les matrices biologiques et c'est la molécule la plus potentiellement bioaccumulable.

ANNEXE

Annexe : Comparaison de l'analyse du tamoxifen dans les organismes vivants par EA-IRMS et par HPLC-MS-MS

Etude exploratoire réalisée en collaboration avec l'Institut des Sciences Analytiques (ISA) du CNRS

Au démarrage de cette thèse, les protocoles analytiques pour l'analyse des résidus médicamenteux par une approche classique (extraction + dosage par HPLC-MS-MS) n'étaient pas disponibles. Pour cette raison, et notamment pour la possibilité de travailler avec de très petites prises d'essai, nous avons développé une méthode spécifique dite des « isotopes stables ».

Cette méthode qui a donné satisfaction pour nos travaux, pose toutefois la question de la possible surévaluation de la concentration en tamoxifen « strict », qui pourrait être liée au fait que chaque atome d'azote présent dans les tissus est assimilé à une molécule de tamoxifen, alors qu'il pourrait s'agir partiellement de métabolites du tamoxifen, voire d'un produit de dégradation final. La dégradation jusqu'à un produit de dégradation final, nous parait exclue ou minoritaire, à l'échelle de temps à laquelle on a travaillé, compte-tenu du caractère très stable de la molécule (DellaGreca et al., 2007). A l'inverse, les métabolites peuvent être pris en en compte par la mesure puisque tous les métabolites connus du tamoxifen possèdent un atome d'azote : 4-hydroxytamoxifen, N-dedimethyltamoxifen, 4-hydroxy-N-demethyltamoxifen, tamoxifen-N-oxide ou N-desmethyltamoxifen (DellaGreca et al., 2007; Kisanga et al., 2005; Gjerde et al., 2005).

Dans ces conditions, on pourrait considérer que « tamoxifen » signifie « tamoxifen + métabolites » dans les résultats de nos expérimentions, ce qui est très intéressant, en évitant d'exclure du raisonnement des molécules qui peuvent contribuer fortement à l'impact environnemental de ce RP. Nous avons attiré l'attention sur ce point dans la discussion et les conclusions de nos différentes publications. Pour consolider ces hypothèses, et compte-tenu de l'avancée récente des techniques analytiques « classiques », nous avons décidé de lancer

fin 2014, en complément de nos travaux initiaux, et en collaboration avec l'ISA du CNRS, une étude exploratoire de comparaison des deux protocoles analytiques portant sur au moins un tissu vivant : les gonades de poisson. Ce tissu a été choisi en raison de la concentration importante en tamoxifen à l'issue de nos expériences, mais aussi en raison de la quantité de biomasse disponible. Ce travail exploratoire a permis de comparer les deux valeurs de BCF obtenues entre elles, mais aussi de les situer par rapport aux valeurs théoriques présentes dans la littérature (tableau ci-dessous). Dans ce tableau nous avons 3 types BCF différents :

- $\mathbf{BCF_{théorique}}$: Ce BCF est obtenu de manière théorique grâce à une méthode basée sur la relation entre structure et l'activité d'une molécule donnée (Jean et al., 2012).
- $\mathbf{BCF_{total}}$: Ce BCF est obtenu à partir des teneurs en tamoxifen mesurées dans les gonades de poissons obtenues par la méthode isotopique décrite au Chapitre 9.
- $\mathbf{BCF_{strict}}$: Ce BCF est obtenu à partir des teneurs en tamoxifen mesurées dans les gonades de poissons obtenue par l'approche « extraction + HPLC-MS-MS » (prise en compte uniquement du tamoxifen, les métabolites étant non dosables dans les tissus vivants par cette technique, dans l'état actuel des connaissances).

$\mathbf{BCF_{théorique}}$	1620 (pH 6) à 385000 (pH9)
$\mathbf{BCF_{total}}$	85600 (Erreur standard : 20400)
$\mathbf{BCF_{strict}}$	8648 à 10758

La plus faible valeur expérimentale est obtenue par la méthode dite « classique ». Elle peut s'expliquer par le dosage exclusif du tamoxifen, mais aussi par la perte de contaminant lors de l'étape d'extraction préalable au dosage par HPLC (contrairement à la méthode des isotopes qui ne nécessite pas d'extraction). Les deux techniques d'analyse pratiquées s'avèrent complémentaires, et nous disposons désormais d'une valeur du BCF du « tamoxifen + métabolites », et d'une valeur du BCF pour le « tamoxifen seul » (c'est toutefois un minimum compte-tenu des pertes potentielles lors de l'extraction).

Ce travail reste maintenant à préciser et à consolider par des études complémentaires, en particulier en dosant les principaux métabolites du tamoxifen par la méthode « classique », et en caractérisant le BCF pour d'autres organes et tissus du poisson et pour les autres organismes de notre chaine trophique (algues, daphnie). Concernant les micro-algues, il est probable que les métabolites seront moins présents compte-tenu de leur arsenal métabolique plus limité dans le domaine, mais cela reste à démontrer expérimentalement.

Ces deux valeurs expérimentales correspondent néanmoins d'ores et déjà à des informations extrêmement intéressantes, et nettement plus robustes que les valeurs théoriques relevées dans la littérature, en vue d'une évaluation des risques écotoxicologiques liées à la présence de l'environnement de ce type molécules.

Bibliographie

Liste des publications citées dans le corps du manuscrit

Bibliographie

Abarnou, A. and Blanchard, M. (2000). *Les contaminants organiques : quels risques pour le monde vivant ?* Ifremer.

Aherne, G., English, J., and Marks, V. (1985). The role of immunoassay in the analysis of microcontaminants in water samples. *Ecotoxicology and Environmental Safety*, 9(1) :79–83.

Arnot, J. A. and Gobas, F. A. (2006). A review of bioconcentration factor (BCF) and bioaccumulation factor (BAF) assessments for organic chemicals in aquatic organisms. *Environmental Reviews*, 14(4) :257–297.

Barnes, K. K., Kolpin, D. W., Furlong, E. T., Zaugg, S. D., Meyer, M. T., and Barber, L. B. (2008). A national reconnaissance of pharmaceuticals and other organic wastewater contaminants in the United States Ů I) Groundwater. *Science of The Total Environment*, 402(2-3) :192–200.

Bartelt-Hunt, S., Snow, D. D., Damon-Powell, T., and Miesbach, D. (2011). Occurrence of steroid hormones and antibiotics in shallow groundwater impacted by livestock waste control facilities. *Journal of Contaminant Hydrology*, 123(3-4) :94–103.

Berthelot, M. and Jungfleisch, E. (1872). Sur les lois qui president ou partage dŠun corps entre deux dissolvants (experiences). (26) :396–407.

Blair, B. D., Crago, J. P., Hedman, C. J., and Klaper, R. D. (2013). Pharmaceuticals and personal care products found in the Great Lakes above concentrations of environmental concern. *Chemosphere*, 93(9) :2116–2123.

Boillot, C., Bazin, C., Tissot-Guerraz, F., Droguet, J., Perraud, M., Cetre, J. C., Trepo, D., and Perrodin, Y. (2008). Daily physicochemical, microbiological and ecotoxicological fluctuations of a hospital effluent according to technical and care activities. *Science of The Total Environment*, 403(1-3) :113–129.

Brackers de Hugo, A., Sylvie, B., Alain, D., Jérôme, G., and Yves, P. (2013). Ecotoxicological risk assessment linked to the discharge by hospitals of bio-accumulative pharmaceuticals into aquatic media : The case of mitotane. *Chemosphere*, (0).

Brodin, T., Fick, J., Jonsson, M., and Klaminder, J. (2013). Dilute Concentrations of a Psychiatric Drug Alter Behavior of Fish from Natural Populations. *Science*, 339(6121) :814–815.

Brown, J. N., Paxéus, N., Förlin, L., and Larsson, D. J. (2007). Variations in bioconcentration of human pharmaceuticals from sewage effluents into fish blood plasma. *Environmental Toxicology and Pharmacology*, 24(3) :267–274.

Burridge, L., Weis, J. S., Cabello, F., Pizarro, J., and Bostick, K. (2010). Chemical use in salmon aquaculture : A review of current practices and possible environmental effects. *Aquaculture*, 306(1-4) :7–23.

Cardoso, O., Porcher, J.-M., and Sanchez, W. (2014). Factory-discharged pharmaceuticals could be a relevant source of aquatic environment contamination : Review of evidence and need for knowledge. *PHARMACEUTICAL PRODUCTS IN THE ENVIRONMENT : FOR A MORE RELIABLE RISK ASSESSMENT*, 115(0) :20–30.

Chèvre, N., Coutu, S., Margot, J., Wynn, H. K., Bader, H.-P., Scheidegger, R., and Rossi, L. (2013). Substance flow analysis as a tool for mitigating the impact of pharmaceuticals on the aquatic system. *Water Research*, 47(9) :2995–3005.

Claessens, M., Vanhaecke, L., Wille, K., and Janssen, C. R. (2013). Emerging contaminants in Belgian marine waters : Single toxicant and mixture risks of pharmaceuticals. *Marine Pollution Bulletin*, 71(1-2) :41–50.

Clément, B. and Zaid, S. (2004). A new protocol to measure the effects of toxicants on daphnid-algae interactions. *Chemosphere*, 55(10) :1429–1438.

Collado, N., Rodriguez-Mozaz, S., Gros, M., Rubirola, A., Barceló, D., Comas, J., Rodriguez-Roda, I., and Buttiglieri, G. (2014). Pharmaceuticals occurrence in a WWTP with significant industrial contribution and its input into the river system. *Environmental Pollution*, 185(0) :202–212.

DellaGreca, M., Iesce, M. R., Isidori, M., Nardelli, A., Previtera, L., and Rubino, M. (2007). Phototransformation products of tamoxifen by sunlight in water. Toxicity of the drug and its derivatives on aquatic organisms. *Chemosphere*, 67(10) :1933–1939.

Eckel, W. P., Ross, B., and Isensee, R. K. (1993). Pentobarbital Found in Ground Water. *Ground Water*, 31(5) :801–804.

Emmanuel, E., Perrodin, Y., Keck, G., Blanchard, J. M., and Vermande, P. (2005). Ecotoxicological risk assessment of hospital wastewater : a proposed framework for raw effluents discharging into urban sewer network. *Journal of Hazardous Materials*, 117(1) :1–11.

Fick, J., Söderström, H., Lindberg, R. H., Phan, C., Tysklind, M., and Larsson, D. G. J. (2009). Contamination of surface, ground, and drinking water from pharmaceutical production. *Environmental Toxicology and Chemistry*, 28(12) :2522–2527.

Gauld, D. T. (1951). The grazing rate of planktonic copepods. *Journal of the Marine Biological Association of the United Kingdom*, 29(03) :695–706.

Gjerde, J., Kisanga, E., Hauglid, M., Holm, P., Mellgren, G., and Lien, E. (2005). Identification and quantification of tamoxifen and four metabolites in serum by liquid chromatography-tandem mass spectrometry. 1082(1) :6–14.

Gros, M., Rodríguez-Mozaz, S., and Barceló, D. (2013). Rapid analysis of multiclass antibiotic residues and some of their metabolites in hospital, urban wastewater and river water by ultra-high-performance liquid chromatography coupled to quadrupole-linear ion trap tandem mass spectrometry. *Journal of Chromatography A*, 1292(0) :173–188.

Gunnarson, L., K., E., J., and A. (2008). Evolutionnary Conservation of Human Drug Target in Organisms used for Environmental Risk Assessment. *Environ. Sci. Technol*, 42 :5807–5813.

Hansch, C. and Leo, A. (1979). *Substituent constants for correlation analysis in chemistry and biology*. Wiley-Interscience publication. Wiley.

Heberer, T. and Stan, H.-J. (1997). Determination of Clofibric Acid and N-(Phenylsulfonyl)-Sarcosine in Sewage, River and Drinking Water. *International Journal of Environmental Analytical Chemistry*, 67(1-4) :113–124.

Hunt, E. and Bischoff, A. (1960). Inimical effects on wildlife of periodic application of DDD to Clear lake. *Califrornia Fish and Game*.

ISO (1995). Qualité de l'eau - Détermination de la mobilité de Daphnia magna Straus, Cladocera, Crustacea, essai de toxicité aiguë.

Jacobsen, P. and Berglind, L. (1988). Persistence of oxytetracycline in sediments from fish farms. *Aquaculture*, 70(4) :365–370.

Jaffal, A., Givaudan, N., Betoulle, S., Terreau, A., Paris-Palacios, S., Biagianti-Risbourg, S., Beall, E., and Roche, H. (2011). Polychlorinated biphenyls in freshwater salmonids from the Kerguelen Islands in the Southern Ocean. *Adaptation of Forest Ecosystems to Air Pollution and Climate Change*, 159(5) :1381–1389.

Jean, J., Perrodin, Y., Pivot, C., Trepo, D., Perraud, M., Droguet, J., Tissot-Guerraz, F., and Locher, F. (2012). Identification and prioritization of bioaccumulable pharmaceutical substances discharged in hospital effluents. *Journal of Environmental Management*, 103(0) :113–121.

Jobling, S., Nolan, M., Tyler, C. R., Brighty, G., and Sumpter, J. P. (1998). Widespread Sexual Disruption in Wild Fish. *Environmental Science & Technology*, 32(17) :2498–2506.

Kidd, K. A., Blanchfield, P. J., Mills, K. H., Palace, V. P., Evans, R. E., Lazorchak, J. M., and Flick, R. W. (2007). Collapse of a fish population after exposure to a synthetic estrogen. *Proceedings of the National Academy of Sciences*, 104(21) :8897–8901.

Kinney, C. A., Furlong, E. T., Kolpin, D. W., Burkhardt, M. R., Zaugg, S. D., Werner, S. L., Bossio, J. P., and Benotti, M. J. (2008). Bioaccumulation of Pharmaceuticals and Other Anthropogenic Waste Indicators in Earthworms from Agricultural Soil Amended With Biosolid or Swine Manure. *Environmental Science & Technology*, 42(6) :1863–1870.

Kisanga, E. R., Moi, L. L. H., Gjerde, J., Mellgren, G., and Lien, E. A. (2005). Induction of hepatic drug-metabolising enzymes and tamoxifen metabolite profile in relation to administration route during low-dose treatment in nude rats. *The Journal of Steroid Biochemistry and Molecular Biology*, 94(5) :489–498.

Kümmerer, K. (2001). Drugs in the environment : emission of drugs, diagnostic aids and disinfectants into wastewater by hospitals in relation to other sources - a review. *Chemosphere*, 45(6-7) :957–969.

Kümmerer, K., Al-Ahmad, A., Bertram, B., and WieSSler, M. (2000). Biodegradability of antineoplastic compounds in screening tests : influence of glucosidation and of stereochemistry. *Chemosphere*, 40(7) :767–773.

Kolodziej, E. P., Harter, T., and Sedlak, D. L. (2004). Dairy wastewater, aquaculture, and spawning fish as sources of steroid hormones in the aquatic environment. *Environmental science & technology*, 38(23) :6377–6384.

Kolpin, D. W., Furlong, E. T., Meyer, M. T., Thurman, E. M., Zaugg, S. D., Barber, L. B., and Buxton, H. T. (2002). Pharmaceuticals, Hormones, and Other Organic Wastewater Contaminants in U.S. Streams, 1999-2000 : A National Reconnaissance. *Environmental Science & Technology*, 36(6) :1202–1211.

Lai, K. M., Scrimshaw, M. D., and Lester, J. N. (2002). Prediction of the bioaccumulation factors and body burden of natural and synthetic estrogens in aquatic organisms in the river systems. *Science of The Total Environment*, 289(1-3) :159–168.

Lajeunesse, A., Gagnon, C., Gagné, F., Louis, S., Cejka, P., and Sauvé, S. (2011). Distribution of antidepressants and their metabolites in brook trout exposed to municipal wastewaters before and after ozone treatment - Evidence of biological effects. *Chemosphere*, 83(4) :564–571.

Langford, K. H. and Thomas, K. V. (2009). Determination of pharmaceutical compounds in hospital effluents and their contribution to wastewater treatment works. *Environment International*, 35(5) :766–770.

Lapworth, D., Baran, N., Stuart, M., and Ward, R. (2012). Emerging organic contaminants in groundwater : A review of sources, fate and occurrence. *Environmental Pollution*, 163(0) :287–303.

Lin, A. Y.-C., Wang, X.-H., and Lin, C.-F. (2010). Impact of wastewaters and hospital effluents on the occurrence of controlled substances in surface waters. *Chemosphere*, 81(5) :562–570.

Loos, R., Gawlik, B. M., Locoro, G., Rimaviciute, E., Contini, S., and Bidoglio, G. (2009). EU-wide survey of polar organic persistent pollutants in European river waters. *Environmental Pollution*, 157(2) :561–568.

Loos, R., Locoro, G., Comero, S., Contini, S., Schwesig, D., Werres, F., Balsaa, P., Gans, O., Weiss, S., Blaha, L., Bolchi, M., and Gawlik, B. M. (2010). Pan-European survey on the occurrence of selected polar organic persistent pollutants in ground water. *Water Research*, 44(14) :4115–4126.

Mater, N., Geret, F., Castillo, L., Faucet-Marquis, V., Albasi, C., and Pfohl-Leszkowicz, A. (2014). In vitro tests aiding ecological risk assessment of ciprofloxacin, tamoxifen and cyclophosphamide in range of concentrations released in hospital wastewater and surface water. *Environment International*, 63(0) :191–200.

Nelis, H. J., Léger, F., Sorgeloos, P., and De leenheer, A. P. (1991). Liquid chromatographic determination of efficacy of incorporation of trimethoprim and sulfamethoxazole in brine shrimp (Artemia spp.) used for prophylactic chemotherapy of fish. *Antimicrobial Agents and Chemotherapy*, 35(12) :2486–2489.

Norpeth, K., Nehrkorn, A., Kirchner, M., Holsen, H., and Teipel, H. (1973). Investigations on the problem of solubility and stability of steroid ovulation inhibitors in water, waste water and activated sludge.

OECD (2006). OECD GUIDELINES FOR THE TESTING OF CHEMICALS : Freshwater Alga and Cyanobacteria, Growth Inhibition Test.

Orias, F. and Perrodin, Y. (2013). Characterisation of the ecotoxicity of hospital effluents : A review. *Science of The Total Environment*, 454-455(0) :250–276.

Ort, C., Lawrence, M. G., Reungoat, J., Eaglesham, G., Carter, S., and Keller, J. (2010). Determining the fraction of pharmaceutical residues in wastewater originating from a hospital. *Water Research*, 44(2) :605–615.

Papa, E., Dearden, J., and Gramatica, P. (2007). Linear QSAR regression models for the prediction of bioconcentration factors by physicochemical properties and structural theoretical molecular descriptors. *Chemosphere*, 67(2) :351–358.

Perrodin, Y., Christine, B., Sylvie, B., Alain, D., Jean-Luc, B.-K., Cécile, C.-O., Audrey, R., and Elodie, B. (2012). A priori assessment of ecotoxicological risks linked to building a hospital. *Chemosphere*, (0).

Ramade, F. (2002). *Dictionnaire encyclopédique de l'écologie et des sciences de l'environnement*. 2ème edition.

Ramade, F. (2011). *Introduction à l'écochimie : les substances de l'écosphère à l'Homme*. Lavoisier.

Sanchez, W., Sremski, W., Piccini, B., Palluel, O., Maillot-Maréchal, E., Betoulle, S., Jaffal, A., Aït-Aïssa, S., Brion, F., Thybaud, E., Hinfray, N., and Porcher, J.-M. (2011). Adverse effects in wild fish living downstream from pharmaceutical manufacture discharges. *Environment International*, 37(8) :1342–1348.

Sanderson, H., Johnson, D. J., Wilson, C. J., Brain, R. A., and Solomon, K. R. (2003). Probabilistic hazard assessment of environmentally occurring pharmaceuticals toxicity to fish, daphnids and algae by ECOSAR screening. *Toxicology Letters*, 144(3) :383–395.

Santos, L. H. M. L. M., Araújo, A. N., Fachini, A., Pena, A., Delerue-Matos, C., and Montenegro, M. C. B. S. M. (2010). Ecotoxicological aspects related to the presence of pharmaceuticals in the aquatic environment. *Journal of Hazardous Materials*, 175(1-3) :45–95.

Sarmah, A. K., T., M. M., and Boxall, A. B. (2006). A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. *Chemosphere*, 65 :725–759.

Schultz, M. M., Furlong, E. T., Kolpin, D. W., Werner, S. L., Schoenfuss, H. L., Barber, L. B., Blazer, V. S., Norris, D. O., and Vajda, A. M. (2010). Antidepressant Pharmaceuticals in Two U.S. Effluent-Impacted Streams : Occurrence and Fate in Water and Sediment, and Selective Uptake in Fish Neural Tissue. *Environmental Science & Technology*, 44(6) :1918–1925.

Selassie, C., Verma, R. P., and Abraham, D. J. (2003). History of Quantitative Structure-Activity Relationships. In *Burger's Medicinal Chemistry and Drug Discovery*. John Wiley & Sons, Inc.

Silva, B. F. d., Jelic, A., López-Serna, R., Mozeto, A. A., Petrovic, M., and Barceló, D. (2011). Occurrence and distribution of pharmaceuticals in surface water, suspended solids and sediments of the Ebro river basin, Spain. *Chemosphere*, 85(8) :1331–1339.

Tauxe-Wuersch, A., De Alencastro, L. F., Grandjean, D., and Tarradellas, J. (2006). Trace determination of tamoxifen and 5-fluorouracil in hospital and wastewaters. *International Journal of Environmental Analytical Chemistry*, 86(7) :473–485.

Ternes, T. A. (1998). Occurrence of drugs in German sewage treatment plants and rivers. *Water Research*, 32(11) :3245–3260.

Thomas, K. V. and Hilton, M. J. (2004). The occurrence of selected human pharmaceutical compounds in UK estuaries. *Marine Pollution Bulletin*, 49(5-6) :436–444.

Vazquez-Roig, P., Andreu, V., Blasco, C., and Picó, Y. (2012). Risk assessment on the presence of pharmaceuticals in sediments, soils and waters of the Pego-Oliva Marshlands (Valencia, eastern Spain). *Science of The Total Environment*, 440(0) :24–32.

Verlicchi, P., Al Aukidy, M., Galletti, A., Petrovic, M., and Barceló, D. (2012). Hospital effluent : Investigation of the concentrations and distribution of pharmaceuticals and environmental risk assessment. *Science of The Total Environment*, 430(0) :109–118.

Verlicchi, P., Galletti, A., Petrovic, M., and Barceló, D. (2010). Hospital effluents as a source of emerging pollutants : An overview of micropollutants and sustainable treatment options. *Journal of Hydrology*, 389(3-4) :416–428.

Weissbrodt, D., Kovalova, L., Ort, C., Pazhepurackel, V., Moser, R., Hollender, J., Siegrist, H., and McArdell, C. S. (2009). Mass Flows of X-ray Contrast Media and Cytostatics in Hospital Wastewater. *Environmental Science & Technology*, 43(13) :4810–4817.

Williams, T. D., Caunter, J. E., Lillicrap, A. D., Hutchinson, T. H., Gillings, E. G., and Duffell, S. (2007). Evaluation of the reproductive effects of tamoxifen citrate in partial and full life-cycle studies using fathead minnows (Pimephales Promelas). *Environmental Toxicology and Chemistry*, 26(4) :695–707.

Yang, Y., Fu, J., Peng, H., Hou, L., Liu, M., and Zhou, J. L. (2011). Occurrence and phase distribution of selected pharmaceuticals in the Yangtze Estuary and its coastal zone. *Journal of Hazardous Materials*, 190(1-3) :588–596.

Yuan, S., Jiang, X., Xia, X., Zhang, H., and Zheng, S. (2013). Detection, occurrence and fate of 22 psychiatric pharmaceuticals in psychiatric hospital and municipal wastewater treatment plants in Beijing, China. *Chemosphere*, 90(10) :2520–2525.

Zenker, A., Cicero, M. R., Prestinaci, F., Bottoni, P., and Carere, M. (2014). Bioaccumulation and biomagnification potential of pharmaceuticals with a focus to the aquatic environment. *Journal of Environmental Management*, 133(0) :378–387.

Zgorska, A., Arendarczyk, A., and Grabinska-Sota, E. (2011). TOXICITY ASSESSMENT OF HOSPITAL WASTEWATER BY THE USE OF A BIOTEST BATTERY. *Archives of Environmental Protection*, 37(3) :55–61.

Contribution à l'évaluation des risques écotoxicologiques des effluents hospitaliers : bioconcentration, bioaccumulation et bioamplification des résidus pharmaceutiques.

Résumé : Les hôpitaux génèrent des effluents riches en résidus pharmaceutiques (RP), fonctions de leurs activités de soins et de diagnostic. Certains de ces RP sont aujourd'hui retrouvés de manière ubiquitaire dans les écosystèmes aquatiques, en raison de leurs propriétés persistantes et/ou de leur émission continue. La variété de ces RP est telle qu'il est nécessaire de les hiérarchiser, en fonction des risques qu'ils représentent pour l'Environnement, à des fins d'étude et de gestion. Un de ces risques est le transfert des RP bioaccumulables (i.e. K_{ow} élevé et faible biodégradabilité) dans les chaînes alimentaires, via les processus de bioconcentration, de bioaccumulation et de bioamplification. L'objectif principal de cette thèse est de caractériser expérimentalement la bioconcentration et la bioaccumulation de molécules identifiées comme prioritaires dans des travaux précédents. Le composé modèle que nous avons choisi est le tamoxifen, molécule utilisée contre le cancer du sein et déjà retrouvé dans l'Environnement. Les organismes étudiés, issus des trois niveaux trophiques de la chaine alimentaire modèle, sont *Pseudokirchneriella subcapitata*, *Daphnia magna* et *Danio rerio*. Pour mesurer la teneur de cette molécule dans les organismes, nous avons développé une méthode d'analyse reposant sur l'utilisation d'une molécule marquée par un isotope stable, le ^{15}N tamoxifen. Nous avons mesuré des facteurs de bioconcentration (BCF) allant de 12800 chez *D. magna* à 85600 dans le foie de *D. rerio* en passant par 21500 chez *P. subcapitata*. Chez ces derniers, nous avons également évalué la part du régime alimentaire dans la bioaccumulation du tamoxifen. Nous avons observé que plus la concentration dans le milieu d'exposition est faible, plus le régime alimentaire contribue à la bioaccumulation. Ces travaux de thèse présentent de nombreuses perspectives que l'on peut regrouper autour de deux axes : connaissance de l'écotoxicité des RP et de l'écotoxicologie isotopique.

Mots clés : Effluents hospitaliers, résidus pharmaceutiques, bioconcentration, bioaccumulation, isotopes stables, tamoxifen

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Contribution to ecotoxicological risk assessment of hospital effluents : bioconcentration, bioaccumulation & biomagnification of pharmaceutical compounds.

Abstract : Hospitals generates effluents rich in pharmaceuticals compounds (PC), notably because of care and diagnostics activities. Some of these PCs are ubiquitous in aquatic ecosystems owing to its persistent properties and/or because of continuous releasing in environment. The diversity of these PCs is so strong that it is necessary to prioritize them, considering risks that PCs represents for the Environment, in order to manage and study these compounds. One of these risks is the transfer of bioaccumulatives PCs (i.e. PCs with high K_{ow} and low biodegradability) along trophic webs, via bioconcentration, bioaccumulation and biomagnification processes. The main objective of this thesis is to characterize bioconcentration and bioaccumulation of molecules identified as priority in previous studies. The model compound choose in our work is the tamoxifen, a molecule used in the treatment of breast cancer and already found in Environment. Organisms studied, typical from three trophic levels of the model trophic chain, are *Pseudokirchneriella subcapitata*, *Daphnia magna* and *Danio rerio*. In order to measure content of tamoxifen in organisms, we developed an innovative analytic method based on the use of stable isotopes labelled tamoxifen : ^{15}N tamoxifen. We succeeded to measure bioconcentration factors (BCF) from 12800 in *D. magna* to 85600 in liver of *D. rerio* including BCF of 21500 in *P. subcapitata*. In this latter, we also assessed the contribution of dietary route to the total contamination of *D. magna* by tamoxifen. We observed that the more the medium concentration was weak, the more the dietary route contribute to the contamination. These works shows numerous perspectives that we can gather inside two axes : ecotoxicity knowledge of PCs an isotopic ecotoxicology.

Key words : Hospital effluents, pharmaceutical compounds, bioconcentration, bioaccumulation, stable isotopes, tamoxifen