Year : 2014

Ecotoxicological approaches to assess the long-term effects of four anticancer drugs and metabolites on Daphnia pulex

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Ecotoxicological approaches to assess the long-term effects of four anticancer drugs and metabolites on *Daphnia pulex*

A thesis submitted to the Graduate Faculty of Geosciences and Environment,
University of Lausanne, Institute of Earth Surface Dynamics, for the

Degree of Doctor of Philosophy

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Lausanne, 2014

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Décanat Géosciences et de l'Environnement
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Titulaire d'une
Maîtrise universitaire ès Sciences en géosciences et environnement
Université de Lausanne

intitulée

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Lausanne, le 8 janvier 2015

Pour le Doyen de la Faculté des géosciences et de l'environnement

Professeur Eric Verrecchia, Vice-doyen

Acknowledgement

I would like to thank my major supervisor Nathalie Chèvre and my other supervisors Patrice Waridel and Thierry Bulcin for their help creating this project and for their guidance and support during my graduate experience. The thanks include all the support, motivation, ideas and advice during my Ph.D. study. I would like to thank Laurent-Arthur Decosterd and Sandra Cruchon who provided lab experiments and advices about result analysis, Florine Baillot and Jules Duruz for their laboratory work, and Pierre Voirol for his recommendations for pharmaceutical consumption and properties. I would like to thank Esther Keller and Serge Santiago who kindly provided the daphnids and the algae that I needed for my lab experiments. I would like to thank the Swiss National Science Fundation and the Faculty of Biology and Medicine Research Commission Fund of the University of Lausanne, which supported this project. I have an important recognition for the thousands daphnids that participated to move science forward in the field.

Many thanks to my colleagues and especially to those with whom I became friends: Vincent Gregorio, Silwan Daouk, Pierre-Jean Copin, Antoine Cogez, Naomi Dumas, Julia Gonzalez, Francesco Femi Marafatto, Nathalie Diaz, Matteodo Magali, Case Van Genuchten, Anna Simanova, Renske Lambert, Florence Bonvin, Catherine Schlegel Rey. They provided incredible energy and happiness, and they helped me remember what life is like outside of work. A special thank to Chiara Perazzolo and Mark Ibberson for the time they took to read and comment the manuscripts, to Marianne Gex-Fabry for her help in statistical analyses, and to Hans-Rudolf Pfeifer's team for their kindness.

I have a general debt to my friends Sabrina Lo Giudice-Ciric, Aline Monin, David Bichsel, Laurent Busenhart, Isabel Cardoso, Régine Mavier, Joao Barreiro, Stève Bonjour, Claude Bernhard, Gérard Clerc, Julie Frigeri, Sergio Tome, Marie Ducotterd, Michel Marthaler, Virginie Crausaz Grégoire Ventura, Jaurès Blanc, who supported me during my studies. I thank my partners and friends of the MAS in toxicology Lucie Dubugnon, Elena Reale, Vincent Perret, Manu Rusconi, Nicolas Roth, Evin Danisman, Daniel Fabian, Myriam Froelich with whom I have shared the privilege of learning while having fun. I also owe much to my friends and squash players Isabelle Wintz, Heath Davies, Philippe Martin, Muriel Jaquet, Esteban Giren, Pierre Ventura, Mark Ibberson, Liam Sinclair, Patrik Castignioli who helped me to let off steam several times a week. A special thank to Ernal Dizdari for his invaluable help and listening.

I would particularly like to thank my mom for her love and support throughout my career. A special thought is for my dad who did not have the chance to see the end of my project. I would like to thank my cousins, aunts, uncles and Marianne Wohlmaier for their encouragements to follow my passion.

You all were involved in making me successful throughout my studies. Thank you and sorry to everyone I have forgotten to mention.

Résumé grand public

Les milieux aquatiques sont exposé continuellement à de plus en plus de substances chimiques qui sont émises par les activités humaines. Les médicaments, par exemple, sont connus pour atteindre l'environnement et provoquer des effets chez les organismes aquatiques. Une fois consommés, ces molécules sont éliminées par l'intermédiaire des urines ou des fèces sous forme originale ou transformée. Ces résidus de médicaments se retrouvent dans les eaux usées et sont dirigés vers une station d'épuration afin d'y être traité. Les stations d'épuration ne sont pas toutes dotées de procédés complexes permettant d'éliminer toutes les substances synthétiques comme les médicaments. Ces molécules sont alors rejetées dans les eaux de surfaces, avec les effluents de la station d'épuration. Une fois dans l'environnement, les effets de ces molécules sont peu connus sur la faune et la flore aquatique.

Les anticancéreux font partie des médicaments qui peuvent être rejetés par les effluents des stations d'épuration et se retrouver dans les eaux naturelles. Parmi ceux-ci, le tamoxifen est utilisée en oncologie pour prévenir et traiter certains cancers du sein. Il est largement prescrit à travers le monde et il a la capacité d'être transformé par le foie en d'autres sous-molécules qui sont également très actives pour lutter contre les cellules cancéreuses. Deux de celles-ci sont le 4-hydroxy-tamoxifen (4OHTam) et l'endoxifen. Tout comme le tamoxifen, le 4OHTam et l'endoxifen sont principalement éliminés par l'intermédiaire des fèces et le tamoxifen a été retrouvé dans des échantillons d'eaux naturelles, à travers le monde. Enfin, un autre anticancéreux qui est éliminé principalement par voie biliaire et qui pourrait potentiellement se retrouver dans les eaux de surface est l'imatinib. Cette molécule cible des cellules tumorales spécifiques et cette spécificité a révolutionné le traitement et la survie des patients souffrant de certains cancers comme la leucémie myéloïde chronique.

Les objectifs de cette thèse ont été d'évaluer les effets du tamoxifen, 4OHTam, endoxifen et imatinib sur organismes aquatiques. Les daphnies ont été choisies car elles représentent des organismes clés de la chaîne alimentaire et leur disparition pourrait entraîner des répercussions importantes sur l'équilibre de l'écosystème. Ces petits crustacés d'environ 3 mm ont été élevés en laboratoire afin d'être exposés à une des quatre molécules anticancéreuse ci-dessus. Ce sont principalement des expériences sur plusieurs générations et à faibles concentrations qui ont été conduites dans notre laboratoire. Une expérience basée sur la modification des protéines a également été entreprise, car il est possible que des protéines soient modifiées alors qu'aucun effet n'a encore été observé chez l'organisme entier. Ce type d'essais permettrait d'identifier de potentiel effets indésirable chez des organismes aquatiques avant que ceux-ci soient affaiblis.

Les résultats obtenus dans cette thèse montrent que le tamoxifen, le 4OHTam et l'endoxifen sont capables de modifier la taille, la reproduction et la viabilité des daphnies à des concentrations qui sont proches de celles pouvant se retrouver dans l'environnement. Ces molécules ont également provoqués

des daphnies anormales, avec des antennes et des queues déformées, des prématurés et des œufs avortés. Le tamoxifen fut la molécule la plus toxique pour les daphnies, suivie du 4OHTam, de l'endoxifen et enfin de l'imatinib. Ce sont donc les effets du tamoxifen, du 4OHTam et de l'endoxifen qui posent le plus de questions quant à l'impact potentiel su la faune et la flore aquatique. Le tamoxifen semble ainsi une molécule à considérer lors des procédures d'évaluation du risque d'une substance pour l'environnement. Nos résultats montrent également que les expériences qui considèrent plusieurs générations de daphnies offrent un meilleur reflet de la réalité environnementale que des essais de courte durée où les générations sont généralement plus élevées. Finalement, nous avons également remarqué qu'il est important de discuter de l'opportunité de mesurer les concentrations qui sont testées lors d'essais en laboratoire afin de ne pas sous-estimer le risque pour la faune et la flore aquatique.

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Summary

The aquatic environment is exposed continuously and increasingly to chemical substances such as pharmaceuticals. These medical compounds are released into the environment after having being consumed and body-excreted by patients. Pharmaceutical residues are synthetic molecules that are not always removed by traditional sewage treatment processes and thus escape degradation. Among pharmaceuticals that escape sewage treatment plants (STPs), the anticancer drugs were measured in STP effluents and natural waters. In the aquatic environment, their long-term effects at low concentrations are sparsely known on non-target species.

Tamoxifen is an anticancer drug that is widely prescribed worldwide for the prevention and treatment of hormone receptor-positive breast cancers. Two of its metabolites, i.e., endoxifen and 4-hydroxy-tamoxifen (4OHTam), have high pharmacological potency *in vivo* and such as tamoxifen, they are excreted via faeces by patients. Tamoxifen was measured in STP effluents and natural waters but, to the best of our knowledge, its metabolites concentrations in waters have never been reported. Imatinib is another and recent anticancer compound that targets specific tumour cells. This pharmaceutical is also body excreted and because of its increasing use in cancer treatment, imatinib may reach the natural water. The effects of tamoxifen and imatinib are unknown upon more than one generation of aquatic species. And the effects of 4OHTam, endoxifen have never been studied in ecotoxicology so far.

The aims of this thesis were threefold. First, the sensitivity of *D. pulex* exposed to tamoxifen, 4OHTam, endoxifen or imatinib was assessed using ecotoxicological experiments. Ecotoxicology is the science that considers the toxic effects of natural or synthetic substances, such as pharmaceuticals, on organisms, populations, community and ecosystem. Acute and multigenerational (2-4 generations) tests were performed on daphnids considering several studied endpoints, such as immobilisation, size, reproduction, viability and intrinsic rate of natural increase. Additional prospective assays were designed to evaluate whether 1) low concentrations of tamoxifen and 4OHTam were able to induce toxic effects when used in combination, and 2) daphnids were able to recover when offspring were withdrawn from solutions carrying the pharmaceutical. Second, the stability of tamoxifen, 4OHTam and endoxifen in incubation medium was evaluated in solution exempted from daphnids. Because the nominal concentrations of tamoxifen, 4OHTam and endoxifen did not correspond to the measured, we provide a predictive method to estimate the concentrations of these chemicals during long-term ecotoxicological tests. Finally, changes in protein expressions were analysed in *D. pulex* exposed 2 or 7 seven days to tamoxifen using ecotoxicoproteomic experiments with a shot-gun approach inducing a peptide fractionation step.

Our results show that tamoxifen, 4OHTam and endoxifen induced adverse effects in *D. pulex* at environmentally relevant concentrations. At very low concentrations, these molecules displayed

unusual and teratogenic effects because morphological abnormalities were observed in offspring, such as thick and short antennas, curved spines, premature neonates and aborted eggs. Tamoxifen was the most toxic compound among the test chemicals, followed by 4OHTam, endoxifen and imatinib. Tamoxifen no-observed effect concentrations (NOECs) that were calculated for size, reproduction and intrinsic rate were below or in the range of the concentrations measured in natural waters, i.e., between 0.12 µg/L and 0.67 µg/L. For instance, the tamoxifen NOECs that were calculated for reproduction were between 0.67 and 0.72 µg/L, whereas the NOEC was < 0.15 µg/L when based on morphological abnormalities. The NOECs of 4OHTam were higher but still in the same order of magnitude as tamoxifen environmental concentrations, with a value of 1.48 µg/L. Endoxifen NOEC for the intrinsic rate of natural increase (r) and the reproduction were 0.4 and 4.3 µg/L, respectively. Daphnids that were withdrawn from tamoxifen and 4OHTam were not able to recover. Also, the reproduction of *D. pulex* was reduced when the treated animals were exposed to the combination of tamoxifen and 4OHTam while no effects were observed when these chemicals were tested individually at the same concentration. Among the anticancer drugs that were tested during this thesis, imatinib was the less toxic molecule towards *D. pulex*. No effects on size and reproduction were observed within two generations, except for the first whose reproduction decreased at the highest test concentration, i.e., 626 µg/L.

Our results also underline the need to use measured or predicted concentrations instead of the nominal during aquatic experiments, particularly when lipophilic molecules are tested. Indeed, notable differences between nominal (i.e., theoretical) and measured concentrations were found with tamoxifen, 4OHTam and endoxifen at all test concentrations. A cost and time sustainable method was proposed to predict the test exposure levels of these chemicals during long-term experiments. This predictive method was efficient particularly for low concentrations, which corresponded to the test concentrations in multigenerational tests.

In the ecotoxicoproteomic experiments a total of 3940 proteins were identified and quantified in *D. pulex* exposed to tamoxifen. These results are currently the largest dataset from *D. pulex* that is published and the results of proteomic analyses are available for the scientific community. Among these 3940 proteins, 189 were significantly different from controls. After protein annotation, we assumed that treated daphnids with tamoxifen had shifted cost-energy functions, such as reproduction, to maintain their basic metabolism necessary to survive. This metabolic cost hypothesis was supported by the presence of proteins involved in oxidative stress. Biomarkers for early detection of tamoxifen harmful effects on *D. pulex* were not discovered but the proteins of the vitellogenin-2 family (E9H8K5) and the ryanodine receptor (E9FTU9) are promising potential biomarkers because their expression was already modified after 2 days of treatment.

In this thesis, the effects of tamoxifen, 4OHTam and endoxifen on daphnids raise questions about the potential impact of tamoxifen and 4OHTam in other aquatic ecosystems, and therefore, about

metabolites in ecotoxicology. Because the NOECs were environmentally relevant, these results suggest that tamoxifen and 4OHTam may be interesting pharmaceuticals to consider in risk assessment. Our findings also emphasize the importance of performing long-term experiments and of considering multi-endpoints instead of the standard reproductive endpoint. Finally, we open the discussion about the importance to measure test exposures or not, during ecotoxicological studies.

Résumé

Les milieux aquatiques sont exposés continuellement à un nombre croissant de substances chimiques, notamment les médicaments issus de la médecine vétérinaire et humaine. Chez les patients, les substances administrées sont utilisées par le corps avant d'être éliminées par l'intermédiaire des excrétas dans le système d'eaux usées de la ville. Ces eaux rejoignent ensuite une station de traitement afin d'y éliminer les déchets. Dans le cas des molécules chimiques, il arrive que les processus de traitement d'eaux usées ne soient pas suffisamment efficaces et que ces molécules ne soient pas dégradées. Elles sont alors libérées dans le milieu aquatique avec les effluents de la station d'épuration. Une fois dans l'environnement, ces résidus de médicaments sont susceptibles d'induire des effets sur la faune et la flore aquatique, dont les conséquences à long terme et à faibles concentrations sont peu connues.

Les anticancéreux sont une famille de médicaments qui peuvent échapper aux traitements des stations d'épuration et qui sont retrouvées dans le milieu aquatique naturel. Parmi ces substances, le tamoxifen est une molécule utilisée dans le monde entier pour prévenir et traiter les cancers hormonaux dépendant du sein, notamment. Une fois ingéré, le tamoxifen est transformé par le foie en métabolites dont deux d'entre eux, le 4-hydroxy-tamoxifen (4OHTam) et l'endoxifen, possèdent un affinité pour les récepteurs aux estrogènes et une efficacité sur les cellules tumorales supérieure au tamoxifen lui-même. Tout comme la molécule mère, ces métabolites sont principalement éliminés par l'intermédiaire des fèces. Le tamoxifen a déjà été mesuré dans les effluents de stations d'épuration et dans les eaux naturelles, mais aucune valeur n'a été reportée pour ses métabolites jusqu'à présent. Un autre anticancéreux, également éliminé par voie biliaire et susceptible d'atteindre l'environnement, est l'imatinib. Cette récente molécule a révolutionné le traitement et la survie des patients souffrant de leucémie myéloïde chronique et de tumeur stromales gastrointestinales. Les effets du tamoxifen et de l'imatinib sur plusieurs générations d'organismes aquatiques, tels que les microcrustacés *Daphnia*, sont inconnus et le 4OHTam et l'endoxifen n'ont même jamais été testés en écotoxicologie.

Cette thèse s'est articulée autour de trois objectifs principaux. Premièrement, la sensibilité des *D. pulex* exposés au tamoxifen, 4OHTam, endoxifen et imatinib a été évaluée par l'intermédiaire de tests aigus et de tests sur deux à quatre générations. La mobilité, la taille, la reproduction, la viabilité et la croissance potentielle de la population ont été relevées au cours de ces expériences. Des tests supplémentaires, à but prospectifs, ont également été réalisés afin d'évaluer 1) la capacité de récupération des daphnies, lorsque leurs descendants ont été placés dans un milieu exempté de tamoxifen ou de 4OHTam, 2) les effets chez les daphnies exposées à une solution contenant de faibles concentration de tamoxifen et de 4OHTam mélangés. Le deuxième objectif a été d'évaluer la stabilité du tamoxifen, 4OHTam et endoxifen dilué dans le milieu des daphnies. Après analyses, les concentrations mesurées ne correspondaient pas aux concentrations nominales (c.-à-d., théoriques) et

il a été nécessaire de développer une méthode efficace de prédiction des niveaux d'exposition lors de tests de longue durée réalisés avec ces trois molécules. Finalement, des changements dans l'expression des protéines chez des daphnies exposées au tamoxifen ont été investigués par l'intermédiaire d'expériences écotoxicoprotéomiques avec une approche dite de shot-gun avec une étape de fractionnement des protéines.

Les résultats obtenus dans cette thèse montrent que le tamoxifen, le 4OHTam et l'endoxifen induisent des effets indésirables chez les daphnies à des niveaux d'exposition proches ou identiques aux concentrations du tamoxifen mesurées dans l'environnement, c'est-à-dire 0.12 et 0.67 µg/L de tamoxifen. Ces molécules ont induit des effets inhabituels tels que la production de : nouveau-nés anormaux, avec des antennes et des queues déformées, des prématurés et des œufs avortés. Le tamoxifen fut la molécule la plus toxique pour les *D. pulex* suivie du 4OHTam, de l'endoxifen et enfin de l'imatinib. Lors des expériences sur plusieurs générations, les concentrations n'ayant statistiquement pas d'effet (c.à.d. NOEC en anglais) sur la taille, la reproduction et la croissance intrinsèque de la population étaient du même ordre de grandeur que les concentrations environnementales du tamoxifen. Par exemple, les NOECs du tamoxifen calculées pour la reproduction étaient de 0.67 et 0.72 µg/L, tandis que celle calculée sur la base des anomalies chez les nouveau-nés était < 0.15 µg/L. Les NOECs du 4OHTam se situaient entre 0.16 et 1.48 µg/L et celles de l'endoxifen pour la croissance intrinsèque de la population, ainsi que pour la reproduction, étaient de 0.4 et 4.3 µg/L, respectivement. Dans l'expérience basée sur la récupération des daphnies, la taille et la reproduction ont diminué bien que la descendance fût placée dans un milieu sans substances chimiques. Les daphnies exposées au mélange de tamoxifen et de 4OHTam ont produit moins de nouveau-nés que les contrôles, alors que ces concentrations n'ont pas induit d'effets lorsque testées individuellement. Finalement, l'imatinib n'a pas montré d'effets sur les deux générations testées. Seule la première génération exposée à la plus haute concentration (626 µg/L) a montré une diminution de la reproduction.

Les résultats obtenus lors de l'évaluation de la stabilité du tamoxifen, 4OHTam et endoxifen dans le milieu des daphnies ont souligné l'importance d'utiliser des concentrations mesurées ou prédites en écotoxicologie. En effet, des différences notables entre concentrations nominales et mesurées ont été observées à toutes les concentrations et l'hypothèse d'un phénomène d'adsorption sur le verre des récipients a été posée. De ce fait, il a été nécessaire d'élaborer une méthode prédictive efficace et acceptable, en terme de temps et de coûts. Une régression polynomiale basée sur des concentrations mesurées et nominales a permis de prédire avec efficacité les faibles niveaux d'exposition utilisés lors d'expériences écotoxicologiques à long terme, sur plusieurs générations.

Suite aux expériences d'écotoxicoprotéomiques, un total de 3940 protéines ont été identifiées et quantifiées chez des daphnies exposées au tamoxifen. Ce nombre est actuellement la plus large série de données publiées et mises à disposition pour la communauté scientifique. Parmi ces protéines, 189

sont significatives et possiblement reliées à des processus de reproduction et de stress. Sur cette base, nous avons émis l'hypothèse que les individus subissant un stress, lié à l'exposition au tamoxifen, ont utilisé leur énergie de base pour favoriser leur survie plutôt que la reproduction. Enfin, la détermination de bio-marqueurs exprimant des dommages précoces des daphnies exposées au tamoxifen n'a pas abouti en tant que telle, mais des protéines prometteuses, telle que la famille de viellogenin-2 (E9H8K5) et le récepteur à la ryanodine (E9FTU9), ont été exprimées après deux jours d'exposition déjà. Ces protéines pourraient faire l'objet d'investigations écotoxicoprotéomiques futures.

Les résultats de cette thèse posent certaines questions quant au risque du tamoxifen, du 4OHTam et de l'endoxifen sur la faune et la flore aquatique et plus particulièrement sur les anticancéreux présents dans l'environnement. Les effets toxiques de ces molécules ont été observés à des concentrations environnementales et sur plusieurs générations. La question de considérer les métabolites, et ainsi les pro-médicaments, en écotoxicologie est soulevée, notamment parce que ces molécules peuvent être plus actives et efficaces que la molécule mère. Les expériences chroniques, sur plusieurs générations sont également à favoriser car elles offrent un meilleur reflet de la réalité environnementale que des essais aigus ou d'une génération. L'utilisation de la protéomique permet d'agrandir les connaissances sur les effets des médicaments à un niveau inférieur de l'organisation biologique et ainsi, de mieux comprendre de potentiels mécanismes d'action ou de déterminer de potentiels biomarqueurs. Finalement, il semble important de discuter de l'opportunité de mesurer les concentrations qui sont testées en écotoxicologie afin de ne pas sous-estimer le risque pour la faune et la flore aquatique.

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Glossary and Abbreviation

4OHTam	4-hydroxyl-tamoxifen
5-FU	5-Fluorouracil
Antagonism	A class of interactive joint action between compounds where the potency effects of the mixture are lower than expected, i.e., where the joint action is interactive
Antimetabolite (cancer treatment)	Class of anticancer drugs that interact with DNA synthesis and thus cell division.
CML	Chronic myeloid leukemia
CYP450	Cytochrome P450
dappu-ERR	Daphnia estrogen-related receptor
DMSO	Organosulfur $(CH_3)_2SO$ dimethyl sulfoxide
Dose-response curve	The dose-response curve is a statistical curve determined for a given organism and a given substance. It is established based on laboratory data that express different concentration-effect relationships for the substance. For a species, the curve predicts the intensity effect (usually expressed in a percentage between 0 and 100) of a substance concentration
EC50	The 50% effect concentration refers to the concentration of a substance where 50% of its maximal effect is observed for a specified endpoint (e.g. immobilisation, death, etc) in a population. This value is statically determined based on the dose-response curve for a given species and a given substance.
Ecdyseroid & Ecdysone	Ecdysteroids are polyhdroxylated ketosteroids that are present in whole arthropod groups. They are major endocrine signaling molecules in crustaceans and other arthropods and are involved in important processes such as molting regulation, embryo development, covering organism formation (cuticle), vitellogenin production, ovulation, etc.
Endoxifen	4-hydroxy-N-desmethyl-tamoxifen
Endpoint	Parameter measured on a given organism during a test exposure, for example, mortality or reproduction. A measurement endpoint designates calculated values such as NOEC or EC50.

EPA (U.S.)	U.S. environmental protection agency
Epigenetic	Gene regulation by factors not coded in the DNA sequence
ERR	Estrogen-related receptors
ERα	Alpha estrogen receptors
ERβ	Beta estrogen receptors
F0, F1, F2, F3	Daphnia generation that are treated with one or several substances. The first generation exposed to a chemical is F0
FDA	Food and Drug Administration
Hsp	Heat shock proteins
LOEC	Lowest observed effect concentration is an experimental value that is determined for a substance. It refers to the lowest test concentration, whose the average response in organism is not statistically different from controls
LogP	Logarithm of the partition coefficient between n-octanol and water $\log(c_{octanol}/c_{water})$
Mode of action	General term used to characterize the action of a molecule on organism. This molecule can have a specific mode of action because a receptor binds to it. In toxicology, the non receptor-mediated mechanisms are the most frequently observed. Most toxic agents act through binding sites in a non-specific manner, i.e. by targeting molecules that are not considered as "receptors" in the pharmacological sense. In addition, toxicants may act indirectly by inducing a change in the immediate biological environment of the target
NOEC	The No-observed effect concentration is an experimental value that is determined for a substance. It refers to the highest test concentration, whose the average response in organism is not statistically different from controls
Nominal concentration	Theoretical concentration is deduced from dilution procedure (stock solutions, prediluted solutions, test solutions), which are applied during experimental work.
OECD	Organisation for economic co-operation and development
Phenotype	Observable traits or characteristics of an organism (e.g., size, behaviour, morphology, etc.)

Pseudopersistent	A continual introduction of pharmaceuticals in the environment by treated and untreated sewage effluents makes these chemicals "pseudopersistent" (Daughton et al. 2002)
SERM	Selective estrogen-response modulator
Signal transduction	The passage of inter- or intra-cellular information (biological activation/inhibition of a function)
STP	Sewage treatment plant
Synergism	Class of interactive joint action between compounds, where the potency effects of the mixture are greater than expected. Synergism can be observed in certain formulations of substances involved in plant protection or biocidal or pharmaceutical products. Synergism was often observed in binary mixture toxicity experiments
Teratogen	Molecules that induce growth retardation or functional abnormalities resulting from nongenetic causes, in fetus and embryo
TKI	Tyrosine kinase inhibitor
Xenobiotic	Unnatural molecule that are found within an organism or a natural compartment (e.g., water, soil, etc.). Usually a xenobiotic is not expected to be present within living-organisms but in the case of pharmaceuticals, it can be intentionally administrated.

Chapter 1

Introduction

General introduction

The aquatic environment is exposed continuously and increasingly to chemical substances, such as pesticides, personal care products, antifouling paint, plasticizers, cosmetics, etc., which are emitted daily by anthropic activities (e.g., manufacturing processes, fishing, agriculture, traffic, industries, leisure, medicine, etc., [1–7]). These substances are not considered as natural and they are called xenobiotics. The potential effects of xenobiotics are of growing concern because living organisms, including humans, are chronically exposed to several of them via food or waters [7–18]. Pharmaceuticals are chemicals among these thousand of xenobiotics that invade the aquatic environment (for reviews see [19,20]). They are present in STP effluents, surface water, groundwater and drinking water (for recent review see [21]). Since the 1990s, several studies have focused on the occurrence of these pharmacologically active compounds because once in the aquatic environment, some of them can induce effects in organisms ([22,23], for recent review on pharmaceuticals see Brausch et al. [20]). These effects were death or physiological dysfunctions in individual organisms (e.g., reproduction, body-length, molte, etc.), but also decline in population growth and alterations in structure and functions of ecosystems [3,5,24,25].

Industries, consumers and prescribers are considered as the three sources of pharmaceuticals release in waters [26–28]. The pharmaceutical industries are the producers of synthetic molecules that are dedicated to prevent, cure, substitute or diagnose physiological dysfunctions. Prescribers are primarily the professional teams (e.g., pharmacists, physicians, etc.) that are entitled to give pharmaceuticals to patients or animals in hospitals, in physician/veterinary offices, in medical services, in pharmacies, etc [29–31]. Consumers are people and animals that receive prescribed or non-prescribed pharmaceuticals. Except from accidentally discharges in the environment, consumers are considered as the main environmental entrance pathway for pharmaceuticals [26]. Once absorbed, the body uses, transforms and eliminates these molecules. Less or more active forms of the pharmaceutical are primarily body-excreted with faeces and urine in soil (animals) or wastewaters (human). These drug residues reach the aquatic environment by runoff or sewage treatment plant (STP) effluents, for instance. Although new STP technologies are effective at removing some pharmaceutical products [32], traditional STP processes are primarily efficient for organic mater. Therefore, some synthetic molecules, such as pharmaceuticals, escape STP processes and are measured in STP effluents and surface waters (e.g., rivers, lakes, estuaries, groundwater [21,22,33–45]).

Among pharmaceuticals that escape STP processes, different anticancer drugs were measured in wastewaters and natural waters. Most of these chemicals induce side effects in treated patients because they do not target abnormal cells only. They are able to disturb physiological function of normal cells, which cause adverse reactions such as mutagenic, carcinogenic, teratogenic or embryotoxic effects [46,47]. Although recent generations of anticancer drugs, such as signal transduction inhibitors, have a

low mutagenic potential, they may interfere with important cell cycle-related functions in exposed organisms (e.g., nucleic acids synthesis, DNA replication, mitosis). For instance, STP poorly biodegraded methotrexate, ifosfamide and cyclophosphamide, and these compounds were often found in STP effluents and natural waters [43,45,47–53]. Anticancer residues may still be active in waters and their specific or general action on tumours and normal cells could induce effects in non-target eukaryote organisms, i.e., in organisms with complex cells that contain a nucleus with genetic material. Consequently, their prolonged presence in surface water may also adversely modulate physiological functions of eukaryotic organisms that compose the flora and fauna [54]. In other words, active anticancer residues may induce genetic and cell cycle changes in aquatic organisms such as algae, invertebrates and vertebrates, which constitute the biodiversity of the environment. Biodiversity is a key factor for humans and environment health and well-being. It ensures the supply of ecosystem services and facilitates ecosystem stability, which is the basis of sustainable development [55,56]. For instance, invertebrates are key species in the food chain and their ability to filter waters improves water quality. Among invertebrates, the freshwater microcrustacean *Daphnia* is primary food supply for most aquatic animals, and it is also involved in the food chain to sustain fish that is consumed by human. Its ability to transform phytoplankton and decaying matter into more usable form represent a vital step for almost any freshwater ecosystem. Daphnids can also be used as sensitive model organism in ecotoxicology to assess the effect of xenobiotics on the aquatic fauna [57,58]. Ecotoxicology is the science that considers the toxic effects of natural or synthetic substances, such as pharmaceuticals, on organisms, populations, community and ecosystem, including the transport, transformation and degradation processes of these compounds [59]. Ecotoxicological experiments can be performed in laboratories with aquatic invertebrates but also with vertebrates and algae. Compared to the number of studies that are performed yearly in rodents and other vertebrates to improve scientific knowledge in pharmaceuticals, which are already on the market or in pre-clinical studies, ecotoxicological experiments are sparse [60], particularly with algae and invertebrates. Invertebrate is, however, an abundant species family that comprises about 95% of all species [61], and crustaceans cover at least 30,000 species [62]. Therefore, invertebrates need to be considered when the effects of chemicals are assessed in the environment.

In ecotoxicology, treated organisms such as daphnids are exposed to one or more substances during a certain period of time that can be acute or chronic when the period is short (e.g., 2 days) or long (e.g., 21 days), respectively. Acute ecotoxicological experiments are usually performed with higher concentrations than those used in long-term experiments. Although recent efforts, ecotoxicological studies with pharmaceuticals are performed primarily in short-term exposure rather than in chronic exposure [20,63–65]. In 2006, Buerge and al. [47] warned researchers about the serious lack of chronic exposure studies of drug residues on aquatic biocenosis. The same year Brennan and al. [66] drew attention to the paucity of multigenerational testing with oestrogen-mimicking compounds, while

alarming effects were observed even in humans; the classical case of diethylstilbestrol still remains the most illustrative example of an endocrine disruptive compounds that induced side effects on offspring [16]. Acute toxicological assays neglect possible chronic actions of the chemical [67] and these experiments have little relevance concerning the "true" exposure of the organisms in the environment. Indeed, pharmaceutical residues are considered as pseudopersistent in the aquatic environment, because of their continuous release by STP effluents [27], and therefore organisms are exposed to these molecules during long periods. International organisations and agencies such as the Organisation for economic co-operation and development (OECD) and the U.S. environmental protection agency (EPA), recommend standard exposure period depending on the test species [68–72], which includes whole life cycle of the organisms or several generations (i.e., multigenerational exposure) [65,66,73]. In risk assessment, ecotoxicological experiments with pharmaceuticals are therefore preferred at low concentration and during long-term exposure, although the impacts of anticancer drugs on the aquatic fauna and flora at these conditions are sparsely evaluated [63].

A complementary approach could be used to observe effects at lower level of the biological organisation and earlier in time, before these effects are observed at the individual level [74–76]. In ecotoxicology, pollutants are primarily interpreted in terms of ecotoxicological threat when they are linked to phenotype effects. Elucidating the effects of xenobiotics at the cell level allows subtle understanding of the mechanisms that are activated by chemical exposure. Proteins carry out or regulate most biological processes, and analysis that consider proteomes, i.e. all proteins that are expressed by a cell or tissue, seems to be relevant to assess the functional state of an organism [77]. Indeed, a proteomics approache may be able to detect changes in protein expression before these changes affect the homeostasis of the organisms [74,78,79]. In association with metabolic, transcriptional and histopathological data, proteomics represents therefore an appealing strategy in ecotoxicology.

In this introduction, the following subsections contain more details on why anticancer drugs are important to consider in environment and why they may induce effect on non-target species. Three anticancer families are presented: the traditional, the recent and the hormonal anticancer drug families. Four molecules belonging to these families are described in this section, including two potent metabolites. The science of proteomics is introduced as well as its usefulness in ecotoxicology. Finally, the reasons of using the freshwater microcrustacean *Daphnia pulex* are described in the last section of this chapter.

Anticancer drugs

Why and which anticancer drugs?

The incidence of cancers is increasing worldwide related to levels of human development, longer average life expectancy, efficiency of new treatments, etc. The number of new cancer cases is expected to grow to 23.6 million each year by 2030, which would be 68% more cases than in 2012 [80]. This prediction takes in consideration the recent trends in incidence of major cancers as well as the global population growth in the future. An accurate evaluation of anticancer drugs consumption is difficult to obtain in Switzerland. In 2002, Buerge et al. [47] reported a consumption of 55 kg cyclophosphamide and 12 kg ifosfamide in Switzerland. In 2004 in France, other authors calculated a yearly administration of about 5.8 tons of hydroxycarbamide, 1.7 tons of 5-fluorouracile, 580 kg of imatinib, 120 kg of ifosfamide [81]. The Canadian Ministers of the Environment and of Health estimated that 320 Kg/year of tamoxifen was used in Canada in year 2012 [82]. Furthermore, demands for chemotherapy treatment may increase with increasing number of cases, and with the recent treatment improvements, the number of deaths may decrease because patients spend progressively longer periods of their lifetime under treatment.

Oncology represents currently one of the most active fields of pharmaceutical innovation and progress. The increasing use of signal transduction inhibitors provides a typical example illustrating the recent evolution of cancer treatments [83,84]. Agents belonging to this class of anticancer drugs are able to disrupt signalling pathways that maintain cellular proliferation. For instance, imatinib and the next generations of tyrosine kinase inhibitors (TKIs) are recent family of anticancer drugs that target specific mechanisms of tumour cell biology. These TKIs prolong life of patients with chronic myeloid leukaemia compared to other traditional treatment used against this disease [85]. They target the mechanisms that are involved in pathological deregulations of a normal cell proliferation, i.e., key biological pathways responsible *per se* or implicated in cancer development [86]. As TKIs, other anticancer drugs from the recent generation have specific mechanisms of action and a high specificity for key biological pathways. These properties increase their antitumour efficiency and limit their overall toxicity. Numbers of these recent anticancer agents are small molecules that interact with membrane-bound receptor kinases, intracellular signalling kinases, epigenetic mechanisms such as DNA methylation and histone acetylation, and/or the tumour microenvironment. The traditional anticancer drugs are less specific and they can be cytotoxic. Cytotoxic anticancer drugs are systemic anti-proliferative agents that target dividing cells and not specific cells [87]. They include antimetabolites, alkylating agents, DNA-complexing agents, mitosis inhibitors or hormones, such as cyclophosphamide, ifosfamide, busulfan, methotrexate, tamoxifen, etc. [88]. Usually, they interfere with cell divisions, DNA synthesis and/or repair mechanisms [86]. Among the traditional class of anticancer compounds some have also endocrine disruptive activities. This class of drug is often used

in cancers associated with endocrine disorder, and they include aromatase inhibitors (letrozole, anastrozole, exemestane), selective estrogen-response modulator SERM (tamoxifen), progesterone-like drugs (megestrol acetate), antiandrogens (flutamide, bicalutamide), etc. These anticancer drugs are prescribed in medicine therapies, but they are also used in sport and veterinary fields to enhance performance or growth [89–91]. Once in the environment, these pharmaco-active molecules could be considered as potential endocrine disruptors, according to the WHO definition [92] : "A potential endocrine disruptor is an exogenous substance or mixture that possesses properties that might be expected to lead to endocrine disruption in an intact organism, or its progeny, or (sub)populations". More precisely, endocrine disruptive substances, such as certain anticancer drugs, may act at the pre-receptor, the receptor and the post-receptor levels of the endocrine system in non-target organisms (for details see Annexe 1). When a xenobiotic interferes with this endocrine system, the physiology and thus the normal development of the organism can be disrupted, which is considered as an adverse effect that would not have occurred in absence of this compound [93]. For instance, some industrial compound residues, such as pesticides (atrazine, DDT, endosulfan, dieldrin, etc), antifouling compounds (tributyltin "TBT"), fungicides (vinclozolin), phtalates, PCBs and its congeners, phenolic chemicals (bisphenol-A) are known to induce endocrine responses in aquatic organisms. In mollusks, alligators and fish, some of these chemicals influence sexual determination, differentiation, and development, which cause adverse effects such as feminization, imposex (simultaneous presence of male and female reproductive organs), decrease in fecundity and embryos production, or morphological abnormalities [17,94–99]. Due to these evidences, hormone-like industrial compounds have received more attention than hormonal drugs [7], although these pharmaceutical were found in the aquatic environment under active forms [100,101]. In general however, anticancer drugs are body excreted and are found in wastewaters and in natural waters, like cyclophosphamide and ifosfamide for instance [47–49,102]. These traditional anticancer drugs have enzymatic targets that are present in numerous living organisms, and once in the aquatic environment, they may pose a risk to the aquatic flora and fauna, and also to human [103].

The chosen anticancer drugs

In this study, tamoxifen and two of its potent metabolites were chosen among the traditional anticancer family that have hormone disruptive effects in vertebrates. Conversely, the anticancer drug imatinib was chosen because it is a new anticancer molecule that has no endocrine disruptive activities in vertebrates. The properties of these four molecules and their pharmacological effects are explained in the next subsections.

Tamoxifen and its potent metabolites 4-hydroxy-tamoxifen and endoxifen

Tamoxifen is a synthetic non-steroidal anticancer drug that is prescribed to slow down growth of hormone-dependent tumours. It was the first hormonal agent approved by the US Food and Drug Administration (FDA) for the prevention of breast cancer, for the treatment of ductal carcinoma in situ, and for the treatment of pre-menopausal breast cancer. It is also the main hormonal treatment prescribed for early and advanced male breast cancers [104]. In 1970, tamoxifen was available for the treatment of advanced breast cancer in postmeopausal women [105] but its approval has differed depending on the countries. In 1978, tamoxifen was accepted as endocrine therapy for postmenopausal women with advanced cancers and became the drug of choice for these cancers in 1980. Tamoxifen approval was mainly due to its similar efficiency, but less toxic, than diethylstilbestrol [105].

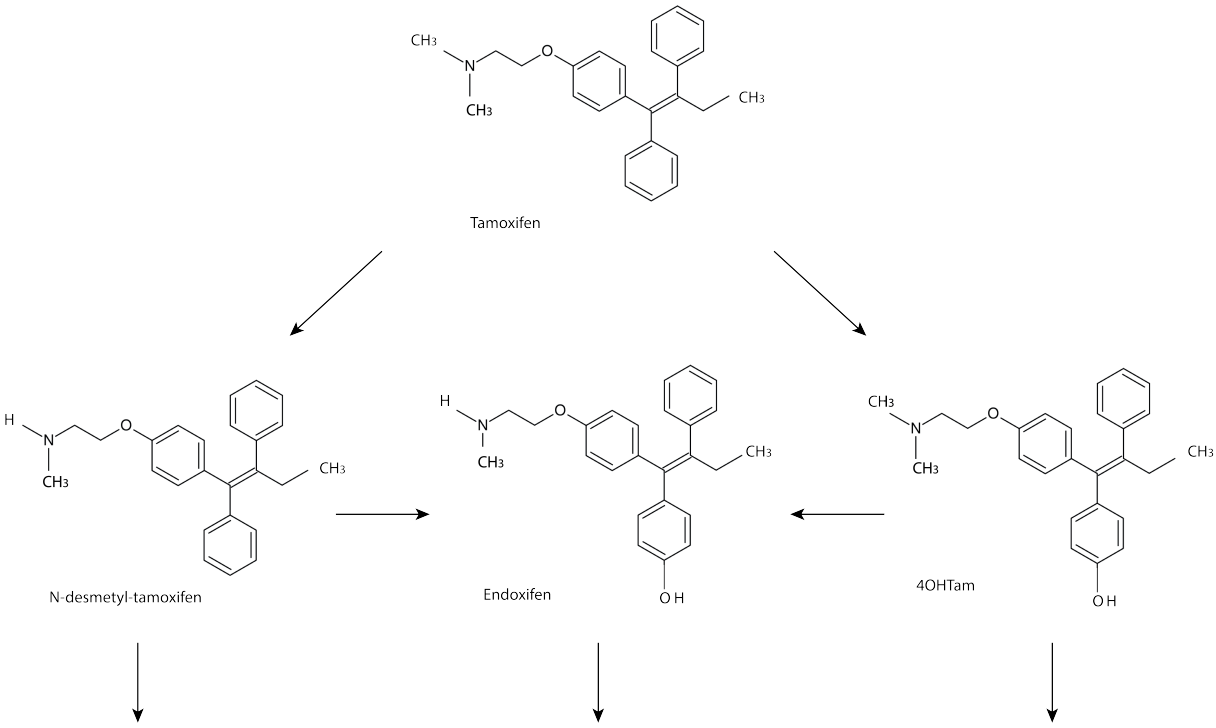

Figure 1.1: Molecular structure of tamoxifen, 4OHTam and endoxifen. Tamoxifen is extensively metabolized (predominantly by the cytochrome P450 system) into several primary and secondary metabolites

Tamoxifen is considered as a selective estrogen receptor modulator (SERM) because it exerts agonist and antagonist estrogenic effects depending on the targeted tissue [16]. When the effects are mediated by alpha estrogen receptors (ERα), tamoxifen acts as a partial agonist or a partial antagonist. When the effects are mediated by beta estrogen receptors (ERβ), this drug behaves as a partial agonist. Tamoxifen anti-estrogenic effect (inhibiting agent) is predominant on breast, vagina and central nervous system tissues while its estrogenic-like effects (stimulating agent) are reported to be mostly present in cholesterol metabolism and other tissues including the endometrium, bone and liver. In

stroma breast cells, its main action is cytostatic since it blocks cell cycle in G1 phase and then induces a decrease of tumour cellular proportion and proliferation [106]. Tamoxifen also controls cell replication through nuclear interaction or growth factors mechanisms (increases the production of transforming growth factor β (TBFβ) or decreases IGF1, TGFα and EGF growth factor expression). Also, tamoxifen is often used in laboratory experimentation with Cre genetically modified animals. It is an inducing agent that trigs the gene expression of specific tissue, usually from mouse mutants. It is administrated at a specific time point in the embryonic development or adult life [107].

Pharmacology of tamoxifen

Patients orally take 20-40 mg of tamoxifen citrate per day [108], which corresponds to 0.4-0.8 mg/kg for an average 50 kg woman, sometimes after loading doses of 80 to 200 mg during 1 to 7 days [109]. The treatment is usually taken for 5 years or according to its efficacy and safety, in the case of metastatic hormone-dependent breast cancer. Longer durations might even become recommended [110]. Following a single oral dose of 20 mg tamoxifen citrate, the peak concentration in plasma is about 40 µg/L (range 35 to 45 µg/L) and occurred 4 - 7 hours after dosing [111]. The native substance half-life is about 7 days [112]. Tamoxifen is more than 99% bound to plasma proteins, predominantly to albumin [113]. A steady-state plasma concentration of 140 and 160 µg/L is reached after a chronic administration of 20 and 30 mg of tamoxifen citrate twice daily, respectively [113,114]. This steady-state is achieved after 4 weeks [112] but it can be reached faster when high loading doses are administrated [109]. The volume of distribution is high, i.e., 50-60 l/kg, which indicates that tamoxifen accumulates in organs either by active transport or by specific binding to tissue molecules. In patients with breast cancer, the concentrations of tamoxifen were 10 to 60 times higher in liver, lung, pancreas, brain and adipose, than in serum [115].

Tamoxifen is metabolized by hepatic P450 cytochromes into several metabolites [116]. Its main metabolites are: N-desmethyl-tamoxifen, 4-hydroxy-tamoxifen (**4OHTam**), tamoxifen N-oxide, and alpha-hydroxy-tamoxifen. Finally, 4-hydroxy-N-desmethyl-tamoxifen (**endoxifen**) results as a secondary metabolite of tamoxifen that is primarily formed from N-desmethyl-tamoxifen by the cytochrome CYP2D6. The molecular structures of tamoxifen, 4OHTam and endoxifen are shown in Figure 1.1. Note that mutations in CYP2D6 prevent the formation of endoxifen and thus may reduce tamoxifen response in patients [117]. Endoxifen serum level is 6 to 12 fold higher than 4OHTam, but pharmacological activity of both metabolites is 30 to 100 fold more potent than the parent molecule [104,118–120]. More specifically, 4OHTam and endoxifen express an affinity to ERs 100 fold higher than the original molecule and than other metabolites. Their potency to suppress cell proliferation in breast cancer is also 30 to 100 fold greater than the latters. *In vitro*, both metabolites have equivalent potency to bind ERα and ERβ, and to suppress ER-dependent human breast cancer cell line.

Tamoxifen anticancer action in breast tumour may also be explained by antagonist properties on the estrogen-related receptors (ERRs) [121]. In mammals, the ERRs belongs to the nuclear receptors superfamily (ERRα, ERRβ and ERRγ in mammals), which regulate transcription via estrogen response elements and estrogen-related response elements [121]. The amino acid sequence of human ERRs is over 60% identical to the human ERα and ERβ [122]. Although they share common transcriptional target genes, their function are different [123–125]. For instance, ERRs are unable to bind natural estrogenic ligands such as 17β-estradiol [126]. Its metabolite 4OHTam has a higher potency and affinity to ERs than tamoxifen [118–120], and also a higher affinity to ERRα and ERRγ. Tamoxifen and 4OHTam are considered as antagonist of ERRα and ERRγ [121] and none of them bind ERRβ [124,127].

Tamoxifen and its metabolites ultimately undergo fecal excretion (i.e. biliary route [109]). To be better excreted within the bile, tamoxifen and 4OHTam are mainly conjugated into glucuronide acids. Deglucuronidated tamoxifen and metabolites undergo enterohepatic cycle [128], which prolongs their half-life in patients. Kisanga et al. [129] analyzed the excretion of tamoxifen and its metabolites in human urine and bile after a *per os* dose of ^{14}C-tamoxfien. 4OHTam and endoxifen were both found in urine and feces. The 4OHTam was first detected followed by endoxifen (three days later than 4OHTam). However, 4OHTam was the dominant metabolite excreted in the bile and might result from a first-pass, contrary to endoxifen that only appeared when tamoxifen had reached the central compartment.

Physical-chemical properties of tamoxifen, 4OHTam and endoxifen

Tamoxifen is a tripheylethylene drug. It is a white crystalline solid from petroleum ether that is easily soluble in methanol but very slightly soluble in water [130]. Tamoxifen is not readily biodegradable [131] and it has a high bioaccumulation potential with an experimental logP of 7.1 [132]. The physical state of tamoxifen as human pharmaceutical is tablets. In pharmaceutical formulations, tamoxifen is invariably present as its citrate salt. Each tablet contains 15.2 mg of tamoxifen citrate, which is equivalent to 10 mg of tamoxifen [82]. The metabolites 4-hydroxy-tamoxifen (4OHTam) and endoxifen are formed in liver cells. Physical and chemical properties of tamoxifen, 4OHTam and endoxifen are summarised in Table 1.1.

Table 1.1: Physical and chemical properties of tamoxifen, 4OHTam and endoxifen

	Property	Type	Data	Reference
Tamoxifen	CAS		10540-29-1	Government Canada, 2014
	Molecular formula		$C_{26}H_{29}NO$	Government Canada, 2014
	Molecular weight (g/mol)		371.51	Government Canada, 2014
	Boiling point (°C)	Modelled	468.2	Government Canada, 2014
	Density (kg/m^3)	Experimental	N/A	Government Canada, 2014
	Vapour pressure (Pa)	Modelled	4.62×10^{-6}	Government Canada, 2014
	Log P	experimental	7.1 (experimental)	DrugBank 2013
	Water solubility (mg/L)	Experimental	< 0.5	CEREP, 2010
4OHTam	CAS		68047-06-3	Government Canada, 2014
	Molecular formula		$C_{26}H_{29}NO_2$	Government Canada, 2014
	Molecular weight (g/mol)		387.51	Government Canada, 2014
	Boiling point (°C)	Modelled	503.04	Government Canada, 2014
	Density (kg/m^3)	Experimental	N/A	Government Canada, 2014
	Vapour pressure (Pa)	Modelled	4.14×10^{-9}	Government Canada, 2014
	Log P	experimental	6.3 (calculated)	Pubchem.ncbi.nlm.nih.gov
	Water solubility (mg/L)	Experimental	< 0.5	CEREP, 2010
Endoxifen	CAS		110025-28-0	Government Canada, 2014
	Molecular formula		$C_{25}H_{27}NO_2$	Government Canada, 2014
	Molecular weight (g/mol)		373.49	Government Canada, 2014
	Boiling point (°C)	Modelled	501.85	Government Canada, 2014
	Density (kg/m^3)	Experimental	Not available	Government Canada, 2014
	Vapour pressure (Pa)	Modelled	4.32×10^{-9}	Government Canada, 2014
	Log P	experimental	6.3 (calculated)	Pubchem.ncbi.nlm.nih.gov
	Water solubility (mg/L)	Modelled	< 1.5	CEREP, 2010

CEREP, 2010 [130], DrugBank, 2013 [132], Government Canada, 2014 [82]

Toxicity of tamoxifen in terrestrial organisms

During acute exposure experiments, it was observed that a single oral dose of 2.5 and 3 g/kg of tamoxifen was lethal on 50% (LD50) of the treated rats and mice, respectively [133]. There is no available data on acute over dosage in humans by ingestion, inhalation, skin exposure, eye contact, parenteral exposure or other. In 1992 however, Trump et al. [134] run a study where high doses were given in advanced metastatic cancer women. In this study, patients took an initial dose of > 400 mg per body surface area in m^2 (mg/m^2) followed by a treatment of 150 mg/m^2 twice a day. These doses induced acute neurotoxicity symptoms, such as tremor, over-reflexes, unsteady gait and/or dizziness, 3 to 5 days after the loading dose. The neurotoxic symptoms were not permanent and decreased 2 to 5 days after the treatment was stopped. Cardiac symptoms, i.e., prolongation of the QT interval on the electrocardiogram, were also observed in patients who received loading dose > 250 mg/m^2, followed by maintenance doses of 80 mg/m^2 twice a day. The maximum tolerated dose determined in that study was 300 mg/m^2/day tamoxifen.

Table 1.2: Tamoxifen toxicity in vertebrates including human

	Compound	Species	Dose	Unit	Endpoint	Effect(s)	Duration	References/cited in
Humans	**Tamoxifen citrate**	Women/men	≤ 180	mg	sides effects	Eyes (retinal damage and keratitiss)	> 1 year	Buckley and Goa. 1989
	Tamoxifen citrate	Women	20	mg/day	pregnancy	Fetal, neonatal disorder	months	IARC, 1996
	Tamoxifen citrate	Women	1 and 5	mg/day	cell proliferation, serum biomarkers	Suppressing breast cell proliferation, decreasing activity in modulating serum biomarkers		Decensi et al., 2003
	Tamoxifen citrate	Women	1	mg/day	blood proteins and markers	IGF-I declined, antithrombin III, CRP	12 months	Decensi et al., 2007
	Tamoxifen citrate	Women	10	mg/day	Ki-67, Estrogen receptor (1D5), progesterone receptor positivity in breast epithelium	Reduced: monoclonal antibody Ki-67 (MIB-1), estrogen receptor (1D5) and progesterone receptor positivity (PgR 636) in the breast epithelium of carcinoma	14 days	Sousa et al. 2006
	Tamoxifen citrate	Women	5	mg/day	biomarker, mammographic density	Insulin-like growth factor I (IGF-I), uterine effects, breast neoplastic events	2 years	Decensi et al., 2009
	Tamoxifen citrate	Women	1 and 5	mg/day	biomarkers	Marker (Ki-67 expression), cancer blood biomarkers (insulin-like growth factor-I, sex hormone-binding globulin), cardiovascular disease, bone fracture risk	4 weeks	Decensi et al., 2003
Animals	**Tamoxifen**	Monkey	2	mg/kg BW	DNA	DNA-adducts in multiple tissues	30 days	Schild et al. 2003
	Tamoxifen	Monkey	3	mg/kg-day	Reproduction	Inhibition of pregnancy establishment	3 to 12 days	Ravindranath et al., 1986
	Tamoxifen Citrate	Dog	0.1	mg/kg	tolerance	Well tolerated	chronic	IARC, 1996
	Tamoxifen Citrate	Mice	0.1	mg/kg	tolerance	Well tolerated	chronic	IARC, 1996
	Tamoxifen Citrate	Mice	> 0.1	mg/kg	sides effects	Endometrium (hyperplasia)	chronic	IARC, 1996
	Tamoxifen Citrate	Mice	5	mg/kg/day	LOAEL	Benign tumors, reproductive system	13-15 months	[a]
	Tamoxifen Citrate	Rabbit	0.5	mg/kg/day	LOAEL	Fetotoxicity	chronic	[a]
	Tamoxifen Citrate	Rabbit	2	mg/kg/day	LOAEL	Not Teratogenic	chronic	[a]
	Tamoxifen Citrate	Rat	0.1	mg/kg	tolerance	Well tolerated	chronic	IARC, 1996
	Tamoxifen Citrate	Rat	> 0.1	mg/kg	sides effects	Endometrium (epithelium hypertrophy)	chronic	IARC, 1996
	Tamoxifen Citrate	Rat	147	mg/kg	LOAEL	Female reproductive system	7 weeks	[a]
	Tamoxifen Citrate	Rat	0.04	mg/kg BW	reproduction	Oocyte implantation	2 weeks	IARC, 1996
	Tamoxifen Citrate	Rat	0.025	mg/kg BW	highest dose	Successful pregnancy		IARC, 1996
	Tamoxifen Citrate	Rat	0.04	mg/kg/day	LOAEL	Fertility, Fetotoxicity	chronic	[a]
	Tamoxifen Citrate	Rat	0.16	mg/kg/day	LOAEL	Fertility, Fetotoxicity	chronic	[a]
	Tamoxifen Citrate	Rat	5	mg/kg/day	incidence of tumour	Tumors, liver	2 years	[b]
	Tamoxifen Citrate	Rat	45	mg/kg/day	LOAEL	Tumors, liver	6 months	[a]
	Tamoxifen Citrate	Rat	5	mg/kg/day	LOAEL	Tumors, liver	24 months	[a]
	Tamoxifen	Rat	22.6	mg/kg-day	Carcinomas incidence	100% incidence of cancer (hepatocellular carcionomas), first carcinoma observed at 6 month already	12 monts	Hard et al., 1993
	Tamoxifen	Rat	11.3	mg/kg twice a day	incidence of carcinomas	67% incidenc of carcinomas	12 montrs	Hard et al., 1993

[a] Pfizer pharmaceutical company: www.pfizer.com/products
[b] www.druginfonet.com/tamoxfen.htm
LOAEC: lowest-observed-adverse-effect concentration

Buckley and Goa, [135], Decensi et al., [136], Hard et al., [137], IARC, [105], Ravindranath et al., [138]Schild et al. [139], Sousa et al., [140].

Table 1.2 summarised the chronic effects assessed during laboratory experiments or clinical studies. The international agency for research on cancer (IARC, [105]) also reviewed the long-term toxicity of tamoxifen in vertebrates. It is know for instance that approximately 4 % of the patients stop tamoxifen therapy due to side effects, mainly such as hot flushes, tachycardia, nausea and vomiting. A chronic exposure to about 0.1 mg/kg is well tolerated by mice, rats and dogs [105]. In rats, repeated doses of higher doses of tamoxifen induced endometrial epithelium hypertrophy, while endometrial hyperplasia was observed in mice. Chronic exposure to tamoxifen also induced different types of ocular toxicity [105,135].

Toxicity of tamoxifen in aquatic organisms

Tamoxifen is known to escape degradation process by STPs [48,49,141,142] and this pharmaceutical was found in the aquatic environment, including groundwater [50,53,143]. The concentrations of tamoxifen in sewage and natural waters are summarised in Table 1.3. We hypothesis its metabolites undergo the same scenario because they are also body-excreted in faeces and because they have similar properties as tamoxifen. Therefore, tamoxifen and its metabolites may enter continuously in surface waters, being considered as pseudopersistent compounds in the environment. To the best of our knowledge, no literature reports aquatic concentration levels or effects of its metabolites in aquatic species (e.g., endoxifen and 4OHTam).

Table 1.3: Tamoxifen concentrations in waters

Location	Concentration (µg/L)	Reference
STP effluents	0.02 - 0.37	Ashton et al., 2004; Langford and Thomas, 2009; Roberts and Thomas, 2006
Surface waters	0.01 - 0.21	Roberts and Thomas, 2006; López-Serna et al., 2012
Groundwater	0.01 - 0.02	López-Serna et al., 2012, Reh et al., 2013

Ashton et al., [51]; Langford and Thomas,[52]; López-Serna et al., [50], Roberts and Thomas, [53], Reh et al., [143]

Some aquatic species are known to be sensitive to tamoxifen, such as fish and crustacean. Table 1.4 summarises the concentrations that induced adverse effect on aquatic species. Various morphological and developmental effects were induced in sea urchin embryos after exposure to 3.715 to 3715 µg/L of tamoxifen but other adverse outcomes were observed in crustaceans and algae [82,144–146].

Table 1.4: tamoxifen toxicity in aquatic species (algae, invertebrates and vertebrates)

	Species	Effect(s)	Endpoint	Exposure (µg/L)	Duration	References
Algae	*M. aeruginosa*	growth rate	LOEC	130	21 days	Unpublished AstraZeneca study
	S. capricornutum	growth rate	LOEC	8	21 days	Unpublished AstraZeneca study
Invertebrates	*B. calyciflorus*	population growth inhibition	EC50	250	48 hours	DellaGreca et al. 2007
	T.s platyurus	lethal	LC50	400	24 hours	DellaGreca et al. 2007
	C. dubia	population growth inhibition	EC50	0.81	7 days	DellaGreca et al. 2007
	D. magna	lethal	LC50	1530	24 hours	DellaGreca et al. 2007
	D. magna	reproduction	LOEC	90	21 days	Unpublished AstraZeneca study
	D. magna	length	NOEC	30	21 days	Unpublished AstraZeneca study
	D. magna	reproduction	NOEC	50	21 days	Unpublished AstraZeneca study
	A. tonsa	inhibition of naupliar development	EC10	8.7	5 days	Andersen et al. 2001
	A. tonsa	inhibition of naupliar development	EC50	49	5 days	Andersen et al. 2001
	P. lividus, S. granularis	fertilization, malformations	-	3715	-	Pagano et al., 2001
	P. lividus, S. granularis	fertilization	-	3.715	-	Pagano et al., 2001
Vertebrates	*L. macrochirus*	? (acute)	LC50	150	96 hours	Unpublished AstraZeneca study
	S. gairdneri	? (acute)	NOEC	180	96 hours	Unpublished AstraZeneca study

EC10: the concentration of a substance that induced effect on 10% of the test organisms
EC50: the concentration of a substancethat induced effec effect on 50% of the test organisms
LC50: the concentration of a substance that is lethal to 50% of the test organisms
LOEC: lowest-observed-effect concentration
NOEC: no-observed-effect concentration

Andersen et al. [144], DellaGreca et al. [145], Pagano et al. [146], Unpublished AsraZeneca study, in [82]

Imatinib

Imatinib (Gleevec®) was the fourth pharmacologically active molecule that was chosen in this thesis. Imatinib was the first tyrosine kinase inhibitor (TKI) that reached the market. This molecule belongs to the recent class of anticancer drug, with specific properties in tumour cells. This specificity has revolutionized the treatment and the survival of patients suffering from chronic myeloid leukaemia or gastrointestinal stromal tumour [147,148]. While the number of people susceptible to receive this pharmaceutical was initially restricted to about 60 per year in Switzerland, it has been increasing by tenfold over the last decade (personal communication, Thierry Buclin) and this number of treated patient may continue to grow. The reason of this increase is precisely due to survival of patients who would otherwise died. Following imatinib several other TKIs were marketed, such as gefitinib, sunitinib, nilotinib, dasatinib, sorafenib and lapatinib. These new generations of molecules are used against various cancers but with a broad prevalence for chronic myelogenous leukemia and gastrointestinal stromal tumours. Because these molecules demonstrated a definite efficacy on survival of advanced cancer patients - however less impressive than for imatinib in its specific indications - a progressive increase in their use and in their release in the environment may also be expected to occur.

Pharmacology of imatinib

Imatinib mesylate is mainly used to treat Philadelphia chromosome positive chronic myeloid leukemia (MCL) and gastrointestinal stromal tumours (GIT) with KIT mutations [147,149]. Imatinib mesylate is an oral anticancer drug precisely designed to selectively inhibit certain protein tyrosine kinase that are involved in oncogenesis. These protein tyrosine kinases control the activation of transduction pathways, which regulates cellular processes, such as growth, differentiation and apoptosis [150]. Dysregulated tyrosine kinases can display adverse molecular responses. In the chronic myeloid leukemia (CML) with Philadelphia chromosome aberration, the translocation of this chromosome leads to the fusion of ABL gene and BCR gene [151]. This fused tyrosine kinase Bcr-Abl induced unusual cell activity and thus the disease. Imatinib acts on cells that express the Bcr-Abl and inhibits the activity of this fused tyrosine kinase, which prevents tumours proliferation and growth [147]. In the case of GIT, imatinib inhibits KIT (and thus signal transduction) in a similar manner to its inhibition of Bcr-Abl [152,153]. Besides, imatinib targets other tyrosine kinases, such as platelet-derived growth factor receptor (PDGF-R). Therefore, imatinib is a specific molecule that act on definite tyrosine kinase domains and not on normal healthy cells [149]. This specificity has revolutionized the treatment of patients, primarily for the CML.

In patients treated with 400 mg/day of imatinib, its steady-state in plasma is about 979 µg/L after 29 days [148]. Cytochrome P450 system metabolises imatinib in different metabolites. The N-demethylated piperizine derivative, also named CGP 74588, is an active metabolite of imatinib, which has a similar potency to its parent compound [152,154]. Imatinib is the predominant component in plasma, followed by CGP 74588 [154]. Imatinib dose is predominately excreted in faeces (68%) and in urine (13%). Approximately 25% of the dose is excreted in unchanged form and < 13% was found in the form of CGP 74588 [154,155].

Physical-chemical properties of imatinib

Imatinib is a benzamide drug classified as protein kinase inhibitors and antineoplastic agent [155]. The molecular structure of imatinib is shown in **Figure 1.2**. This molecule is very soluble in water [155]. **Table 1.5** summarises the physical and chemical properties of imatinib and imatinib mesylate. The physical state of imatinib is tablets and its pharmaceutical formulation is imatinib mesylate. The daily dose of imatinib mesylate is between 400 and 800 mg, which corresponds to between 478 and 956 mg of imatinib (factor of conversion: 1.2) [108].

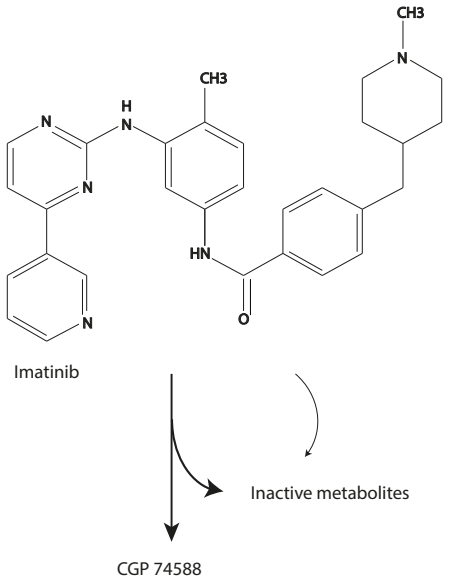

Figure 1.2: Molecular structure of imatinib mesylate, $C_{29}H_{31}N_70$. Thick and thin arrows are the primary and the minor routes of metabolite formation, respectively

Table 1.5: physical and chemical properties of imatinib and imatinib mesylate

	Property	Type	Data	Reference
Imatinib	CAS		152459-95-5	
	Molecular formula		$C_{26}H_{31}N_7O$	
	Molecular weight (g/mol)		493.6	Drugbank imatinib
	Log P	Experimental	3	
	Water solubility (mg/L)	Predicted	14.2 (pH not indicated)	
Imatinib mesylate	CAS		220127-57-1	
	Molecular formula		$C_{26}H_{31}N_7O \bullet CH_4SO_3$	
	Molecular weight (g/mol)		589.7	Bin Peng et al. 2005
	Log P	Experimental	1.99	
	Water solubility (mg/L)	Experimental	50 (at pH 1.99)	

Drugbank, 2013 [155], Peng et al., [152]

Toxicity of imatinib in aquatic organisms and environmental concentrations

To the best of knowledge, the concentrations of imatinib in the aquatic environment have never been monitored up to now, and a single study assessed the effects of this chemical in non-target species. Parrella et al. [156] predicted a concentration of 0.005 µg/L, based on patients excretion rate. Then, they exposed four aquatic invertebrates to imatinib and shown that D. magna and C. dubia were the most sensitive species among the test animals, with NOECs of 3 and 0.3 µg/L, respectively [156]. The results of this study are summarised in **Table 1.6.**

Table 1.6 Imatinib acute and chronic ecotoxicity in aquatic invertebrates. The results come from the study of Parrella et al. [156]

	Species	Effect(s)	Endpoint	Exposure (µg/L)	Duration
	Acute				
Acute	*C. dubia*	mortality	LD50	32	24 hours
Acute	*B. calyciflorus*	mortality	LD50	3.8	24 hours
Acute	*T. platyurus*	mortality	LD50	43.3	24 hours
Chronic	*D. magna*	imobilisation	EC50	12	48 hours
Chronic	*C. dubia*	reproduction	EC50	115	7 days
Chronic	*B. calyciflorus*	population growth inhibition	EC50	740	48 hours
Chronic	*D. magna*	reproduction	EC50	308	21 days
Chronic	*C. dubia*	reproduction	NOEC	0.3	7 days
Chronic	*B. calyciflorus*	population growth inhibition	NOEC	70	48 hours
Chronic	*D. magna*	reproduction	NOEC	3	21 days

EC50: the concentration of a substance that induced effec effect on 50% of the test organisms
LD50: the concentration of a substance that is lethal to 50% of the test organisms
NOEC: no-observed-effect concentration

Ecotoxicoproteomics

The aim of toxicoproteomics is to understand changes that occur at the protein level of a cells or an organism in response to toxin exposures, such as pollutants or drugs. Nowadays, toxicoproteomics is used in environmental toxicology to find early ecotoxicological markers at the protein level. This field of science is called ecotoxicoproteomics.

Proteome refers to the techniques used to study proteins, which are encoded by a given genome, on a large scale. Contrary to genome, the proteome is highly dynamic and changes continuously as a response to numerous intra and extracellular signalling [157]. Proteomics can be more broadly defined as "the effort to establish the identities, quantities, structures, and biochemical and cellular functions of all proteins in an organism, organ, or organelle, and how these properties vary in space, time, and physiological state" [158]. Proteomics is closer to physiology than genomics because post-translational regulation of proteins can reduce the correlation between mRNA abundance and protein activity [157]. Proteomics has the ability to characterize a wide number of proteins in biological samples, and it is increasingly used to search for novel biomarkers of human disease [159]. Also, this method allows human studies that otherwise could have not been carried out at overtly toxic exposures.

Toxicoproteomics is the field of science that focuses on changes at the protein level, in cells or in organisms that are exposed to toxic compounds. The purpose of this science is to better understand the toxicological mechanisms and the potential effects of a compound on living organisms. Toxicoproteomics can be used in environmental toxicology to find early markers at the protein level resulting from chemical exposure [74,160]. With omic- technologies, different levels of the biological organization (from cell to the organism for example) could be considered in ecotoxicology to increase understanding of the environmental effects of micropollutants. Ecotoxicoproteomics may link toxicant responses that were observed at the protein level with the responses at the organism level, i.e., a higher level of the biological organization. While standard ecotoxicological tests commonly focus on survival and reproduction, omic- studies try to give faster responses to environmental changes. Indeed, protein responses may appear earlier in time than the response at the organism level, and at lower (environmental) stressor concentrations than those used in chronic ecotoxicology experiments.

Ecotoxicoproteomic studies were already performed on microorganisms (bacteria, fungi), plants, invertebrates (molluscs, microcrustaceans, worms, insects) and vertebrates (freshwater and seawater fishes). For instance, specific proteomics signatures were associated with the exposure to sublethal concentrations of various marine pollutants (diallyl phthalate, PBDE-47, and bisphenol-A) in the blue mussel (*Mytilus edulis*) [75]. The authors also used identified proteins to better understand the possible mechanisms of toxicity in the blue mussel. They concluded that the main cause of the observed changes in protein expression was adaptive responses from oxidative stress. Heckmann and al. [161] also provided an interesting example of the application of "omics" technology to drug in ecotoxicology. The authors used a biology approach to link acute transcriptomic responses with chronic phenotypic stress responses, which occurred on *Daphnia magna* exposed to ibuprofen, a nonsteroidal anti-inflammatory drug. Similarities in the mode of ibuprofen action were shown in eicosanoid metabolism between vertebrates and invertebrates. In their study, they found that the reduced fecundity of daphnids resulted from disruption of the juvenile hormone metabolism. This study demonstrated that it is possible to use acute molecular responses to investigate the mode of action of xenobiotics and to assess their chronic impact on non-target organisms.

However, for an efficient application of proteomics in organisms commonly used as model in ecotoxicology, a comprehensive protein sequence database of these organisms is required. Fortunately, a draft genome sequence of *D. pulex* was recently released (see the website: wfleabase.org). Froehlich et al. [74] showed that LC-MS/MS proteomics analyses were an efficient tool for *D. pulex* and *D, longicephala* studies. Their data demonstrated proteomics to be very promising for ecotoxicological investigations into *Daphnia* species, although few publications have inquired the proteome of daphnids up to know. In this study, we decided to use *D. pulex* as aquatic model to perform ecotoxicological and ecotoxicoproteomic experiments

Ecotoxicology

Daphnids in general

Daphnids are crustacean from the Branchiopoda class, suborder Cladocera, and more communally named water fleas because of their fast and spastic movements that look like small jumps. During their life, these organisms have the particularity to use two reproductive strategies (http://animaldiversity.ummz.umich.edu/site/accounts/information/Daphnia_pulex.html).

1. The parthenogenesis reproduction (i.e. asexual reproduction) is used during periods that are not stressful for the organisms, i.e., when the environmental parameters do not change and allow species to survive without negative pressure, such as resource decreases or temperature and photoperiod changes. During these periods, eggs undergo a single maturation division in the ovary [162]. The females produce broods of genetically identical diploid offspring (i.e. clones) [163] and only females are produced.
2. The sexual reproduction is induced when environmental conditions are stressing for the daphnids, e.g., decrease in food resources or temperature. The females produce broods of male offspring. Once adult, males will mate "females that have produced a limited number of haploid eggs" [163]. The fertilized eggs, also called *ephippium* [164] or ephippia, will pass through the stressing period and hatch when favorable conditions come back.

Daphnids are aquatic arthropods whereas respiration occurs through gills or upon the surface of the whole body [162]. The thoraco-abdominal part is almost fused with the head. A large compound eye with small hyaline lenses is located in the frontal part of the head (Figure 1.3). Two pairs of antennae and two mandibles that are followed by two pairs of maxillae emerge from the head. The first pair of antennae (antennules) looks like small stiff and play a role in olfactory. The second pair of antennae is much larger and visible and allows jerky swimming (Figure 1.4). Muscles from the neck can move the antennae. The carapace has a bivalve appearance because it covers the body, except the ventral part that is open. Eggs are released through this opening abdominal part. The posterior extremity ends by a spine [162].

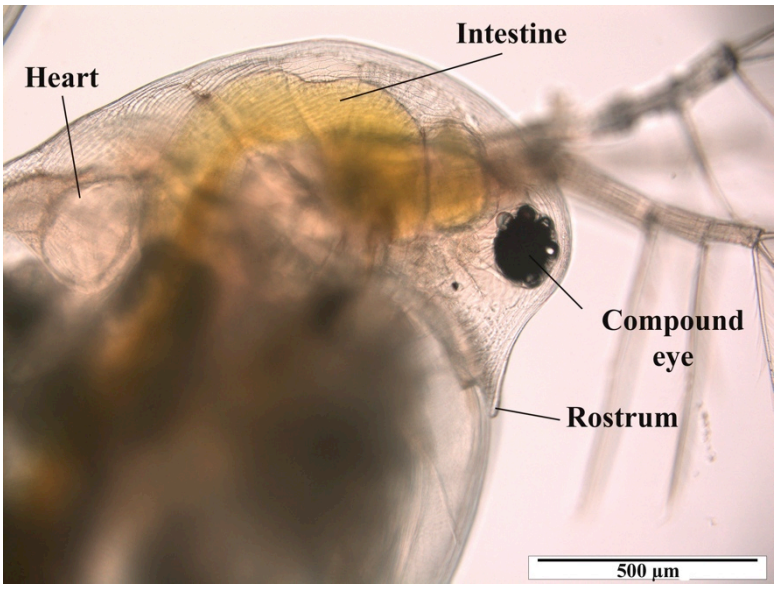

Figure 1.3 *Daphnia pulex* head. Photos were taken using an Olympus polarisation microscope BX51 with a digital imaging system (Colorview)

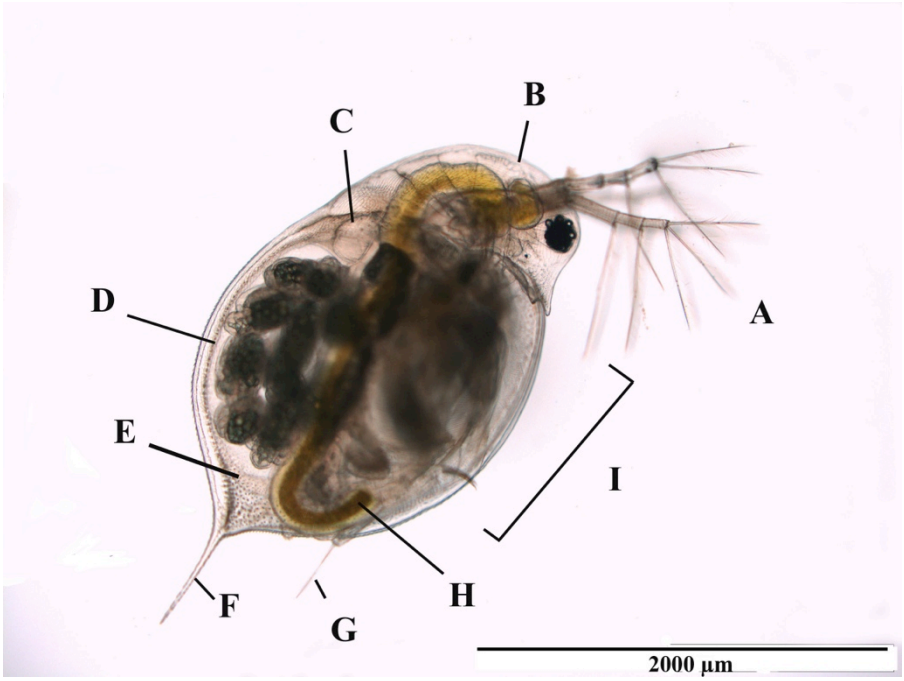

Figure 1.4 *Daphnia pulex*. A = second pair of antennae, B = head, C = heart, D = brood chamber with eggs, E = shell, F = apical spine, G = abdominal setae, H = intestine; I = thoracic appendages with filter setae from the filter apparatus. Photos were taken using an Olympus polarisation microscope BX51 with a digital imaging system (Colorview)

Daphnia pulex

The *Daphnia pulex* is a common species that is spread mainly over the entire North American continent and found in various habitats [165]. The average life span of *D. pulex* reared in our laboratory conditions is approximately 45 days (annexe 2). Molting (ecdysis, shedding) is an important process that allows daphnids to grow. Growth occurs immediately after each shedding while the new carapace is still flexible. Molting is strongly linked to reproduction and this process is active over the entire daphnids life. When sexual maturity is reached, i.e., about 6.5 days after birth (± 1 day) in our laboratory, the exoskeleton shedding comes with the passage of eggs from ovaries to the brood chamber [163,166]. When the first clutch of eggs is fully developed in the ovary, the first offspring (= single instar) is produced. The released young are similar in form to the adults. During each adult instar, daphnids undergo four distinct periods that occur in few minutes: the birth of young, molting, growth, and the release of new eggs into the brood chamber where they will hatch [162]. Indeed, eggs are dismissed into the brood chamber within minutes after molting and the juveniles are released just before the next female molt. *D. pulex* commonly produces 3 to 9 eggs and usually has 18-25 adult instars.

In our laboratory, *D. pulex* from the *arenata* strain was reared in conditions that maintained parthenogenetic reproduction: total hardness 90 ± 5 mg/l as $CaCO_3$; pH 7.9 ± 0.2; conductivity adjusted to 25°C, 286 ± 14 µS/cm; dissolved oxygen > 5 mg/L (annexe 3). Stock daphnids as well as individuals exposed to chemicals were reared in glass beakers placed in a Coolstore® environmental chamber (16: 8 h light: dark photoperiods at 21 ± 1°C). *D. pulex* was fed with the unicellular algae *Pseudokirchneriella subcapitata* (annexe 4). To ensure lipid need, suspension of Tetramin® tropical fish food was also given daily to daphnids [72,167].

D. pulex estrogen-related receptor (ERR) and tyrosine kinase proteins

D. pulex is the first crustacean to have its complete genome sequenced (see the website: wfleabase.org) and since, Thomson et al. [168] identified twenty-five nuclear receptor genes. A single copy of the estrogen-related receptor (dappu-ERR, NR3 subfamily) gene was found, which was close structurally to their human homologs ERRα, ERRβ and ERRγ, and close also to human ERα and ERβ [168]. In *D. pulex*, the endogenous ligands and the functions of the ERR family are unknown [168], but they may be involved in estrogen signalling and metabolism pathway [122,169]. In drosophile for instance, this gene is expressed in embryo's development [170]. The presence of dappu-ERR may explain why daphnids could be sensitive to tamoxifen and its metabolites. Imatinib may also induce physiological disruptions in *D. pulex* during long-term exposure because tyrosine kinase family is present in this species, such as insulin-like peptides and insulin receptor, which belongs to an ancient

transmembrane receptor tyrosine kinase superfamily [171–173]. A variety of related tyrosine kinases are also present in eukaryote cells [171], such as in cells of aquatic or terrestrial invertebrates [173–175], but the tyrosine signaling pathway has been extensively characterized in the fruitfly *Drosophila melanogaster* and less in other invertebrates. For instance, Brogiolo et al. [172] reported that insulin receptor plays a role in growth control that was conserved from insects to humans.

Long-term and multigenerational ecotoxicity experiments

Daphnids are key organisms in the food chain and they produce clones by parthenogenesis reproduction. Clones are very attractive in ecotoxicology and in proteomics because confounding genetic differences are avoided [176], which eases the observation and the comparison of phenotype modifications between treated individuals of any strain. Daphnids are an attractive model for multigenerational testing as they have a short time of reproduction. Furthermore, daphnids have a long history as model organisms in ecology, in evolution and in environmental studies (see the website: www.wfleabase.org). In ecotoxicology, they are also classically used [72,177] because of their high sensitivity to a large number of chemicals, their key place in the trophic chain (primary consumer) and their ease of handling [178–180]. Daphnids are suitable organisms for ecotoxicological tests [177] and their relatively short life and amenability to laboratory culture ease long-term tests. In the last few years, daphnids were already used successfully to highlight the negative effects of drugs on aquatic organisms. However, few results are available in the literature about the chronic toxicity (i.e., $\geq$ 1 generation) of xenobiotics on daphnids. For instance, Brennan et al. [66] performed a multigenerational study on *D. magna* with the endocrine disruptors diethylstilbestrol and 17 β-estradiol, a synthetic and a natural estrogen, respectively. The authors revealed a significant decrease in fertility over consecutive generations exposed to diethylstilbestrol, and an increase in adult daphnids mortality in the second generation (F2) that was exposed to 17 β-estradiol, compared with the first (F1). This difference in mortality between F1 and F2 may suggest that sensitivity increased in the second generation and that potential genetic or epigenetic damages were induced. Sanchez et al. [181] showed that the F1 generation of treated animals did not recover completely after having being transiently exposed to a pesticide. Finally, Campiche et al. [182] also reported transgenerational effects on springtails exposed to pesticides.

Regarding anticancer drugs, their long-term effects on aquatic species are also rarely assessed. Hensel and al. [183] reported a higher sensitivity of daphnids during chronic exposure to methotrexate than in acute. In this experiment, the NOEC of methotrexate was indeed > 330 times lower than the acute EC50. A two-generational study was performed on the freshwater microcrustacean *D. magna* exposed to falsodex, a selective estrogen receptor modulator (SERM) that is prescribed in breast cancer. In this study Clubbs and Brook [184] reported NOEC values of 10 and 100 µg/L (nominal concentrations) in

the first and second generation, respectively. In a seven days experiment on the cladoceran crustaceans *C. dubia* exposed to the SERM tamoxifen, the concentration that induced effect on 50% of the test organisms (EC50) was 0.81 µg/L (nominal concentration). Five anticancer drugs (5-fluorouracil, capecitabin, cisplatin, etoposide and imatinib) were tested in a 21 days experiment that was performed on the rotifer *B. calyciflorus* and the crustaceans *D. magna* and *C. dubia* [156]. The toxicity of the chemicals was: cisplatin < 5-fluorouracil < imatinib < etoposide < capecitabin with EC10 that range from 0.25 µg/L for cisplatin to 2800 µg/L for capecitabin.

These few examples show the importance of investigating multigenerational effects of chemical compounds. And this is particularly true in the case of pseudopersistent chemicals that may chronically interact with the physiological functions of aquatic species. Therefore, the prolonged presence of anticancer compound exposes the fauna and flora to potential long-term effects, which can adversely modulate eukaryotic organisms homeostasis [20,54] and also being magnified over generations.

Thesis objectives and hypotheses

The general objective of this thesis was to assess multigenerational effects of anticancer drug compounds on aquatic key species. This thesis is a multidisciplinary study, which involves ecotoxicity, toxicology and proteomic fields. Four objectives were met to increase knowledge on ecotoxicoproteomics with pharmaceuticals:

1. Is *D. pulex* sensitive to anticancer drugs or to some of their metabolites? The first goal was to determine which relevant anticancer compounds would be used in ecotoxicology, among the recent and the traditional anticancer families. Imatinib belongs to the recent family, while tamoxifen belongs to the traditional and also to the hormonal anticancer classes.

2. Which effects are induced by anticancer drug exposure on daphnids, and are the effective concentrations environmentally relevant? Ecotoxicological experiments were performed to determine the effective concentrations of the chosen anticancer drugs. Acute and long-term experiments, over generations, were performed to observe the effects of these chemicals on *D. pulex*. The experiments were carried out with imatinib, tamoxifen, 4-hydroxytamoxifen and endoxifen in controlled environmental conditions.

3. Are the effects observed at the organism level associated with sub-organism changes in the expression of certain proteins? Protein changes in daphnids exposed to tamoxifen were analysed and described using short and mid-term test exposure. These protein changes were compared to phenotype effects on treated daphnids. The biological levels studied in this thesis are summarised in Figure 1.5.

4. Do protein changes provide suitable biomarkers for an early detection of drug-related stress of daphnids? This question links ecotoxicological experiments with proteomic analyses to find early evidence of chronic stress in daphnids.

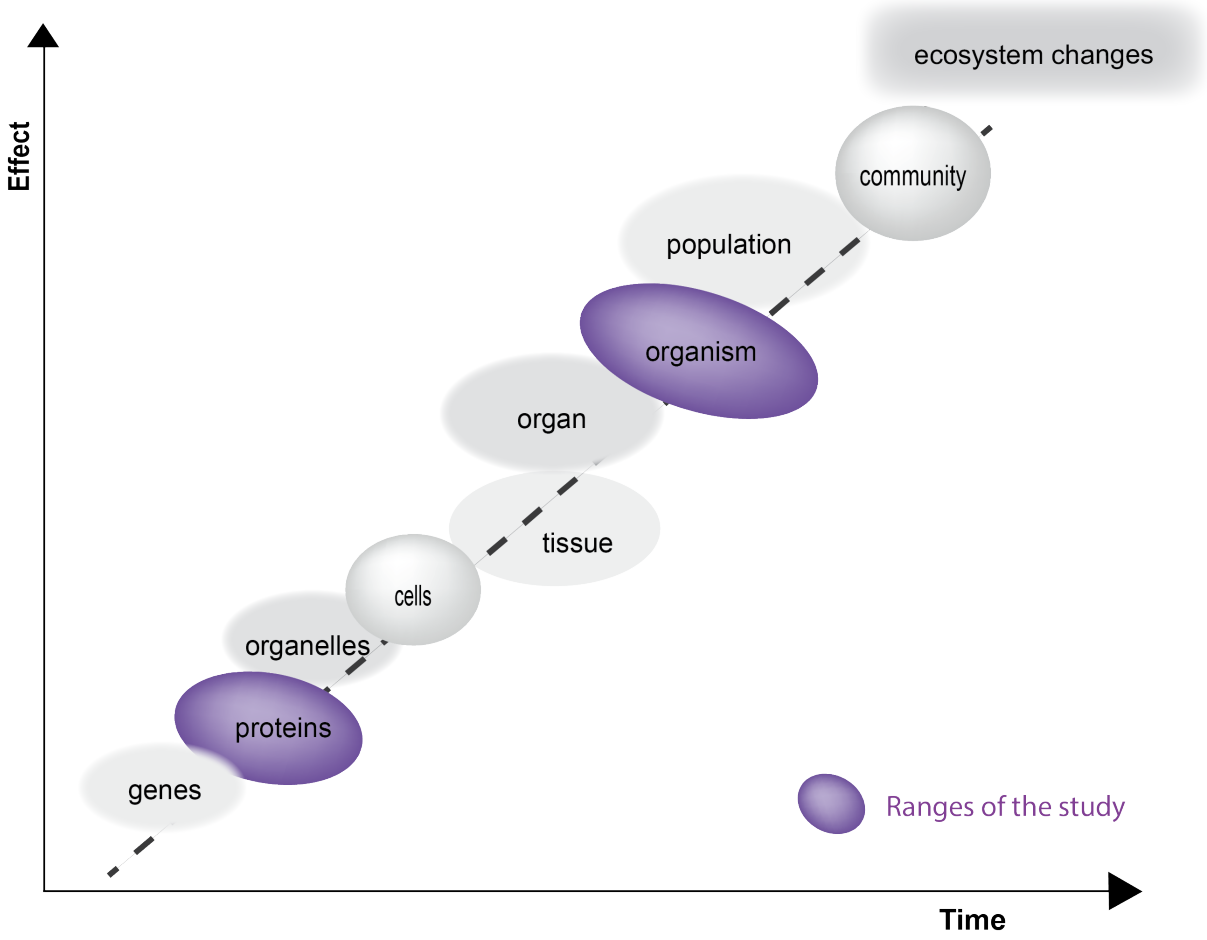

Figure 1.5: Consequences of pharmaceutical toxic effect at different level of the biological organisation, and relationship with the temporal scales of the response. Adapted from Lemos and al. [157]

Outlines of the thesis

This thesis comprises results of different laboratory experiments that are presented in independent chapters.

Chapter 2: Is *D. pulex* sensitive to tamoxifen?

The aim of the first experimental study was to investigate whether *D. pulex* was sensitive to tamoxifen, using non-standardised acute and chronic experiments. The viability, the reproductive performance and the morphology of offspring were considered in quantitative or qualitative assessment. While the acute test has already shown unusual effects in treated daphnids, such as erratic swim or evisceration, the chronic assay shown embryo abnormalities on offspring, in particular, aborted eggs, premature neonate and body-malformations. The chronic effects of a solvent, the DMSO, that were used in ecotoxicological experiments to dilute tamoxifen and its metabolites are also presented in the first part of this chapter.
In this chapter, the need to consider measured test concentrations rather than nominal was also underlined, particularly in ecotoxicological experiment with lipophilic molecules, such as tamoxifen. Tamoxifen stability in incubation media was assessed at different concentrations and times. Due to large differences between nominal and measured concentrations, and because sampling and analyses in long-term ecotoxicological experiments are time consuming and costly to be performed, a good prediction method was developed and proposed. This cost-effective method allowed us to predict test exposure levels that were calculated from measured concentrations.

Chapter 3: Is *D. pulex* sensitive to tamoxifen metabolites (4OHTam and endoxifen)?

In this chapter, two metabolites of tamoxifen, i.e., 4-hydroxy-tamoxifen (4OHTam) and endoxifen, were considered because these metabolites are potent anticancer molecules in humans and because their effects in aquatic organisms were unknown. Therefore, the main objective of this study was to assess the sensitivity of *D. pulex* towards 4OHTam and endoxifen in a two-generational experiment in which each generation was exposed to the chemical during 21 days. The reproduction, the size, the viability and the estimated population growth (r) were impaired in treated daphnids. The toxicity was higher with 4OHTam than with endoxifen. The stability of the chemical in the incubation medium was also assessed because the measured concentrations in test solutions did not correspond to the nominal. A predictive method, such as the method proposed with tamoxifen, was developed and used to predict the exposure level in the ecotoxicological experiment with 4OHTam and endoxifen. This predictive method was efficient and easier to apply than a regular follow up of the test solutions during long-term ecotoxicity studies.

Chapter 4: Multigenerational effects of tamoxifen and 4OHTam. Are the toxic effects magnified over four generations of *D. pulex*?

The chapter four reports the design and the results of a multigenerational experiment that was performed with the anticancer drug tamoxifen and its metabolite 4OHTam. Multigenerational studies are sparse in ecotoxicology and it was necessary to design the experimental framework prior to starting the experiment. The experimental design was based on the previous two-generational experiments that were performed with 4OHTam and endoxifen (see chapter 2 and 3). Tamoxifen and 4OHTam were chosen because they were more toxic than endoxifen in acute and long-term studies and because magnified effects over generations were suspected in these previous experiments. The aim of this study was to assess whether the sensitivity of daphnids increased over generations. The size, the reproduction, the viability and the intrinsic rate of natural increase (r) were followed within four generations of *D. pulex* exposed 14 days each to tamoxifen or 4OHTam. These studied end points were also observed in two prospective experiments. First, offspring from treated mothers were withdrawn from chemical to determine whether these descendants were able to recover. Second, neonates from treated and untreated parents were exposed to tamoxifen and 4OHTam used in combination. The results showed that tamoxifen and 4OHTam induced several effects in treated organisms. The effects on the four generations exposed to 4OHTam were not magnified, while they were over the successive generations exposed to tamoxifen. Also, the descendants withdrawn from the chemical solution were not able to recover because their size and reproduction performance were decreased at the end of the experiment. The reproductive performance of daphnids exposed to mixture of tamoxifen and 4OHTam was reduced, whilst no effect was observed when chemical test concentrations were tested individually.

Chapter 5: Is *D. pulex* sensitive to imatinib?

Imatinib is the fourth anticancer drug that was tested in this thesis. This pharmaceutical belongs to the recent generation of anticancer compound that has very specific mode of action in tumour cells. This drug was mainly chosen in prediction to its increased consumption worldwide. The aim of this study was to observe the sensitivity of two generations of *D. pulex* towards imatinib. The range of test concentration was chosen using a preliminary chronic experiment on a single generation exposed 21 days to this chemical. The viability, the size, the reproduction and the intrinsic rate of natural increase were not impaired in *D. pulex* at the concentrations that were chosen in this experiment, except at the highest concentrations where the reproductive performance was decrease in the first treated generation.

Chapter 6: Ecotoxicoproteomics with tamoxifen

The aim of this ecotoxicoproteomic study was to identify and quantify proteins that were differentially expressed in daphnids exposed to tamoxifen. Because little were published on the extraction of daphnia proteins [74], it was necessary to design and to test an extraction procedure, prior to starting ecotoxicoproteomic experiments. The objective of this preliminary part was to determine an easy and efficient technic procedure to obtain sufficient qualitative and quantitative biological material. Therefore, this chapter presents the extraction procedure in daphnids as well as the protein analysis results after daphnids exposure to tamoxifen.

Chapter 7: general discussion and conclusion

The different results that were found during the ecotoxicological and proteomic experiments are summarised and discussed in this last chapter. The significance of these results in the aquatic system is considered and possible additional laboratory experiments are proposed to improve knowledge on the anticancer drugs and their metabolites in aquatic species. Four levels of action are reported to prevent pharmaceutical releases in the aquatic environment. Also, the potential impact of pharmaceutical on the ecosystem, including human, is briefly discussed.

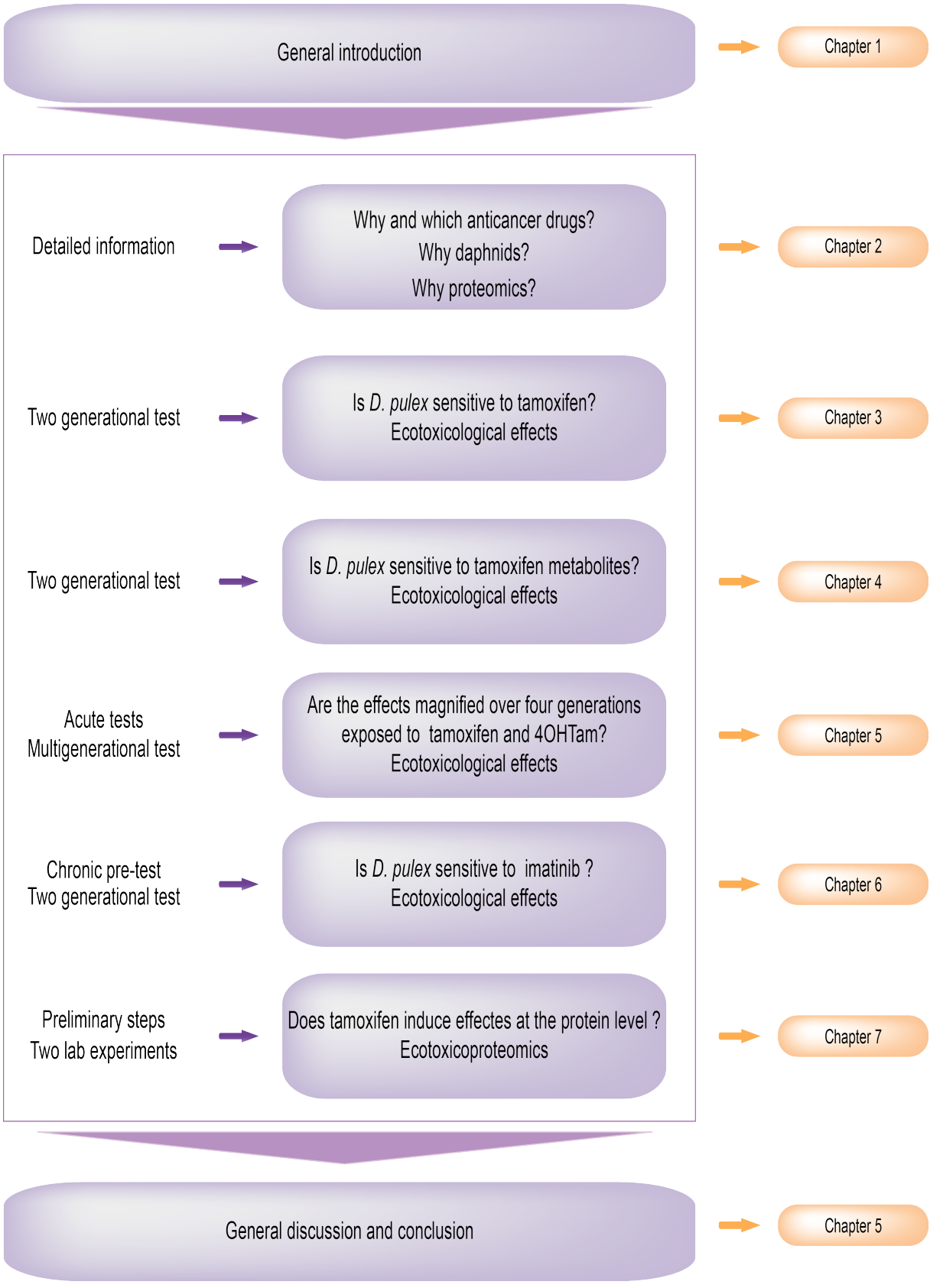

Figure 1.6: Conceptual approach and outline of the thesis

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Chapter 2

Is *D. pulex* sensitive to tamoxifen?

In this chapter the effects of the anticancer drug tamoxifen were assessed on *D. pulex* during acute and long-term experiments. Prior to these experiments, the effects of the solvent (i.e., DMSO) that was used to dissolve tamoxifen in water were evaluated in a two-generational study. No effects were observed in the DMSO at the percentage that would have been used during ecotoxicological experiments with tamoxifen. Therefore, experiments with this chemical and also with its metabolites were also conducted. The sensitivity of daphnids toward tamoxifen was observed using a non-standard ecotoxicological method. More precisely, quantitative or qualitative assessments were undertaken based on the following endpoints: size, viability, reproductive performance and morphology of the treated organisms and their offspring. Unusual effects in treated daphnids, such as erratic swim-pattern or evisceration, were observed in the acute experiments. In the chronic assay, tamoxifen induced morphological abnormalities on offspring, such as aborted eggs, premature neonates and body-malformations.

An additional experiment was carried out to assess tamoxifen stability in test solutions and at different times. Large differences were observed between nominal and measured concentrations. These differences lowered tamoxifen exposure during ecotoxicological experiments, and we assumed that adsorption phenomenon of tamoxifen on glass flasks was one of the reasons of the chemical loss. A regular follow up of the concentrations would have been required to ascertain tamoxifen exposure during ecotoxicological experiments, but because this follow up were cost and time consuming, it was necessary to develop a sustainable method to predict tamoxifen exposure during multigenerational experiments. Therefore, subsamples of some test solutions were analysed and the measured concentrations were plotted against their nominal levels. This polynomial regression was used to establish the so-called predicted concentrations.

The first part of this chapter presents the two-generational experiment that was performed with DMSO. This preliminary steps was required to determine DMSO concentrations to be used in the two-generational assay with tamoxifen. The second part of this chapter reports the toxic effects of tamoxifen in the two-generational study, the stability of tamoxifen in daphnia medium over times and the method to predict tamoxifen exposure in ecotoxicological experiments.

Long-term toxicity of DMSO as a solvent carrier in *D. pulex* experiments (addendum)

Introduction

Tamoxifen and its metabolites are molecules practically insoluble in water and they need to be dissolved initially in solvent carrier to prepare stock solutions. These stock solutions would later be diluted in daphnia medium to prepare the test solutions that would be used in ecotoxicological experiments. The organosulfur $(CH_3)_2SO$ dimethyl sulfoxide (DMSO) is a solvent that is recommended to dissolve tamoxifen and its metabolites [1]. To the best of our knowledge, long-term effects on *D. pulex* exposed to DMSO have never been reported. Therefore, the reproductive effects of this solvent were assessed before ecotoxicological experiments were carried out with tamoxifen and its metabolites. The aim of this experiment was to ensure that no reproductive effects would be induced in treated organisms exposed ≥ 10-d to DMSO.

Materials & Method

Test organisms

D. pulex from the *arenata* strain was mass-cultured in conditions that maintain parthenogenetic reproduction (total hardness 90 ± 5 mg/l as $CaCO_3$; pH 7.9 ± 0.2; conductivity adjusted to 25°C, 286 ± 14 µS/cm; dissolved oxygen > 5 mg/L). Stock daphnids and treated individuals were reared in glass beakers placed in a Coolstore® environmental chamber (16: 8 h light: dark photoperiods at 21 ± 1°C). Daphnids were fed daily with both 0.2 mgC/daphnia of *Pseudokirchneriella subcapitata* algae and suspension of Tetramin®[2,3]. The treated and untreated medium was renewed every two days.

A potassium dichromate ($K_2Cr_2O_7$) assay was performed at the beginning and at the end of the exposure period of each generation. The mean concentration that immobilised 50% of the treated individuals (EC50) were consistent with those regularly obtained in our laboratory (i.e., mean EC50 ± standard deviation: 0.75 ± 0.25 µg/mL). The position of the test tubes in the environmental chamber was randomised to minimise systematic errors.

Two-generation testing procedure

Two generations of *D. pulex* (i.e., F0 and F1) were exposed to the following percentage of DMOS: 0, 0.0003, 0.0008, 0.002, 0.03, 0.12%. Eighteen neonates (< 24-h, > 3rd brood) were randomly selected from a single stock of mothers to form F0. These neonates were pipetted and placed in separate glass

beakers with 50 mL of medium (three replicates per concentration). On the twenty-first day of maternal exposure, eighteen neonates (< 24-h) were collected to form the generation F1. One neonate per beaker (three replicates) was transferred to 50 mL of corresponding maternal concentration medium. They were reared during eleven days. Every day since treated animals started to reproduce, neonates were removed from glass beakers and counted. Then, they were discarded except on day 21.

Statistics

The reproductive performance of the daphnids was expressed as the average number of neonates per adult at the end of the exposure period. The results were compared using a one-way ANOVA test followed by the Bonferroni correction ($\alpha < 0.05$). Calculations were carried out with GraphPad Prism (version 4.00 for Mac OS X, GraphPad Software, San Diego California USA, www.graphpad.com).

Results and discussion

Figure 2.1 summarised the reproductive performances of *D. pulex* when they were exposed to DMSO during 21st and 11th days. In each experiment, the average number of neonates was statistically not different from the blank ($p < 0.05$). During the multigenerational experiment that was performed with tamoxifen and its metabolite 4OHTam (see chapter 4), two DMSO-controls were carried out at the DMSO percentages of 0.002% and 0.01%. The reproductive performance of the two first generations is summarised in Figure 2.2. In both experiments, the reproduction of treated daphnids was comparable with the controls. These results are in accordance to Barbosa et al. [4], who reported low chronic toxicity of DMSO on *D. magna*. Therefore, DMSO was considered as a solvent of choice for long-term assays and was used in all ecotoxicological experiments.

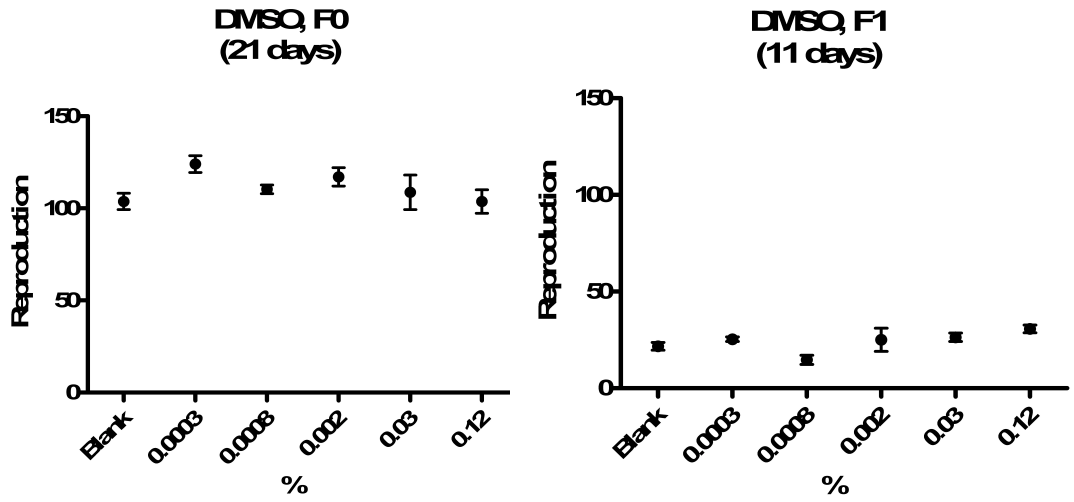


Figure 2.1: Reproduction of two generations (F0 and F1) of *D. pulex* exposed 21 and 11 days to different percentages of DMSO (mean ± SD, n = 18 for F0 and for F1). The reproduction is the mean number of neonates that were produced during the exposure period.

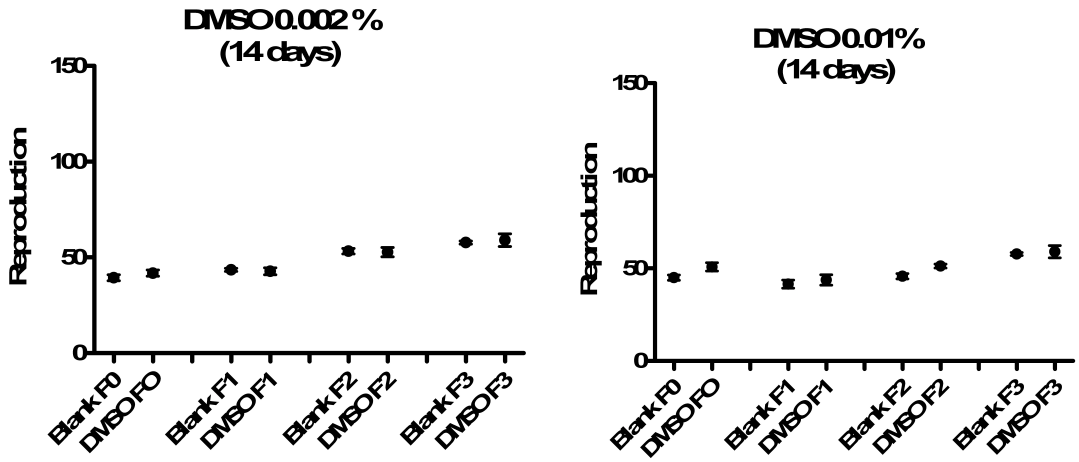


Figure 2.2: Reproduction of two generations (F0 and F1) of *D. pulex* exposed 14 days each to DMSO (mean ± SD, n = 48 for F0 and for F1).

Tamoxifen preliminary test to determine the long-term test concentrations

The concentrations that were chosen for the two-generational study with tamoxifen, which is presented next, were determined based on previous ecotoxicological experiments. These preliminary tests were performed in accordance with the experiments that are presented in this thesis. Briefly, acute pre-tests (48 hours of exposure, no replicates, n = 86) had preceded the acute experiment (48 hours of exposure, 3 replicates, n = 167). The studied end points were death and viability after 2 days of exposure. In the chronic pre-test, daphnids were exposed 21 days to tamoxifen (no replicate, n = 10), and the studied end points were death and the total neonates produced per individual. Figure 2.3 summarised two points: 1) the predicted and the nominal concentrations that were used during each test (for more information on predicted concentrations see subsection "Predicted tamoxifen concentrations in ecotoxicity tests"), 2) the occurrence of adverse effects in *D. pulex*. The dotted zones are uncertainty areas in which toxic effect may be possible. In this figure, the so -called chronic test represents the two-generational experiment that is presented in the next section (see: "Tamoxifen, a molecule to consider in ecotoxicology?"). In this two-generational experiment, the range of test concentrations was determined based on the acute and the pre-chronic test results that are summarised in Figure 2.3.

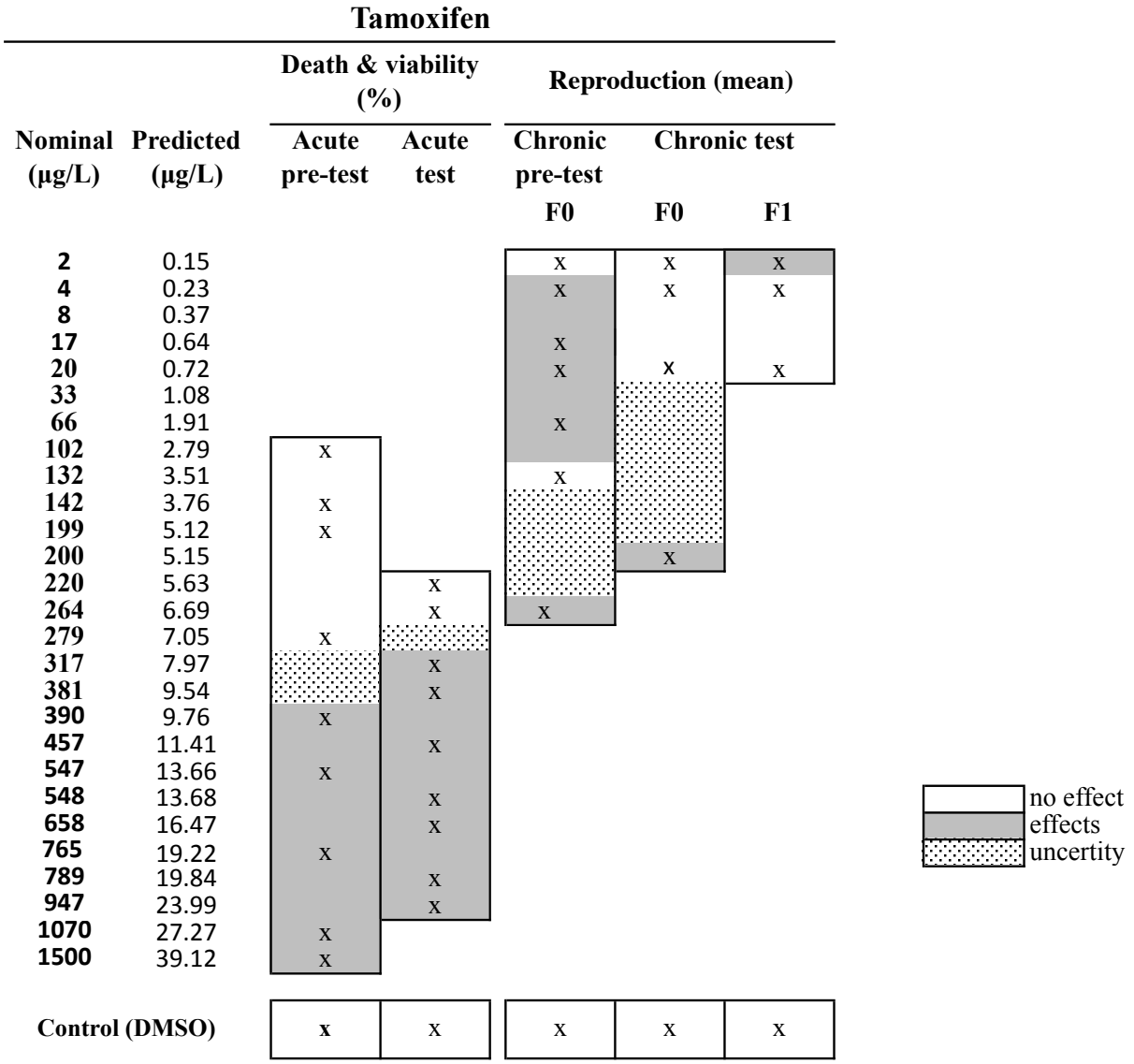


Figure 2.3: Nominal vs predicted concentrations of tamoxifen used in ecotoxicological experiments (acute and chronic tests and pre-tests), and occurence of effects on *D. pulex* exposed to this chemical. In the acute pre-test (n = 86) and the acute test (n = 167), the studied end points were death and viability after 2 days of exposure. In the pre-chronic (21 days, n = 10) and chronic experiments (21 and 12 days for F0 and F1, respectively), the studied end points were death and the total neonates produced per individual (n = 18 and 17 for F0 and F1, respectively). The dotted zones are uncertainty areas in which toxic effect may be possible.

Tamoxifen, a molecule to consider in ecotoxicology?

Abstract

Tamoxifen is widely prescribed worldwide for the prevention and treatment of hormone receptor-positive breast cancer. Tamoxifen undergoes faecal excretion and is poorly removed by sewage treatment plants (STPs). It has been found in STP effluents and in natural waters in concentrations up to 0.3 µg/L and 0.2 µg/L, respectively. The aim of this study was to assess the acute and long-term (i.e., over two generations) toxicity of tamoxifen on *Daphnia pulex*, a freshwater microcrustacean crucial for the aquatic food chain. While the acute effect concentration 50% (EC50) was 13 µg/L, in chronic exposure tamoxifen decreased reproduction in daphnids already at a concentration of 5.15 µg/L. These EC50 estimates were obtained through measurement of actual exposure concentrations, shown to significantly deviate from calculated levels for such a hydrophobic molecule. In addition, tamoxifen induced teratogenic effects on the offspring, such as miscarriages and morphological abnormalities. The latter occurred at lower concentrations than those effects that disrupted reproduction. The no observed effect concentration (NOEC) that was calculated for reproduction was 0.72 µg/L, whereas the NOEC was < 0.15 µg/L when based on morphological abnormalities. These effects raise questions regarding the potential impact of tamoxifen and related anticancer drugs on aquatic ecosystems, in particular with respect of their growing therapeutic use.

Introduction

Unprecedented amounts and varieties of pharmaceuticals are released continuously into the aquatic environment (for review, see [5,6]). The daily human consumption of pharmaceuticals to treat or prevent medical ailments amounts to thousands of tons of active substances worldwide [7]. After metabolism, these pharmaceuticals are excreted from the body in their intact or metabolised forms into sewage water networks and are transported to a sewage treatment plant (STP). Because conventional STPs are less efficient for chemicals than for organic matter [8], several pharmaceuticals escape STP degradation processes and are discharged into the aquatic environment with STP effluents [9–13]. These chemical residues often carry specific pharmacological activities that are susceptible to interacting with biological processes, which cause growing concerns regarding their potential adverse effects on the health and integrity of our living environment [14,15].

Among other pharmaceuticals, the presence of anticancer agents in aquatic systems is of special concern primarily because of their potential mutagenic, carcinogenic, teratogenic and/or embyotoxic properties [16,17]. These agents indeed do not exclusively target abnormal cells but interact as well with normal cells in the body. For instance, cytostatic agents that are used to treat malignant neoplastic diseases have the potential to increase the incidence of secondary tumours in animals and humans [18]. Moreover, demands for chemotherapy treatment have grown by 10% per year in industrial countries [19], which also indicates that the total amount of chemotherapy pharmaceuticals that are discharged in the environment is constantly increasing. For many years, researchers have highlighted the requirement to assess the risk of these chemicals to the aquatic environment [5,8,9,11,20,21]. Similarly, Dang et al. [22] overviewed the scientific efforts that have been devoted to hormone analogues that are found in surface water because these compounds induce endocrine-disrupting effects primarily in the reproductive system of living organisms, such as daphnids and fish. This group of molecules includes pharmaceuticals that are used in oral contraception, hormone replacement therapy, bone disorders and cancer treatments.

Tamoxifen is a chemotherapeutic anti-oestrogen drug compound that is widely prescribed worldwide for the prevention and treatment of hormone receptor-positive breast cancer [18,23,24]. Basically, tamoxifen is considered a selective oestrogen receptor modulator that behaves not only as both a partial agonist and antagonist on alpha oestrogen receptors (ERα) but also as a partial agonist on beta oestrogen receptors (ERβ). Its action on breast cancer cells might also be explained by its ability to bind one of the three estrogen-related receptors (ERRs) described in humans [25]. In stromal breast cells, tamoxifen's primary action is cytostatic [26]; however, tamoxifen is also able to control cell replication through nuclear interactions or through mechanisms involving growth factors. This pharmaceutical might also relieve Duchenne muscular dystrophy, which is a striated muscle disease that currently has no treatment [27]. In animals, tamoxifen is sometimes given in poultry as a growth

promoter or to laboratory rodents to trigger gene expression in specific tissues [28,29]. Tamoxifen has been recognised to cause various side effects, such as DNA-adducts formation, epigenetic dysregulation, genotoxicity and carcinogenicity, as recently acknowledged by the International Agency for Research on Cancer [30–37].

Regarding tamoxifen use in humans, patients are orally administered 20-40 mg of tamoxifen per day [31] sometimes after loading doses of 80 to 200 mg for 1 to 7 days [38]. The treatment is usually taken for 5 years or according to its efficacy and safety in the case of metastatic hormone-dependent breast cancer. Longer durations might even become advised [39]. Tamoxifen is partly metabolised in the organism and ultimately undergoes faecal excretion through the biliary route with an enterohepatic cycle [38,40]. Studies reported that approximately 30% of the dose was excreted in its unconjugated form in faeces, of which tamoxifen and its 4-hydroxy-tamoxifen metabolite were the predominant compounds [40,41]. Tamoxifen is not readily biodegradable [42] and has a high bioaccumulation potential with an experimental logP of 7.1 [43]. Similar to other anticancer agents, tamoxifen escapes STP degradation processes [44–46] and was measured in STP effluents in concentrations ranging from 0.02 to 0.37 µg/L [47–49]. Tamoxifen was also found in the natural aquatic environment with concentrations ranging from 0.01 to 0.21 µg/L [49,50]. Due to its bioaccumulation potential and its toxicity to non-human organisms, tamoxifen was recently identified as requiring priority assessment [51].

The aim of this study was thus to assess the acute and long-term (i.e., over two generations) toxicity of tamoxifen on *Daphnia pulex*. Daphnids are aquatic organism extensively used as test animals in ecotoxicology because of their sensitivity to xenobiotics, their key role in the trophic chain, and their short life cycle that allows experiments on several generations. Although daphnids lack ERs, a single copy of the ERR gene (dappu-ERR, 3 subfamily) was recently found in *D. pulex* [52], which may result in sensitivity to tamoxifen. To assess this sensitivity, non-standard endpoints, beyond reproduction, were considered in the acute and two-generation experiments. Indeed, Kim et al. recently highlighted the importance of studying the effects of pharmaceuticals on non-target species based on non-traditional endpoints [53]. Some of these endpoints are qualitative but they may be considered as relevant for risk assessment. Furthermore, because tamoxifen has low solubility in water (< 0.5 mg/L [54]) and tends to be adsorbed on surfaces of the glass flasks, we were confronted to the challenge of estimating its actual water concentration during our experiments. Therefore, we proposed a cost-effective method to predict real exposure levels from nominal concentrations.

Materials & Methods

Chemicals

Tamoxifen was purchased from Sigma/Fluka (>99%, lot: 011M1682V). For the ecotoxicological experiments, two stock solutions were prepared in pure DMSO (www.gaylordchemical.com/index.php?page=102b-dmso-solubility-data) at nominal concentrations of 10 g/L and 2.5 g/L for acute and two-generational tests, respectively. Another stock solution was prepared at a nominal concentration of 1.6 E+07 µg/L for the experiment that was dedicated to assess the stability of tamoxifen in daphnia medium (see subsection "Tamoxifen stability in medium"). The three stock solutions were prepared in glass flasks with plastic caps a day before tests were performed and were stored in the dark at -80°C for no longer than the test duration. These stock solutions were thawed at room temperature before each use.

Tamoxifen experiments

Test organisms

Individuals from the *arenata* strain of *D. pulex* were kindly provided by the Eawag Department of Aquatic Ecology (www.eawag.ch/forschung/eco/index_EN). These organisms were fed daily with both 0.2 mgC/daphnia of *Pseudokirchneriella subcapitata* algae and a suspension of the tropical fish food Tetramin® [55,3]. Individuals were mass-cultured in conditions that maintained parthenogenetic reproduction as follows: Elendt M4 [3] with a total water hardness of 93 ± 3 mg/L $CaCO_3$ [56]; pH 7.8 ± 0.3; conductivity adjusted to 25°C, 285 ± 15 µS/cm; and dissolved oxygen, >5 mg/L. These chemical parameters were measured regularly, and also at the beginning and end of the experiments to ascertain the quality of the medium. Stock daphnids, as well as individuals that were exposed to tamoxifen, were reared in a Coolstore® environmental chamber, with 16 : 8 h light : dark photoperiods, at 21 ± 1°C. A potassium dichromate ($K_2Cr_2O_7$) assay was performed at the beginning and end of each test to verify daphnia sensitivity, and the mean concentration that induced 50% effects (EC50) were consistent with our previous laboratory results (mean EC50 ± standard deviation: 0.75 ± 0.25 µg/mL). The position of the test tubes in the environmental chamber was randomised to minimise systematic errors.

Acute toxicity experiment

The tamoxifen acute toxicity assay was performed over 48 hours exposure. One hundred and sixty-seven neonates (< 24 h, > 3rd brood) were randomly placed in glass tubes at the following tamoxifen concentrations: 5.63, 6.69, 7.97, 9.54, 11.41, 13.68, 16.47, 19.84 and 23.99 µg/L (five individuals per

vessel, 3 replicates). These levels of exposure were predicted from measured concentrations (see subsection "Predicted tamoxifen concentrations in ecotoxicity tests") and they correspond to the following nominal concentrations: 220, 264, 317, 381, 457, 548, 658, 789, 947 µg/L. Two controls were performed in parallel: a blank (i.e., with no drug or solvent) and a solvent control that contained DMSO 0.009%, which corresponded to the maximum solvent percentage that was used for the test concentrations.

The studied endpoints were death and viability. Daphnids were counted as nonviable when the organisms showed evident signs of damage that could be observed by the naked eye, such as tissue deterioration (even if the organisms were able to swim), or by the immobilisation of living daphnids > 20 seconds in the middle of the tube, despite gentle agitation. These endpoints were observed after 24 and 48 h of exposure.

Two-generation toxicity experiment

The experimental design of the tamoxifen chronic test is shown in Figure 2.4. Twenty-one neonates (< 24 h, > 3rd brood) were randomly isolated from a single stock of mothers to form the first generation (F0) that was exposed to tamoxifen. One individual per glass beaker (three replicates per concentration) was placed in 50 ml of the following predicted concentrations of tamoxifen: 0.15, 0.23, 0.72, or 5.15 µg/L, which corresponded to the nominal concentrations of 2, 4, 20, 200 µg/L, respectively (see subsection "Predicted tamoxifen concentrations in ecotoxicity tests"). Daphnids were exposed continuously to the same concentration for 21 days. At the 6th laying (i.e., approximately the 17.5th day), eighteen neonates were randomly selected to form the second generation (F1). Because a significant decrease in reproduction was already recorded for the F0 parents, the 5.15 µg/L concentration was not tested on the F1 generation. The neonates were individually placed in 50 mL of the corresponding maternal concentration for 12 days (three replicates per concentration). Indeed, preliminary chronic experiments showed that a 12-d exposure period was sufficient for highlighting the reproductive tendency. For F0 and F1 experiments, two controls were performed in parallel: a solvent control with 0.0008% of DMSO and a blank; both controls were composed of one individual per glass beaker (three and six replicates, respectively). Daphnids were fed daily with 0.2 mgC/daphnia of *P. subcapitata* algae and Tetramin® [55,3].

The mortality (i.e., the number of dead parents) and the number of neonates in each incubation beaker were recorded daily. Then, the neonates were discarded, except those of the 6th laying. At each concentration, the reproductive performance of daphnids was expressed as the average number of neonates per adult that was released during the entire exposure period. Morphological abnormalities of offspring (e.g., body-deformed individuals) from the F0 generation were discovered during the experiment. Thus, randomly chosen neonates from each beaker were examined using an Olympus

polarisation microscope BX51 with a digital imaging system (Colorview). When morphological abnormalities were found, the day and the type of abnormalities were recorded. In addition to this qualitative assessment, small and incompletely developed neonates, which are designated here as "premature neonates", were isolated and exposed to the same maternal concentration to assess their viability and reproductive ability (one premature neonate at 0.15 µg/L and two at 0.72 µg/L of tamoxifen). Because only one or two premature neonates per brood were released, no replicates were performed.

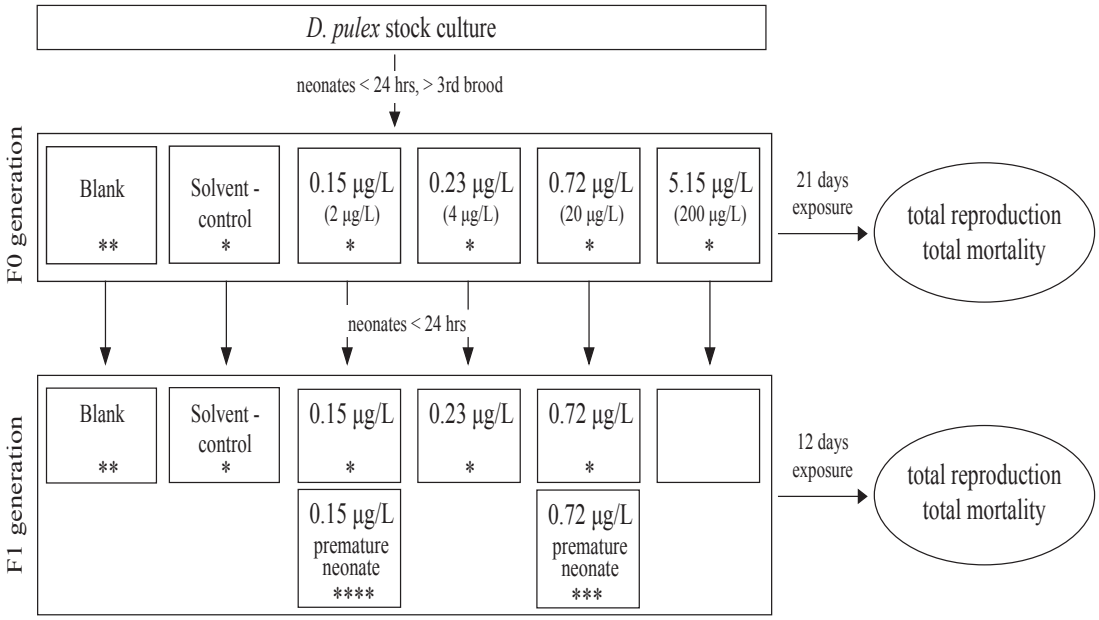


Figure 2.4: Design of a two-generation toxicity study on *D. pulex*. A single organism per beaker was exposed to increasing concentrations of tamoxifen. The exposure is expressed in predicted concentrations and their corresponding nominal concentrations are given once in brackets. On the 6th laying, normal and premature neonates were kept to form the second generation. The concentration at 5.15 µg/L was not tested because significant reproductive effects were already observed in F0. * three replicates ** six replicates, * one and **** two premature neonates were exposed to 0.15 and 0.72 µg/L, respectively**

Predicted tamoxifen concentrations in ecotoxicity tests

One hundred and forty-six aliquots of tamoxifen solutions (without daphnia and food) were collected and analysed during ecotoxicological experiments that were performed on daphnids over a period of 18-months in our lab. Aliquots of the tamoxifen solutions were collected in PP microtubes immediately after preparation (t0) or after 48 h (t48) of exposure to the environmental chamber conditions. The aliquots were immediately frozen at –80°C until analyses. Tamoxifen concentrations

were determined using liquid chromatography which was coupled to a triple stage tandem mass spectrometry system (LC-MS/MS) using an adaptation of a method that was developed and validated at the Laboratory of Clinical Pharmacology at CHUV (University Hospital Center Lausanne, Switzerland) [57]. The limit of quantification (LOQ) of the LC-MS/MS assay for tamoxifen is 0.5 ng/mL. This multiplex method also allows the assay of the metabolites E/Z- endoxifen, N-demethyltamoxifen and 4-hydroxy-tamoxifen with LOQs of 0.5, 1.0 and 0.2 ng/mL, respectively.

Tamoxifen stability in medium

Due to the large differences that were observed between the nominal and measured concentrations of tamoxifen in the incubation medium, we performed an additional experiment to assess its concentration changes over time. Two methods were performed in parallel in the absence of daphnids, with the experimental design shown in Figure 2.5. Two final solutions (50 mL each) were prepared in duplicate with both methods, which were either supplemented with or without daphnia food. The dilutions were prepared using the incubation medium M4 [3] in laboratory glassware.

In method 1, the nominal tamoxifen concentrations in final solutions were 3.2 and 320 µg/L with DMSO percentages of 0.00002 and 0.002%, respectively. These solvent percentages were kept as low as possible to avoid any influence of DMSO if ecotoxicity tests would have been run on the organisms. However, in this case, the concentrations of tamoxifen were above its reported solubility level in water (< 0.5 mg/L [54]). In method 2, the nominal tamoxifen concentrations were 1.6 and 160 µg/L. The DMSO percentage was maintained at 0.1% in the final solutions to ensure a sufficient solubility for tamoxifen. For the final solutions that were supplemented with food, the quantity of proteins and lipids corresponded to the diet of a single daphnia that would have been reared in 50 mL of medium volume (i.e., 25 µl of the tropical fish food Tetramin® and 0.2 mgC/daphnia of *P. subcapitata* [58]). All final solutions were stored for 48 h in a Coolstore® environmental chamber, with 16:8 h light:dark photoperiods, at 21 ± 1°C. These conditions correspond to those conditions that were used in our ecotoxicological tests.

Finally, the final test solutions were collected in PP microtubes (400 µg/L, 3 replicates) immediately after preparation (t0), and after 12 h (t12), 24 h (t24) and 48 h (t48) of exposure to the environmental chamber conditions. These solutions were then frozen and eventually analysed as described in subsection “Predicted tamoxifen concentrations in ecotoxicity tests”.

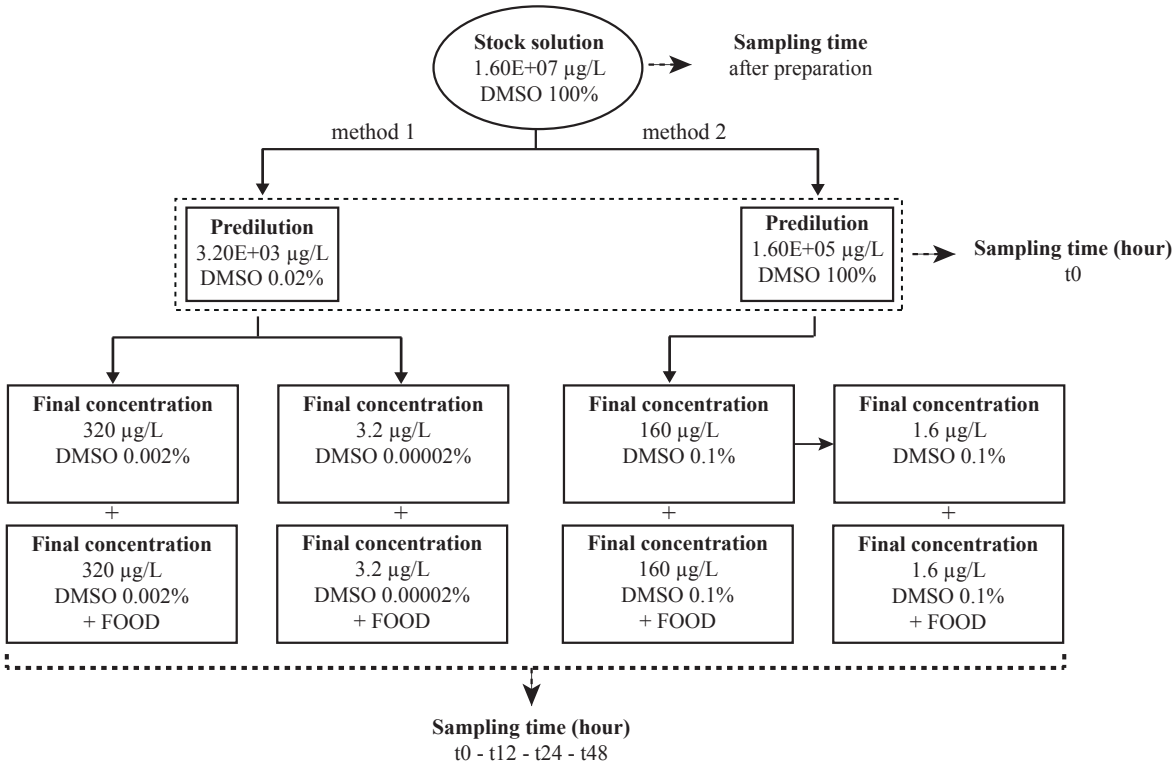


Figure 2.5: Experimental design of the analytical experiment that was performed with tamoxifen (nominal concentrations). Each step was performed using laboratory glassware. The volume of final concentrations was 50 ml. Sampling was performed at four different times. Food refers to the algae and lipids diet for a single daphnia

Statistics

Ecotoxicity experiments

For the acute toxicity test, dose-response curves were plotted using nonlinear regression (sigmoidal dose-response), from which the acute effect concentration 50% (EC50) and its confidence intervals were calculated. For the two-generation test, reproduction was first compared between the blank and solvent controls using a one-way ANOVA with Bonferroni correction ($\alpha < 0.05$). Because no significant differences were observed, the solvent control was used as a unique control for each ANOVA comparison with the Bonferroni correction ($\alpha < 0.05$). Calculations were performed using the software GraphPad Prism (version 4.00 for Mac OS X, GraphPad Software, San Diego California USA, www.graphpad.com).

Tamoxifen stability

Regarding tamoxifen stability in daphnia medium, the mean of the measured concentrations was compared either over time or between solutions that were supplemented with or without daphnia food. Statistically significant differences in means between times or solutions were calculated at the 95.0% confidence level using Fisher's least significant difference procedure (Statgraphics Centurion software, version 16.07 for Windows, Virginia USA, www.statgraphics.com).

Results

Predicted tamoxifen concentrations in ecotoxicity tests

Tamoxifen is poorly soluble in water and tends to be adsorbed on surfaces of the glass flasks. Therefore, tamoxifen concentration was measured in some of the incubation media (without daphnia and food) to ascertain the actual drug exposure of daphnids during the acute and two-generation experiments. All measured concentrations were plotted against their respective nominal concentration and were fitted using a nonlinear regression analysis (Figure 2.6). The equation of the fitted model was:

$$Ln(cP) = -3.67305 + 1.0503*Ln(nC) + 0.0363642*Ln(nC)^2 \quad (2.1)$$

Where cP = predicted concentration; nC = nominal concentration; and unit = µg/mL (r^2 = 0.93, residual plots were used to check that the assumptions for regression analysis were met). This regression can be used to extrapolate the actual tamoxifen exposure concentration from nominal concentrations. In the absence of evidence for systematic effects of time or daphnia food, the results of both acute and two-generation toxicity experiments are expressed as a function of the predicted concentrations that were calculated using Equation 2.1.

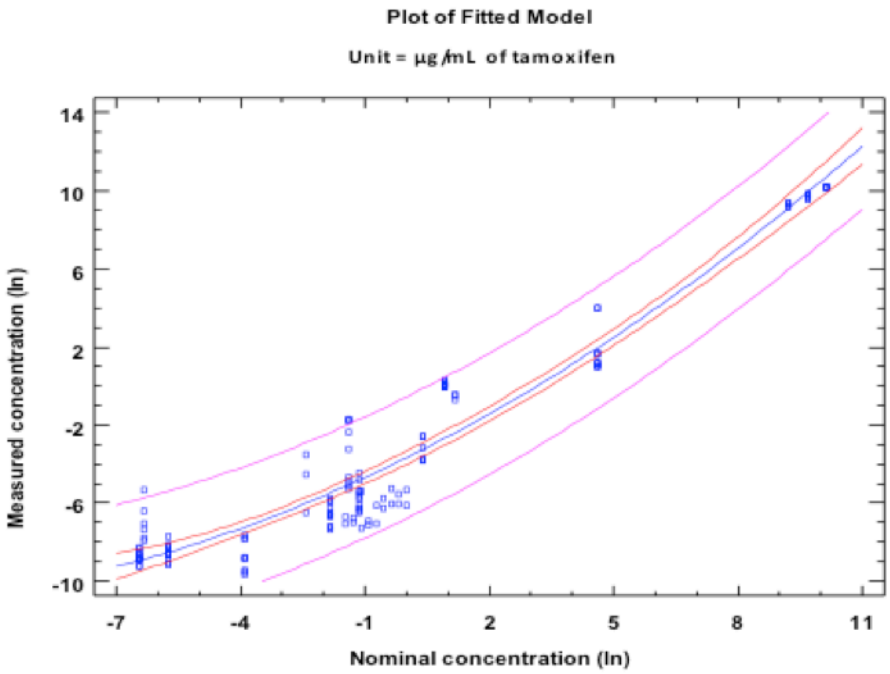


Figure 2.6: Relation between measured and nominal concentrations of tamoxifen (natural log). One hundred and forty-six samples were taken in solutions without daphnia and food. The graph shows the 95.0% prediction intervals for new examination (i.e. predicted concentration) and 95.0% confidence intervals for the mean of observations. The prediction and confidence intervals correspond to the inner and outer bounds, respectively, on the graph of the fitted model. Unit = µg/mL

Tamoxifen stability in medium

Comparison between nominal and measured concentrations

Major differences were highlighted between the nominal and measured concentrations of tamoxifen, following either dilution method 1 or 2 (Table 2.1). With method 1 (solvent < 0.1%), all mean measured concentrations were at least 90% lower than the nominal, excepted for the pre-dilution solution (solvent = 0.02%), whose concentration decreased only by 81.7%. With method 2 (solvent = 0.1%), the solutions with 1.6 and 160 µg/L had measured concentrations between 74.4 and 99.5% lower than the nominal. In solutions of 1.6 µg/L, the discrepancy increased with time, and the concentrations that were measured after 24 h were 90% lower than the nominal. With methods 1 and 2, intra-concentration variability was found primarily in solutions that were freshly prepared (t0). Conversely, the solutions with 100% of solvent, i.e., the stock and pre-diluted solutions of method 2, had mean measured concentrations 9.1% and 5.8% higher than the nominal, respectively.

Table 2.1: Nominal and measured concentrations of tamoxifen using two different methods. Method 1 was performed with a low DMSO percentage that did not increase significantly tamoxifen solubility, whereas method 2 maintained both tamoxifen solubility and a high DMSO percentage. The final concentrations were prepared with (regular characters) or without (bold characters) the addition of daphnia food. Δ is the difference between nominal and measured concentrations in %

	TAMOXIFEN	Time (h)	DMSO (%)	Nominal (µg/L)	Measured mean (µg/L)		SD		Δ concentration (%)	
	Stock solution	0	100	1.60E+07	**1.51E+07**		**1.65E+06**		**5.8**	
Method 1	pre-diluted solution	0	0.02	3200	**585.608**		**82.245**		**81.7**	
	concentration 1	0	0.002	320	**8.023**	8.738	**3.615**	2.697	**97.5**	97.3
	concentration 1	12	0.002	320	**3.665** *	3.366	**0.850**	0.510	**98.9**	98.9
	concentration 1	24	0.002	320	**2.559** *	6.551	**1.252**	6.914	**99.2**	98.0
	concentration 1	48	0.002	320	**1.808** *	2.049	**0.168**	0.459	**99.4**	99.4
	concentration 2	0	0.00002	3.2	**0.309**	0.169 ★	**0.132**	0.065	**90.3**	94.7
	concentration 2	12	0.00002	3.2	**0.142**	0.149	**0.035**	0.018	**95.6**	95.3
	concentration 2	24	0.00002	3.2	**0.227**	0.148	**0.100**	0.048	**92.9**	95.4
	concentration 2	48	0.00002	3.2	**0.198**	0.145	**0.038**	0.052	**93.8**	95.5
Method 2	pre-diluted solution	0	100	1.60E+05	**1.45E+05**		**9.39E+03**		**9.1**	
	concentration 1	0	0.1	160	**2.004**	8.135 ★	**0.729**	3.383	**98.7**	94.9
	concentratios 1	12	0.1	160	**1.367**	1.957 *	**0.082**	0.521	**99.1**	98.8
	concentration 1	24	0.1	160	**2.502**	1.473 *	**0.907**	0.427	**98.4**	99.1
	concentration 1	48	0.1	160	**0.726**	0.547 *	**0.049**	0.156	**99.5**	99.7
	concentration 2	0	0.1	1.6	**0.179**	0.410 ★	**0.070**	0.188	**88.8**	74.4
	concentration 2	12	0.1	1.6	**0.186**	0.219 *	**0.017**	0.027	**88.4**	86.3
	concentration 2	24	0.1	1.6	**0.138**	0.135 *	**0.036**	0.020	**91.4**	91.5
	concentration 2	48	0.1	1.6	**0.165**	0.155 *	**0.080**	0.047	**89.7**	90.3

* $p < 0.05$ when compared to t0

★ $p < 0.05$ when compared to solution without food addition

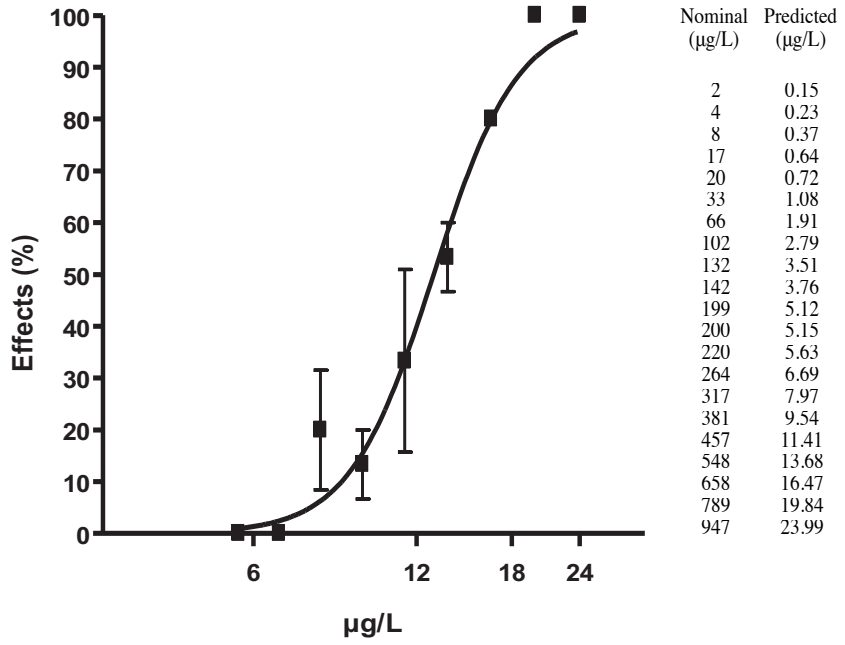
Comparison between the measured concentrations

The mean measured concentrations were not significantly different in five of the eight final solutions, when compared with the initial concentration at t0 (Table 2.1). Only for the solutions of 1.6 µg/L (with food), 160 µg/L (with food) and 320 µg/L (without food) did the mean measured concentrations decrease by up to 67%, 93% and 77% at t12, t24, and t48, respectively.

At t12, t24 and t48, no significant differences in concentrations were observed between most solutions supplemented with or without daphnia food. Only two solutions that were supplemented with food showed significantly higher concentrations than the corresponding solutions without food, namely the solutions of 1.6 and 160 µg/L at t0 (by 56% and 75%, $p < 0.05$, respectively), while an opposite trend was found at 3.2 µg/L (mean measured concentration 45% lower on supplementation with food).

Acute toxicity experiments

Daphnid viability was assessed using two parameters: the immobilisation of the organisms for >20 seconds and visual signs of tissue damage. The concentration that impaired the viability in 50% of the daphnids was 13 µg/L (CI 95%: 12.1 to 13.8, Figure 2.7). At concentrations of 19.8 µg/L and higher, organisms with internal tissues hanging from abdomen were observed. The tissues appeared as long fibres or as cluster of cells. Surprisingly, daphnids with these severe signs of damage were still able to swim. However, we hypothesised that daphnids whose organs were hanging were animals that could not escape predators in the environment and were counted as nonviable. Already at the lowest concentration, few daphnids swam with low energy or stopped moving for 15-20 seconds before suddenly resuming normal, energetic swimming.



Nominal (µg/L)	Predicted (µg/L)
2	0.15
4	0.23
8	0.37
17	0.64
20	0.72
33	1.08
66	1.91
102	2.79
132	3.51
142	3.76
199	5.12
200	5.15
220	5.63
264	6.69
317	7.97
381	9.54
457	11.41
548	13.68
658	16.47
789	19.84
947	23.99

Figure 2.7: Tamoxifen acute experiment in D. pulex. The effects are death and viability (mean ± SEM, n = 167). The EC50 is 13 µg/L. The exposure levels are expressed in predicted concentrations and their corresponding nominal concentrations are given on the right.

Two-generation toxicity experiments

Figure 2.8 shows the reproductive performance of both generations after 12 days of exposure to tamoxifen. The F0 and F1 generations began to release offspring approximately on the 6th day after birth, as expected. For the F0 organisms that were exposed to 5.15 µg/L of tamoxifen, the total number

of neonates decreased significantly ($p < 0.05$) compared with the solvent controls, namely by 58.2 and 40.0% after 12-d and 21-d of exposure, respectively (Table 2.2). No significant difference was found for other concentrations. In addition to reproductive impairment, aborted eggs and/or morphological abnormalities were observed in offspring from the F0 parents that were exposed to lower concentrations, i.e., 0.15, 0.23 and 0.72 µg/L of tamoxifen. The occurrence of these abnormal events and a qualitative example of abnormalities are provided in Table 2.2 and Figure 2.9, respectively. The first embryonic developmental abnormalities appeared on the 7th day of exposure at 0.23 and 0.72 µg/L, as well as after 13 days at 0.15 µg/L. Dead neonates were recorded at 0.72 µg/L but not at the other concentrations. Aborted eggs were observed in a single beaker of solvent control on the 7th day, whereas a single premature neonate was pipetted in a blank on the 15th day. Interestingly, no abnormalities were observed at 5.15 µg/L (with the exception of the significant drop on reproduction) in the F1 generation either.

Table 2.2: Reproduction and occurrence of abnormal events in the *D. pulex* generation F0. The reproduction is the total number of neonates produced after 21-d of exposure. The number of living individuals are compared to the total of tested individuals, * occurrence of abnormal events, such as aborted eggs or morphological abnormalities.

	Tamoxifen (µg/L)	Exposure period (day)																					Reproduction (mean)
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	
F0 generation	**0.15**	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	**3/3**[a]	**3/3**[a]	**3/3**[a]	**3/3**[a]	3/3	3/3	3/3	3/3	3/3	125
	0.23	3/3	3/3	3/3	3/3	3/3	3/3	3/3	**3/3**[a]	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	**3/3**[a]	3/3	**3/3**[a]	3/3	3/3	3/3	117
	0.72	3/3	3/3	3/3	3/3	3/3	3/3	3/3	**3/3**[a]	**3/3**[a]	**3/3**[a]	3/3	3/3	3/3	2/3	2/3	**2/3**[a]	2/3	**2/3**[a]	2/3	**2/3**[a]	2/3	2/3	110
	5.15	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	2/3	2/3	2/3	2/3	2/3	2/3	46
	Blank	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	5/6	5/6	**5/6**[a]	5/6	5/6	5/6	5/6	5/6	5/6	113
	Solvent	3/3	3/3	3/3	3/3	3/3	3/3	3/3	**3/3**[a]	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	110

[a] occurrence of abnormal events

The reproduction of the F1 generation that was exposed to 0.15 µg/L was statistically higher than both solvent controls and the F0 generation, by 17.6 and 43.1%, respectively. At 0.23 µg/L, an individual died on the 8th and 10th days of the experiment, and the third individual was lost on the 3rd day. The reproduction of premature neonates (F1) that were exposed for 12-d to tamoxifen was also assessed. At 0.15 and 0.72 µg/L, their reproductive capacity was reduced by 39 and 35.6%, respectively, when compared with their mature sisters exposed to the same concentrations, and by 2.3 and 3.3%, respectively, when compared with solvent controls (Figure 2.8).

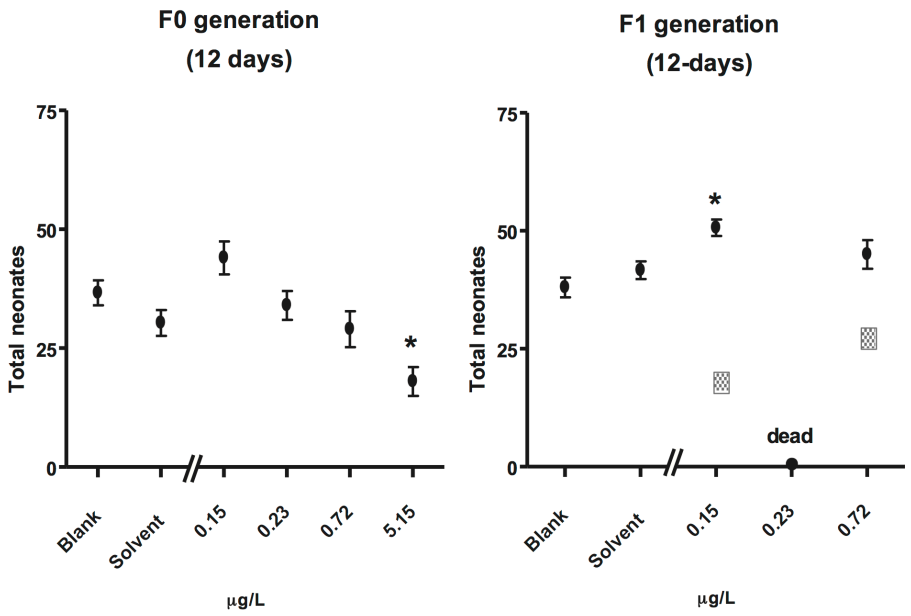


Figure 2.8: Reproduction of F0 and F1 generation of *D. pulex* in the two-generation study. F0 was exposed 21 days to the chemical, while F1 was exposed 13 days (mean ± SD, n = 21 for F0, and n = 18 and 3 for F1 normal and F1 premature neonates, respectively). The concentrations are predicted from measured concentrations. Reproduction is the total number of neonates per female. The grey squares are the reproduction effects on F1 premature neonates * p < 0.05

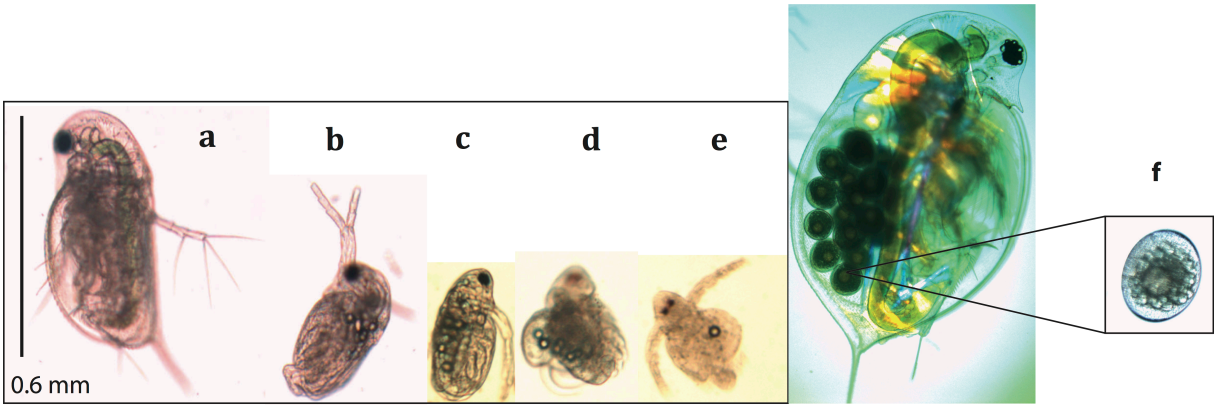


Figure 2.9: Reproductive and developmental toxicity effects of tamoxifen on *D. pulex* offspring. (a) Neonate < 24 h from control (blank); (b) premature neonate with temporary abnormal development: 2-fold smaller than a 24 h neonate from controls, able to swim; (c-d-e) neonates with abnormal morphology: 2 to 3-fold smaller than blank, aged < 24 h, dead or not able to swim, body-deformed, found on the bottom of the beakers; (f) spontaneous aborted egg found on the bottom of the beaker

Discussion

The results of our experiments show that tamoxifen induces acute toxic effects on *D. pulex* at the µg/L order of exposure. In the literature, a single study reported the acute effects of this pharmaceutical on *D. magna* but at higher concentrations [59]. The authors estimated an EC50 of 1530 µg/L, which is approximately 118-fold the EC50 that was observed in our experiment. Although differences in sensitivity may be observed in species that belong to the same *Daphnia* genus, no major differences in overall sensitivity towards several chemicals were found between *D. pulex* and *D. magna* [60]. Therefore, this discrepancy might be rather due to the use of nominal instead of measured concentrations. In our study indeed, the acute EC50 based on nominal concentrations would have been 516 µg/L instead of 13 µg/L, which is, therefore, much closer to the EC50 that was found in the DellaGreca study. This comparison highlights the importance of controlling the tested concentrations with analytical measurements, whenever possible. If a difference exist between measured and nominal exposure, it is crucial to express the results in terms of measured concentrations or of concentrations predicted from analytical results, rather than in terms of nominal levels (i.e. theoretical concentrations that were deduced from dilution procedures). In this study, we decided not to use nominal concentrations and we question the use of these values in ecotoxicology. Indeed, we raise the issue of assessing actual test exposure, as already advocated here and there in the toxicological literature, including for human trials [61], or purely *in vitro* assays, when physicochemical properties of chemicals, such as lipophilicity, volatility, stability, etc., may lead to chemical losses and may affect toxicity assessments [62]. Eventually, the use of nominal values can lead to an underestimation of the risk of chemicals to the aquatic flora and fauna. Unfortunately, the nominal concentrations (i.e., without analytical verification) remain often used in publications. A survey of the 2012 publications in the journal Environmental Toxicology and Chemistry showed that among seventy-three tested chemicals, the concentrations of 45% were not measured, although 23% of them were suspected to be poorly hydrophilic (LogKow > 3), as it is the case for tamoxifen. The major reason why nominal concentrations of lipophilic molecules are still often used is probably the burden of analyses. Indeed, analysing each test concentration is time and cost consuming, particularly in long-term ecotoxicological experiments. Therefore, an efficient prediction method might represent an interesting alternative to derivate effect levels close to real exposure concentrations. For instance, Groothuis et al. [62] recommend an algorithm for appropriate and feasible dose measurement to improve toxicological risk assessment. In this study, we propose a predictive method for tamoxifen exposure that can be applied to any long-term aquatic experiment. First, we assessed the stability of tamoxifen in daphnia medium (without daphnids but in the presence and absence of food). Then, we randomly selected the test solutions that were intended to be analysed in the two-generation experiment. The concentrations that were measured prior to this long-term test, i.e., during pre-tests, acute tests and stability experiment, were also considered in this model. The measured concentrations of tamoxifen that were

determined during these experiments were plotted against their respective nominal level to establish the relationship between the nominal and measured concentrations. A nonlinear regression was finally used to predict the non-measured test concentrations. Although this method does not fully override the need for systematic and regular sampling, this approach spares workload and still prevents the use of nominal concentrations and, thus, the risk of misleading conclusions that is based on overestimated exposure levels.

As expected indeed, the analytical results showed major differences in concentrations between the nominal and measured concentrations. The quantity of tamoxifen that was measured in final solutions ranged between 26% and 0.3% of the respective nominal, in accordance with a significant loss of the compound in the medium. Because the concentrations of tamoxifen degradation products that were measured in these solutions were not relevant, the differences between the nominal and the measured concentrations cannot be explained by a chemical degradation. Therefore, we hypothesise that this lipophilic pharmaceutical was adsorbed on glass surfaces, which reduced the amount of free tamoxifen in solutions. Chamart et al. [63] showed that 20% of the tamoxifen adsorbed on glass material after 15-minutes contact time, when diluted in the minimum essential medium (MEM, [64]). This result is consistent with our findings, which revealed large differences between nominal and actual concentrations already at the initial time point (t0). This phenomenon was observed regardless of the DMSO percentage (method 1 and 2) in our study. Besides the lack of solvent influence, the presence of daphnia food in the incubation medium did not change tamoxifen concentrations in 87.5% of the total samples that were analysed. Conversely, the type of medium may influence tamoxifen-free concentrations. Indeed, Chamart et al. [63] observed adsorption of tamoxifen on glass vessels when diluted in the MEM but not when diluted in bi-distilled water. These authors hypothesised that one of the MEM components might trigger adsorption. This may also be the case with the daphnia medium, which is also supplemented with numerous compounds [3]. These results emphasise the requirement to measure the concentration exposure in the actual conditions, when ecotoxicity tests are performed.

In our study, tamoxifen impaired reproduction and induced unusual toxic effects on *D. pulex*. In the acute assay for instance, tamoxifen caused severe internal tissue damage or prolonged immobilisation periods that were followed by sudden and extended energetic swimming. In the chronic assay, tamoxifen reduced reproduction and elicited teratogenic effects, such as abnormal neonates and aborted eggs. Teratogens are known to cause miscarriages and abnormalities in structure or function and growth that may affect embryo or foetus viability [65]. In higher animals, the mechanisms that may induce teratogenic effects are numerous, such as cytotoxicity, mutation, chromosomal damage, protein activity disruption, apoptosis alteration, etc., which, in turn, may lead to hormonal or immunological dysfunctions, implantation failure, foetal structure abnormalities and miscarriages [65,66]. Although our results are qualitative, we hypothesised that similar mechanisms could have been activated in the F0 daphnids that were exposed to tamoxifen, which may explain deformed

offspring, for instance. In vertebrates, tamoxifen is not only an anticancer agent but also a hormonal disruptor that works as a selective oestrogen receptor modulator. Similar to tamoxifen, other hormones or hormone-precursors (e.g., testosterone and androstenedione) and synthetic endocrine disruptors (e.g., 4-nonylphenol and the fungicide propiconazole) were reported to reduce fecundity and to induce developmental toxicities in daphnids [67–70]. In these studies, toxic effects were observed at the mg/L level, whereas tamoxifen already induced these effects at the µg/L level. Kast-Hutcheson et al. [67] conscientiously categorised embryonic development regarding time and maturation stages. Based on their results, we divided the morphological effects of tamoxifen into specific classifications, as described in Figure 2.9. The severity and type of malformations vary with embryonic developmental stages and with the exposure duration. Therefore, according to Kast-Hutcheson et al. [67] and LeBlanc et al. [68], the aborted eggs and morphologically abnormal neonates (dead or alive) that were observed in our study might reflect toxicity at early and late stages of development. Further experiments are however required to confirm the results observed in *D. pulex* and to identify the most sensitive developmental stage.

Because our study primarily focused on reproductive performance of *D. pulex* exposed to tamoxifen, a test period, longer than 12-d, would have been of interest for the teratogenic assessment of the second generation. Indeed, this generation did not produce abnormal neonates during the 12-d of exposure to tamoxifen. The F0 generation released abnormal neonates after the 7^{th} and/or the 13^{th} day of exposure (Table 2.2), which suggests that abnormal neonate production in the F1 generation could have occurred after 12-d of exposure. Interestingly, the reproduction of the daphnids that were exposed to 0.15 µg/L had elevated reproduction compared with controls and other concentrations. Physiological adaptation, such as resistance or higher metabolism-cost, which is intended for species survival, may be a reason for this increase in reproduction [71,72]. Another reason may be the pharmaceutical molecule activity itself. Indeed, tamoxifen has the ability to increase ovulation rates in patients [73], and we wonder whether some analogous mechanism was involved in daphnids that were exposed to this low concentration.

In our study, daphnids showed a higher sensitivity to tamoxifen in the non-standard endpoints relative (severe tissue damage, abnormal neonates, etc.) to the standard endpoints (immobilisation, reproduction [3,74]). Consequently, exposure of aquatic organisms to tamoxifen might be better assessed using our non-standard endpoints. For instance, the standard immobilisation endpoint would not consider daphnids with organs hanging from abdomen because these daphnids were able to swim. In this case, the EC50 would be higher than our EC50 value that was based on viability. Similarly, chronic experiments that only focus on the reproductive endpoint (i.e., total number of neonates) would not consider aborted eggs and abnormally developed neonates. In our study, the morphological abnormalities in embryos appeared at lower concentrations than concentrations that adversely affected reproduction. These results highlight the need to consider non-classical endpoints in ecotoxicological

testing, and we wonder whether current standard testing is appropriate for pharmaceuticals with anticancer and endocrine disrupting properties.

While the no observed effect concentration (NOEC) that was calculated for reproduction was 0.72 µg/L; however, a NOEC of less than 0.15 µg/L was estimated for morphological abnormalities in the F0. This level is below the tamoxifen concentrations that were measured in STP effluents (0.02 to 0.37 µg/L) [47–49] and in aquatic environments (0.01 to 0.21 µg/L) [49,50]. To the best of our knowledge, no NOEC of tamoxifen on daphnids has been reported to date in the open literature. These results raise concerns regarding the integrity of the aquatic flora and fauna and should stimulate further investigations to assess ecotoxicological risks at the population level.

Conclusion

Pharmaceutical residues are released into the aquatic environment every day, which justify growing concerns regarding their potential adverse effects on living organisms. Our data emphasise the importance of measuring the tested concentrations of hydrophobic drugs, such as tamoxifen, to avoid incorrect conclusions and an underestimation of their risk on aquatic systems. At a minimum, some concentrations can be measured and the rest can be predicted, as described in this study.

In chronic experiments, tamoxifen reduced reproduction and showed teratogenic effects. These results raise questions regarding its potential impact on other relevant aquatic species and in experiments over several generations, particularly because the NOEC is low (< 0.15 µg/L). In general, little is known regarding long-term effects of pharmaceuticals on aquatic species [75], although these molecules are continuously present in aquatic systems. Multi-generational testing would be required to answer the question regarding long-term effects of pharmaceuticals.

Furthermore, patients who are treated with tamoxifen primarily excrete the parent molecule and 4-hydroxy-tamoxifen, which is known to be a potent active metabolite in humans [76–78]. This metabolite induced effects on the reproduction, survival and body length of *D. pulex* (chapter 3); however, its toxicity in other aquatic organism is unknown, although developmental effects were observed in organisms that were exposed to tamoxifen at environmental relevant concentrations. There is a clear need to further characterise the risk of tamoxifen and other anticancer compounds or metabolites on the aquatic flora and fauna.

Finally, global cancer rates are expected to increase by 50% by 2020, with 15 million new cases [79]. This increase is closely related to the decline of other life-shortening conditions, such as infections and cardiovascular diseases. In 2000 in France, a survey that was performed by national authorities calculated that there were 800,000 people living with cancer, whereas 28,000 new cancer cases and 150,000 deaths were reported [80]. Five years later, the number of new cases increased; however, the

number of deaths decreased, which indicated that cancer patients spend progressively longer periods of their lifetime under treatment. Therefore, the release of anticancer drugs into aquatic systems is expected to increase, which makes the assessment of their effects on aquatic organisms crucial.

Acknowledgements

This project was funded by the Swiss National Science Foundation (http://p3.snf.ch/project-132344) and was supported by the Faculty of Biology and Medicine Research Commission Fund of the University of Lausanne (UNIL). The authors would like to thank Professor Othmar Müntener from the UNIL Institute of Earth Sciences for his explanations regarding the Olympus polarisation microscope Sandra Cruchon from the Laboratory of Clinical Pharmacology (CHUV, Lausanne) for her excellent analytical work that was performed on all tamoxifen samples, Florine Baillot for the parallel laboratory experiences regarding tamoxifen stability in incubation medium (Ecole de Pharmacie Genève-Lausanne, Universities of Lausanne and Geneva), Chiara Perazzolo for her useful comments on the manuscript, Mark Ibberson (Swiss Institute of Bioinformatics) for his invaluable suggestions to this project, and the statistician Marianne Gex-Fabry (University of Geneva) for her help in statistical analyses.

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Chapter 3

Is *D. pulex* sensitive to the tamoxifen metabolites 4OHTam and endoxifen?

In this chapter, the toxic effects of 4-hydroxy-tamoxifen (4OHTam) and endoxifen, two tamoxifen metabolites, are described and discussed. Endoxifen acute experiments are first presented in this chapter, as an addendum. In humans, 4OHTam and endoxifen interact with the same receptors as tamoxifen but with higher affinity and higher potency than their parent compound. Patients excrete tamoxifen, 4OHTam and endoxifen in faeces and tamoxifen was measured in wastewaters and natural waters. Contrary to their parent drug, the environmental concentrations of tamoxifen metabolites are unknown. The effects of 4OHTam and endoxifen in aquatic organisms have also never been reported yet. In March 2014, the Canadian Ministers of the Environment and of Health [1] have reported the results of a screening assessment of tamoxifen in which they consider 4OHTam and endoxifen as highly toxic to aquatic organisms, with a potential for endocrine disruption. The aim of this chapter was therefore to assess the sensitivity of daphnids toward 4OHTam and endoxifen. A two-generational study was performed in which each generation of daphnids (F0 and F1) was exposed 21 days to 4OHTam or endoxifen. At the end of this chapter, a supplementary material is provided on: 1) the stability of 4OHTam and endoxifen in daphnia medium in test solutions and at different times, 2) a method to predict 4OHTam and endoxifen concentrations in daphnia medium 3) the choice of the concentration that were used in the two-generational study.

Endoxifen acute experiment on *D. pulex* (Addendum)

Introduction

Endoxifen is a secondary metabolite of the anticancer drug tamoxifen. In human, its potency and its affinity to estrogen receptors (ERs) is higher than the parent drug [2–4]. Endoxifen is mainly an ERα ligand, which has antiestrogen activity [3]. The aim of this study was to assess the sensitivity of *D. pulex* towards endoxifen during 48 hours. This acute toxicity experiment was performed to determine the range of effective concentration on daphnids as well as the concentrations that reduce mobility on 50% of the treated animals.

Material and method

Chemical

Endoxifen was purchased from Sigma/Fluka (> 99%, lot: H7904). The day before the experiments, the chemical was dissolved in pure DMSO [5]. A stock solution was prepared at the measured concentrations of 2.73E+06 µg/L. This stock solution was stored in glass bottle, in the dark at -80°C, and it was thawed at room temperature before used.

Test organisms

Individuals from the *arenata* strain of *D. pulex* were mass-cultured in Elendt M4 medium in which the water hardness was reduced to 95 mg/l CaCO3 [6]. They were reared in glass flasks, in a Coolstore® environmental chamber with 16-h light and 8-h dark photoperiods at 21 ± 1°C. These stock daphnids were fed daily with both 0.2 mgC/daphnia of *Pseudokirchneriella subcapitata* algae and suspension of tropical fish food such as Tetramin® [7,8].

Acute experiment

Three hundred and ten neonates (< 24 h, > 3rd brood) were exposed 48 hours to endoxifen in two acute experiments. Briefly, five individuals per vessel (3 replicates) were randomly pipetted in glass tubes at predicted concentrations ranging from 7.64 to 530.74 µg/L of endoxifen. Table 3.1 summarises endoxifen nominal, measured and predicted concentrations. For explanations on predicted concentration, see supporting information “predicted concentrations”. During each acute experiment, two controls were performed: a blank (without chemical) and a solvent control that contained 0.1%

DMSO. This percentage corresponded to the maximum DMSO percentage that was used at the highest test concentration of each experiment. The studied endpoint was the immobilisation of daphnids > 20 seconds, despite a gentle agitation of the tube. This endpoint was observed after 24 and 48 h of exposure. Individuals were not fed during the experiments.

A potassium dichromate ($K_2Cr_2O_7$) assay was also performed at the beginning of the acute experiments and the EC50 (± standard deviation) corresponded to the $K_2Cr_2O_7$ results in our laboratory: 0.6 (± 0.25) µg/mL.

Statistics

The results of both acute experiments were combined and expressed as a sigmoidal dose-response curve, from which the acute EC50s and confidence intervals were calculated. The dose-response curve was generated by the following nonlinear regression:

Y= 100/(1+10^((LogEC50-X)*HillSlope)) (2)

where x is the logarithm of concentration. Y is the response, and Y starts at 0 and goes to 100 with a sigmoid shape. The HillSlope result was 2.5 for endoxifen and r^2 was 0.86.

Table 3.1: Endoxifen acute test concentrations. Predicted concentrations were calculated using a polynomial regression of measured *vs* nominal concentrations (n samples = 202). The measured concentrations are expressed as the mean of triplicate samples

Nominal (µg/L)	Measured mean (µg/L)	SD	Predicted (µg/L)
171			27.80
222			37.09
250	27.41	16.08	42.31
288			49.53
360	28.91	12.58	63.55
375			66.53
487			89.26
530	51.98	33.40	98.21
633			120.09
770	74.10	14.61	150.08
824			162.14
1071			218.92
1110	117.32	9.13	228.11
1392			296.11
1610	214.54	45.93	350.42
1809			401.18
2300	460.61	261.79	530.74
2352			544.79

Results and discussion

The results of both experiments were combined and they are summarised in Figure 3.1. After 48-h of exposure, the calculated EC50 of endoxifen was 233 µg/L, which is higher than tamoxifen and 4OHTam EC50s. Indeed, in previous acute experiments, tamoxifen and 4OHTam EC50s were 9.5 and 42 µg/L, respectively (see chapter 4). These results show that endoxifen was less potent than 4OHTam and tamoxifen in daphnids. This is not the case in human, since endoxifen and 4OHTam have greater pharmacological effect than tamoxifen *in vivo* [9–11], but it is possible that endoxifen does not have similar targets in *D. pulex* as tamoxifen and 4OHTam. This hypothesis is in accordance with Hawse et al. [12], who reported that endoxifen's mechanism of action was different from tamoxifen and 4OHTam in gene transcription, cell cycle arrest and markers of apoptosis, although these three chemicals targeted the same gene, i.e., ERα in vertebrates. Hawse et al. also showed that endoxifen induced different effects depending on the concentrations, which may modulate the antitumor activity in patients. Taking together, we hypothesised that the mechanisms of endoxifen action might also be different with this of tamoxifen and 4OHTam in daphnids.

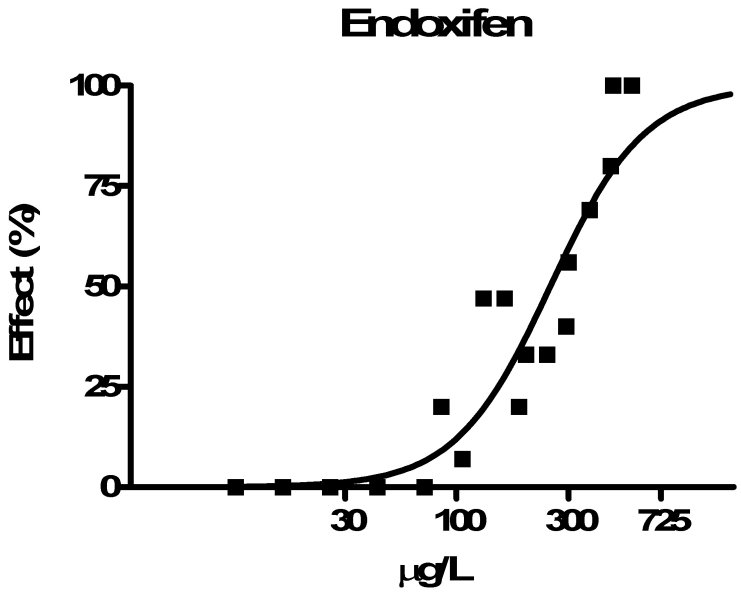

Figure 3.1: Endoxifen acute toxicity experiment. The EC50 (95% IC) is 233 (199, 290) µg/L. The exposures are predicted from measured concentrations (µg/L). The HillSlope was 2.5, r^2 was 0.86, n = 310 (including controls)

The anticancer drug metabolites endoxifen and 4-hydroxy-tamoxifen induce toxic effects on *Daphnia pulex* in a two-generation study

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Submitted to *Science of the Total Environment*, manuscript ID STOTEN-D-14-04632

Abstract

Endoxifen and 4-hydroxy-tamoxifen (4OHTam) are two metabolites of the widely used anticancer drug tamoxifen for the prevention and treatment of breast cancers. Both metabolites have a high pharmacological potency in vivo, attributing prodrug characteristics to tamoxifen. Tamoxifen and its metabolites are body-excreted by patients, and the parent compound is found in both sewage treatment plan effluents and natural waters. The aim of this study was to assess whether medicinal drug residues induce long-term toxicities on *Daphnia pulex*, a freshwater microcrustacean that has already shown some susceptibility to tamoxifen. Two chronic tests of 4OHTam and endoxifen were run in parallel on two generations of *D. pulex* and several endpoints were assessed. Both metabolites induced effects on reproduction, survival and body length. The effects on reproduction were magnified on the second generation compared to the first. The intrinsic rate of natural increase (r) decreased with increasing 4OHTam and endoxifen concentrations, with increased sensitivity observed in the F1 generation. The No-observed effect concentrations calculated for the reproduction of the second generation exposed to 4OHTam and endoxifen were < 1.8 µg/L and 4.3 µg/L, respectively, while the NOECs that were calculated for the intrinsic rate of natural increased were < 1.8 and 0.4 µg/L, respectively. Our study raises questions about prodrug and active metabolites in environmental toxicology assessments of pharmaceuticals. Our findings also emphasize the importance of performing long-term experiments and considering multi-endpoints instead of the standard reproduction outcome.

Introduction

Residues of pharmaceuticals have been detected in surface waters worldwide, leading scientists as well as non-scientists to wonder about their risks to aquatic systems [13]. Aside from veterinary use and industrial releases, the primary source of drugs in water appears to be human consumption [14]. After their absorption, pharmaceuticals are eliminated in either intact or metabolised forms through human excreta. They then follow urban sewage water networks to a sewage treatment plant (STP). New technological treatment processes are able to remove some pharmaceuticals from wastewaters [15], but the most current traditional plants were designed to eliminate organic matter, phosphates and nitrates, and not organic chemicals, such as pharmaceuticals. Therefore, numerous pharmaceuticals and their derivatives with pharmacological activities escape STP processes and continuously reach the aquatic compartment [13]. Such bioactive molecules are capable of chronic interactions with the biological processes of aquatic species and their offspring, even at low concentrations. For example, this is the case for the synthetic steroid analogue 17-alpha-ethinylestradiol, for which a predicted no-effect concentration (PNEC) was proposed at the level of 0.1 ng/L [16]. Among other pharmaceutical groups, the anticancer agents are of particular concern because of their potential mutagenic, carcinogenic, teratogenic and/or embryotoxic properties [17].

Tamoxifen is a chemotherapeutic selective estrogen-receptor modulator that is widely prescribed worldwide for the prevention and treatment of hormone-receptor-positive breast cancers [11,18,19]. This anticancer drug has partial agonist and antagonist activity on alpha and beta estrogen receptors (ERα, ERβ) depending on the tissues. Tamoxifen is nearly considered as a prodrug [20] and releases active metabolites, i.e., 4-hydroxy-tamoxifen and endoxifen, after enzymatic and/or chemical transformation *in vivo*.

Tamoxifen was recently found in wastewater effluents and surface waters at concentrations up to 0.37 µg/L and 0.22 µg/L, respectively [21–26]. No data have been reported addressing actual concentrations of endoxifen and 4OHTam in aquatic environments up to now, but we assume that these metabolites could be found in wastewaters and natural waters, such as their parent compound, because they are also body-excreted, mainly via faeces [18,27,28]. Their concentrations in excreta are scarcely known because they have never been studied in a respectable cohort of patients. For instance, the concentrations of tamoxifen, 4OHTam, and endoxifen were measured in faeces of three patients, and these concentrations ranged between 230-1092 µg, 123-579 µg, and 92-189 µg, respectively [27]. Because information is lacking, it is difficult to estimate their concentration in wastewaters and STP effluents. However, both metabolites share similar physical-chemical properties with their parent compound, which suggests that they may escape sewage treatment processes [26]. Furthermore, it is known that pharmaceutical metabolites can be found in STP effluents and natural water, such as the anti-inflammatory metabolites carboxy-ibuprofen and hydroxyl-ibuprofen [29,30], the antidepressant

metabolites norfluoxetine and desmethylsetraline [31], and the analgesic metabolite salicylic acid [32,33]. The salicylic acid was found also at high concentrations, up to 0.28, 0.37 and 12.7 µg/L in lake, river and STP effluent waters, respectively [31,33].

Tamoxifen metabolites are of particular interest because of their high potency that was discovered only recently, long after tamoxifen commercialisation. In vertebrates, tamoxifen is primarily metabolised by the hepatic P450 cytochromes into hydroxylated and demethylated metabolites, such as N-desmethyl-tamoxifen, 4-hydroxy-tamoxifen (**4OHTam**), tamoxifen N-oxide, and alpha-hydroxy-tamoxifen [34]. In a second step, N-desmethyl-tamoxifen is transformed into 4-hydroxy-N-desmethyl-tamoxifen (**endoxifen**). Although tamoxifen is already an active molecule, the 4OHTam and endoxifen metabolites have an affinity to ERs 100 times that of the original molecule, and their potency to suppress cell proliferation in breast cancer is also 30 to 100 times that of tamoxifen [4,9,35]. Furthermore, tamoxifen and 4OHTam also interact with estrogen-related receptors (ERRs) that are nuclear receptors present in many organisms, including invertebrates [36,37]. ERRs bind and regulate transcription through estrogen response elements (EREs) and estrogen-related response elements (ERREs). In mammals, three types of ERRs exist: ERRα, ERRβ and ERRγ. The ERRs have distinct function than ERs. They are related to ERs but are unable to bind natural estrogenic ligands [38]. *In vitro,* 4OHTam has higher affinity than tamoxifen with ERRβ and ERRγ, and none of them bind ERRα [39]. Finally, the action of tamoxifen and its metabolite via the ERRs may play an important role in breast tumour therapy [36].

In a recent study, Thomson et al. [37] found that a single copy of the gene ERR (dappu-ERR, NR3 subfamily) was present in the freshwater microcrustaceans *Daphnia pulex,* among the twenty-five nuclear receptor genes that were identified in this species [37]. Daphnids lack ERs but their ERR gene is close structurally to the human homologs ERRα, ERRβ and ERRγ, and close also to human ERα and ERβ [37]. Indeed, the phylogenetic relationship of the ERR in *D. pulex* and the ERRs and ERs in *Homo sapiens* belongs to the same sub-familly of nuclear receptors [37]. In *D. pulex*, the endogen ligand and the functions of the ERR family are unknown [37], but they may be involved in estrogen signalling and metabolism pathway [40,41]. In a parallel study, we investigated if *D. pulex* that was sensitive to tamoxifen. We observed reproductive impairments, aborted eggs and/or morphologically abnormal neonates [42]. The No-observed effect concentrations (NOECs) were < 0.15 µg/L and 0.72 µg/L when calculated for morphological abnormalities and for reproduction, respectively. Interestingly, Suillvan and Thummel [43] found that the orphan ERR in drosophile may have embryonic functions and that they are expressed during embyogenesis.

Because of the presence of ERR gene in daphnids, of the high potency of 4OHTam and endoxifen, and of their potential continuous inputs of in the aquatic environments, we hypothesise that tamoxifen metabolites may induce long-term effects on aquatic organisms. Also, 4OHTam and endoxifen were recently considered to be highly toxic to aquatic organisms, with a potential for endocrine disruption

[1]. Tamoxifen was so identified as a priority molecule for assessment because of its inherent toxicity to non-human organisms. The aim of this study was therefore to assess the long-term toxicities of 4OHTam and endoxifen on *D. pulex* that has already shown some susceptibility to tamoxifen [42]. To reach this goal, we exposed the daphnids to low doses of these chemicals during two generations. The primary objective was to observe whether these two anticancer drug metabolites affect the survival, reproduction and/or size of *D. pulex*. Second, we aimed to calculate the intrinsic rate of natural increase (r) to reflect how metabolite concentrations would restrict population growth.

Materials & Methods

Chemicals and drug concentration measurements

Endoxifen and 4-hydroxy-tamoxifen (4OHTam) were purchased from Sigma/Fluka and used without further purification (>99%, lot: 110M4107V for endoxifen and 099K46042 for 4OHTam). Endoxifen and 4OHTam were initially dissolved in pure dimethyl sulfoxide, DMSO [5,44]. A day before the test, stock solutions were prepared in glass bottles at mean measured concentrations of $2.11 \cdot 10^6$ µg/L and $2.04 \cdot 10^6$ µg/L for 4OHTam and endoxifen, respectively. Throughout the test duration, both stock solutions were stored in the dark at -80°C and thawed every two days, i.e., before each use.

Predicted concentrations of 4OHTam and endoxifen

Endoxifen and 4OHTam are lipophilic molecules that are slightly soluble in water. After sampling analyses of these chemicals in Elendt M4 solutions [8], differences between nominal and measured concentrations were observed, primarily with the solutions freshly diluted (see supporting information, SI, part I, for detailed explanations on the stability of 4OHTam and endoxifen in incubation medium).

During ecotoxicological experiments, the OECD [8] suggests to sample test solutions at renewal (t0) and after 48 hours (t48) of exposure. In the two-generational experiment performed in this study, the test solutions were not all measured because this OECD follow up would have required analysis of about 900 samples (i.e., about 8 concentrations sampled at t0 and t48, in triplicate at least, during 2 generations). Also, this requirement may discourage and slow down the science of ecotoxicology to move forward. Therefore, a good prediction method was required to determine the 4OHTam and endoxifen exposures to daphnids, and a polynomial regression of the measured concentrations *versus* the nominal concentrations of each chemical was performed. In this study, exposure levels are predicted concentrations that were calculated from the fitted model of 4OHTam and endoxifen (equation S1 and S2, SI, part I).

Test organisms

D. pulex from the *arenata* strain have been mass-cultured for two years in our laboratory [42]. They are reared in conditions that maintain parthenogenetic reproduction (total hardness 90 ± 5 mg/l as $CaCO_3$; pH 7.9 ± 0.2; conductivity adjusted to 25°C, 286 ± 14 µS/cm; dissolved oxygen > 5 mg/L). Stock daphnids as well as individuals exposed to chemicals were reared in glass beakers placed in a Coolstore® environmental chamber (16: 8 h light: dark photoperiods at 21 ± 1°C).

Two-generation testing procedure

Endoxifen and 4OHTam chronic assays were performed in parallel over two generations of *D. pulex* (F0 and F1). This two-generation study was adapted from previous ecotoxicological experiments, such as acute and chronic pre-tests performed with 4OHTam and endoxifen (SI, part II), and the framework of the experimental design is illustrated in Figure 3.2. Briefly, the parental generation (F0) was randomly selected from a single stock of mothers. Forty neonates (< 24 h, > 3rd brood) were pipetted and placed in separate glass beakers with 50 mL of medium (four replicates per concentration). The exposure concentrations were 1.8, 6.8, 23.8, and 98.9 µg/L and 0.4, 4.3, 51.8, and 202.4 µg/L for 4OHTam and endoxifen, respectively. The DMSO percentage was not analysed. On the fifteenth day of maternal exposure, seventy-four neonates (< 24-h) were collected to form generation F1. Three neonates per beaker (three replicates) were transferred to 150 mL of corresponding maternal concentration medium. Note that on the fifteenth day, the mothers (F0) exposed to 23.8 µg/L of 4OHTam produced only five neonates. These five were distributed between two different beakers to form F1.

Two controls were included for both the endoxifen and 4OHTam chronic tests (four replicates for F0 and F1 generations): a blank (i.e., without solvent or chemicals) and a solvent control referring to the DMSO percentage at the highest endoxifen and 4OHTam concentration in the F0 (i.e., 0.04% of DMSO). Daphnids from the controls were reared in the same environmental conditions as the test organisms. Daphnids were fed daily with 0.2 mg C/daphnia of *Pseudokirchneriella subcapitata* algae and a suspension of Tetramin® tropical fish food [8,45]. At the beginning of the experiment, a potassium dichromate ($K_2Cr_2O_7$) assay was performed to assess daphnia sensitivity. Additionally, a $K_2Cr_2O_7$ assay was conducted every 21 days. Dissolved oxygen, pH and conductivity were measured at least twice a week.

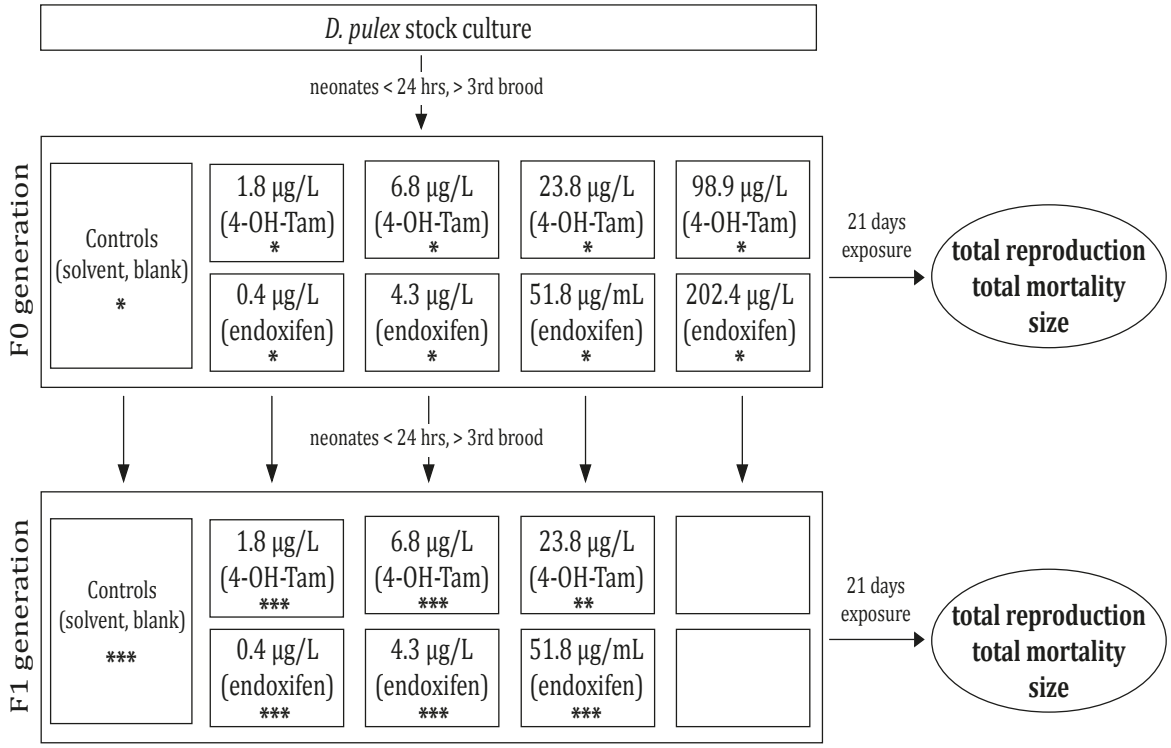

Figure 3.2: Experimental design for the chronic toxicity test on *D. pulex*. On the fifteenth day, neonates were kept to form the second generation (F1) and they were exposed to corresponding maternal concentration medium. The concentrations of 4OHTam at 98.9 µg/L and endoxifen at 202.4 µg/L were not tested on F1 because there were no survivors from F0. * 1 organism/beaker (4 replicates per concentration), * 3 organisms/beaker (3 replicates per concentration), ** 2 or 3 organism/beaker (2 replicates for 23.8 µg/L of 4OHTam)**

Daphnia mortality, reproduction and size

The mortality (i.e., number of dead parents) and number of offspring per beaker were recorded daily (the neonates were then discarded, except on day 15). The reproductive performance of the daphnids in each group was expressed as the average number of neonates per adult during the 21-d exposure period. Visual body-size differences with the controls were noted for the daphnids exposed to chemicals during the experiment. Body-size was defined as the body shape that includes body breadth and length.

Intrinsic rate of natural increase (r)

The intrinsic rate of natural increase r was calculated using mortality and birthrate data and by iteration of the Euler-Lotka equation [46]:

$$\Sigma\, l_x m_x e^{-rx} = 1 \qquad (1)$$

where l_x is the proportion of individuals surviving to age x, m_x is the number of neonates produced per surviving adult at age x, and x is time expressed in days. In accordance with van Leeuwen [47], a 21-day study is sufficient to estimate the intrinsic rate of natural increase, as r calculated in *D. pulex* after 21 days is identical to the estimated r for their entire lifespan. In this work, r was calculated for each replicate on both generations. Similarly to Tanaka [48], the r function of each exposure concentration was fitted according to the following equation:

$$r(x) = r(0)\,[1-(x/\,\alpha)^{\beta}] \qquad (2)$$

where x is the exposure concentration, r(x) is the mean of the intrinsic rate of natural increase under x, α corresponds to the concentration at which r reaches zero (i.e., at which the population stops growing), and β is the curvature shape of the response. The α and β values are estimated with the least chi-squared method (Levenberg-Marquardt algorithm; SPSS Statistics 21:0). The intrinsic mean rate of the control (blank and solvent controls together) was used here as r(0), here equal to 0.4 (Table 3.2).

Statistics

For the two-generation testing, the reproduction and intrinsic rate were first compared between the blank and solvent controls using a one-way ANOVA test followed by the Bonferroni correction ($\alpha < 0.05$). No significant differences were observed between these controls; therefore, both controls were gathered and used as the unique control for each multiple comparison. The calculations were carried out with GraphPad Prism (version 4.00 for Mac OS X, GraphPad Software, San Diego California USA, www.graphpad.com).

Results

Daphnia reproduction and mortality

The effects of endoxifen and 4OHTam on *D. pulex* were assessed for generations F0 and F1 (Table 3.2). With 4OHTam, the reproduction of the F0 and F1 generations displayed a systematic trend following exposure concentrations. The F0 mortality was 100% at the highest concentration, i.e., 98.9 µg/L, and daphnids died on the fourth day, before the age of reproduction was reached (on average the 6.5th day). The average number of neonates produced by the F0 generation exposed to 6.8 and 23.8 µg/L of 4OHTam was significantly reduced ($p < 0.001$) compared to the controls, by about 25 and 56

%, respectively. On the fifteenth day, when neonates were kept to form generation F1, the mothers exposed to 23.8 µg/L of 4OHTam produced only five neonates. The reproduction of F1 was reduced in each test concentration, by about 21, 28 and 73 % for 1.8, 6.8 and 23.8 µg/L, respectively. The no-observed effect concentration for reproduction (NOECrep), which is the highest tested level where the total number of young produced was not significantly different from the controls, was 1.8 µg/L for the F0. For the F1, the NOECrep could not be calculated because effects were observed in each test concentration. Therefore, this NOECrep was below the lowest test concentration, i.e., < 1.8 µg/L.

Table 3.2: Survival, size and fecundity of two *D. pulex* generations (F0 and F1) exposed 21 days each to 4OHTam and endoxifen in the two-generation study (mean ± SD, n = 40 and 74 for F0 and F1, respectively). The exposure refers to predicted concentrations that were calculated from measured concentrations. Reproduction is the total number of neonates per female. Size is capitalized as 0 and 1 for no visual differences and visual differences compared to controls, respectively. The F1 generation was exposed to corresponding maternal concentration medium. * and ** indicate statistical significance in comparison with controls

	Treatment	Exposure (µg/L)	Longevity (days)	size	Reproduction	Intrinsic rate (r)	NOEC reproduction	NOEC (r)
F0 generation	4OHTam	1.8	21 ± 0	0	76 ± 8.2	0.418 ± 0.027	1.8	6.8
		6.8	19.3 ± 3.5	0	64 ± 8.3 **	0.376 ± 0.037		
		23.8	21 ± 0	1	37 ± 6.1 **	0.333 ± 0.033 *		
		98.9	4 ± 0 **	1	0	0**		
	Endoxifen	0.4	21 ± 0	0	92 ± 4.2	0.398 ± 0.039	4.3	4.3
		4.3	21 ± 0	0	88 ± 6.5	0.376 ± 0.023		
		51.8	18.3 ± 3 **	1	28 ± 17.3 **	0.286 ± 0.126 *		
		202.4	4 ± 0 **	1	0	0**		
	Control		21 ± 0		86 ± 10.3	0.394 ± 0.028		
F1 generation	4OHTam	1.8	21 ± 0	0	82 ± 3.4 *	0.359 ± 0.018 *	< 1.8	< 1.8
		6.8	21 ± 0	0	74 ± 7.5 **	0.368 ± 0.014 *		
		23.8	21 ± 0	1	28 ± 0 **	0.309 ± 0**		
		98.9	-					
	Endoxifen	0.4	20.9 ± 0.3	0	105 ± 5.9	0.398 ± 0.011	4.3	0.4
		4.3	21 ± 0	0	95 ± 8.5	0.372 ± 0.026 *		
		51.8	5.2 ± 2.6 **	1	0**	0**		
		202.4	-					
	Control		20.6 ± 1.4		103 ± 10.6	0.404 ± 0.017		

* p < 0.05 (Bonferroni correction) - no neonates for the F1 assay

** p < 0.01 (Bonferroni correction)

For endoxifen, the concentration of 202.4 µg/L was lethal in 100% of the daphnids after four days of exposure. At 51.8 µg/L of endoxifen, mortality as well as reproduction was affected in both the F0 and F1 generations. Half of the F0 individuals were dead after sixteen days of exposure, and the reproduction of the other half was highly significantly reduced ($p < 0.001$) by about 67 % compared to the controls at the end of the exposure period. In addition, a high variability was observed for F0

reproduction at this concentration. For example, one individual that died on the sixteenth day of exposure produced a total of forty neonates, which was higher than the total reproduction of the daphnids that survived until the end of the test. Conversely, another individual that died on the fifteenth day produced only two neonates. The F1 daphnids died between the second and eleventh days of exposure and did not produce any offspring at the concentration of 51.8 µg/L. Seven of them died before the age of reproduction was reached, and the last three did not reproduce before dying. No effect was observed at the two lowest test concentrations, so the reproductive NOEC for endoxifen in both generations is therefore 4.3 µg/L. Finally, the mortality in the solvent controls and blanks of the F0 and F1 generations were the same for both tested chemicals and never exceeded 10%. At the end of the test, their mean number of offspring produced was above 60 neonates, as recommended by the OECD guideline [8].

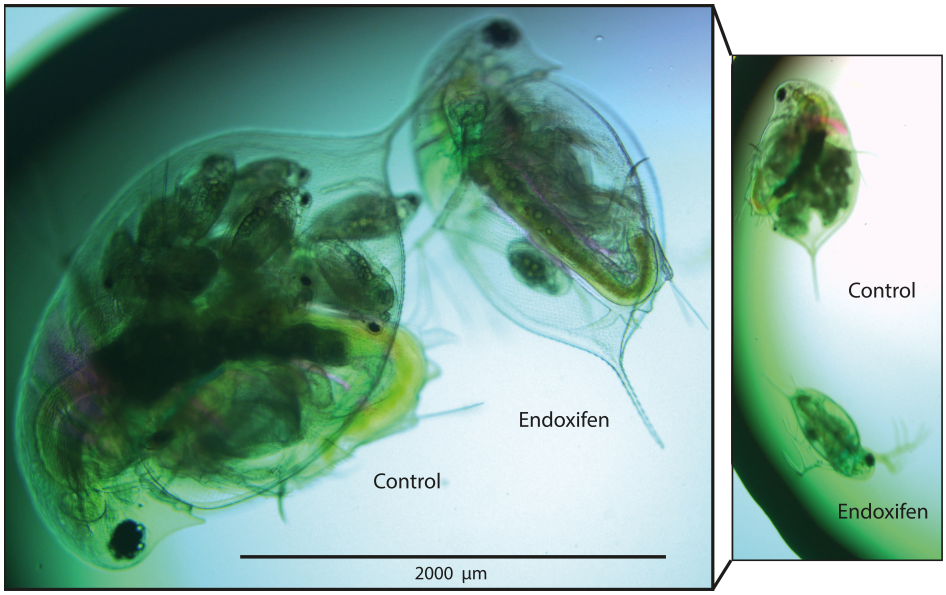

Figure 3.3: Body-size of maternal *D. pulex* after 21 d of exposure to 51.8 µg/L of endoxifen or blank (same generation). On the left, the photo is magnified 4 times, compared to the photo on the right. Photos were taken using an Olympus polarisation microscope BX51 with a digital imaging system (Colorview)

Daphnia size

Differences in body length were observed in the daphnids exposed to 23.8 and 98.9 µg/L of 4OHTam and to 51.8 and 202.4 µg/L of endoxifen. These daphnids were smaller than the controls after 21 d of exposure. As an example, Figure 3.3 shows the visual difference in body-size between two randomly selected individuals exposed for 21 d to 51.8 µg/L endoxifen or to daphnia medium exempted of chemical. The F0 daphnids exposed to the highest concentration of both chemicals exhibited impaired growth. They all died at a size corresponding to a 24-h neonate from the controls (i.e., solvent and blank). No size difference was observed at lower concentrations.

Intrinsic rate of natural increase (r)

The intrinsic rate of natural increase r, which estimates population growth of *D. pulex*, was calculated for the F0 and F1 generations exposed to 4OHTam and endoxifen (Table 3.2). For the F0 individuals exposed to 98.8 and 202.4 µg/L of 4OHTam and endoxifen, respectively, and for the F1 daphnids exposed to 51.8 µg/L of endoxifen, no individual survived and r was zero. The r of the F0 daphnids exposed to 23.8 µg/L of 4OHTam was significantly lower ($p < 0.05$) than the controls, and they were all significantly lower in the F1 ($p < 0.01$ for 1.8 and 23.8 µg/L, $p < 0.05$ for 6.8 µg/L). The r of the F0 and the F1 exposed to 51.8 and 4.3 µg/L of endoxifen, respectively were significantly lower ($p < 0.05$) when compared to the control. The NOECs of 4OHTam that were calculated for r, NOEC(r), were 6.8 µg/L for the F0 and this NOEC was < 1.8 µg/L for the F1. The NOECs(r) of endoxifen were 4.3 and 0.4 µg/L for the F0 and F1, respectively. Figure 3.4 shows how population growth under exposure to endoxifen or 4OHTam decreases with increasing concentrations. The estimation of the second generation exposed to 4OHTam was performed with r = 0 at the concentration of 98.9 µg/L. Indeed, the daphnids exposed to this concentration died in the F0 and therefore, the intrinsic rate is null.

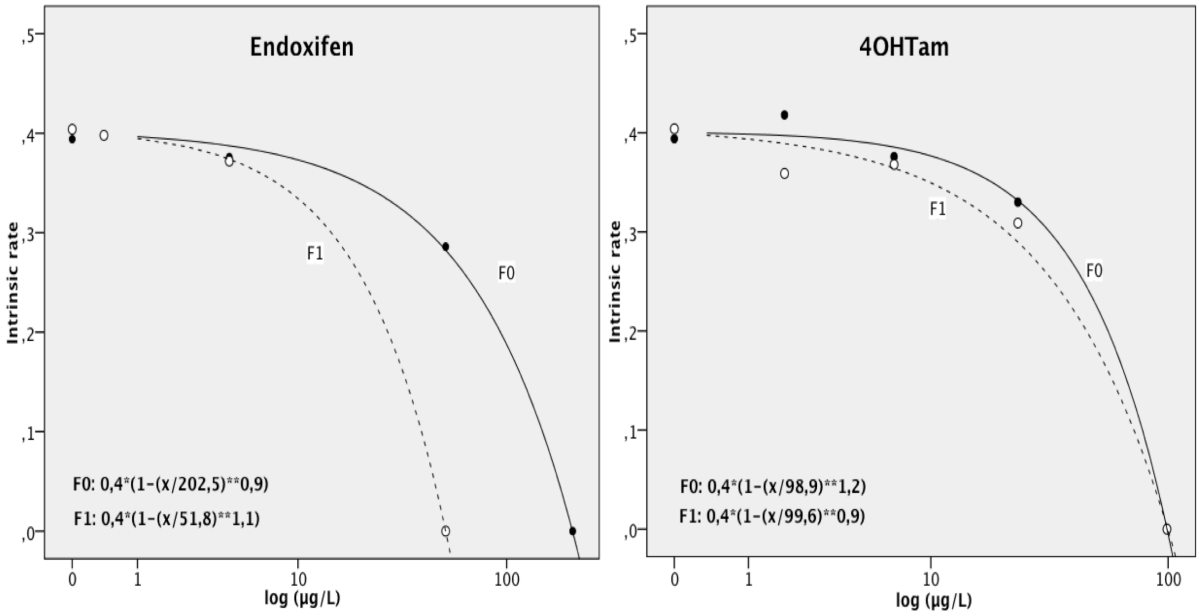

Figure 3.4: Population-level effect of endoxifen and 4OHTam on *D. pulex*: intrinsic rate of natural increase variation as a function of endoxifen or 4OHTam concentrations. ● F0, ○ F1. 4OHTam $r^2 = 0.91$ and 0.72 for F0 and F1 respectively, and endoxifen $r^2 = 0.87$ and 0.73 for F0 and F1 respectively. The estimation of the second generation exposed to 4OHTam was performed with r = 0 at the concentration of 98.9 µg/L

Discussion

In a two-generational study performed on *D. pulex*, the tamoxifen metabolites 4OHTam and endoxifen induced effects on reproduction, survival and body length. These chemicals were able to disrupt physiological mechanisms at the µg/L level, i.e., at levels that were close to the environmental concentrations of their parent compound [21–26].

For both metabolites, the sensitivity of *D. pulex* increased in the second generation. The effects on reproduction and the estimation of population growth were indeed magnified in the F1 exposed to 4OHTam and endoxifen, respectively. For instance, the reproduction of F0 exposed to 23.8 µg/L of 4OHTam dropped, and continued to decline in the F1 generation. Similarly, the reproduction of the survivors exposed to 51.8 µg/L of endoxifen decreased, and their offspring (F1) did not survive when maintained at the same concentration. Several studies have also reported increased sensitivity and decreased fitness of the second generation of daphnids exposed to endocrine disruptors [48–50]. In accordance with the authors, we hypothesise that the second generation of daphnids was weakened by maternal exposure in our study. The F1 individuals exposed to 1.8, 6.8 and 23.8 µg/L of 4OHTam and 51.8 µg/L of endoxifen were probably affected at an early life stage, contrary to their parents, which were not exposed during embryonic development. Our experiment started with F0 neonates from previously unexposed parents, meaning that the first generation was not exposed to the chemical during the oogenesis and embryogenesis processes. Therefore, the F0 generation could better survive and reproduce than neonates pre-exposed to chemicals in the maternal brood chamber. In addition, a potential toxicant transfer from mothers to offspring might not be excluded, as 4OHTam and endoxifen are lipophilic molecules. Although slightly less lipophilic than their parent compound tamoxifen, their calculated XLogP3 (i.e., online logP calculator; Pubchem.ncbi.nlm.nih.gov) is 6.3, which is close to the experimental tamoxifen logP of 7.1 [51]. This high lipophilicity allows molecules to accumulate in fat and pass physiological barriers (e.g., protein membranes, [52]). Because adult daphnids provide energy to their eggs in the form of fat [53,54], a bioaccumulation process is possible in the case of 4OHTam and endoxifen. Supporting this hypothesis, a recent study reported a positive correlation between bioaccumulation and logP for daphnids exposed to xenobiotics from the perfluoroalkyl acid family [55]. Further studies investigating the bioaccumulation of tamoxifen metabolites in daphnids and in neonates would therefore be interesting to perform.

In addition to mortality and reproduction, qualitative differences in body-size were observed for daphnids exposed to 4OHTam or endoxifen. At the two highest concentrations tested, the F0 generation did not grow and remained at a size similar to control < 24-h neonates. The offspring from daphnids that were smaller than controls after 21-d of exposure were also smaller at the end of their respective test period. Other studies have reported size differences in both adult daphnia and their offspring exposed to low chemical concentrations, such as dispersants or pesticide mixtures [56,57].

This size reduction may be crucial for population survival, as Villarroel and al. [58] observed a positive relationship between small body-size and a diminished reproduction rate in *D. magna* exposed to the tetradifon insecticide. In our work, body breadth and length were not measured but most of the daphnids that had apparent reduced size either died or produced fewer neonates than the controls. To our knowledge, studies that report daphnia sizes after xenobiotic exposures are scarce, although this effect seems to reflect daphnids sensitivity to chemicals. Indeed, Hammers-Wirtz and Ratte [57] argued that changes in neonate size reduce individual fitness, enhance mortality or impair the reproduction of daphnids. Therefore, the question of measuring treated daphnids may be posed when the sensitivity and the survival of daphnia population is assessed.

The intrinsic rate of natural increase (r) is an interesting calculation that considers significant parameters, such as reproduction, mortality, time of the first brood, etc. Although, the total number of neonates is often considered a more sensitive endpoint than the r dynamic [59], this calculation provides some evidence that relevant effects may occur at the population level. In the concerted effort that is made by the international scientific community to develop adverse outcome pathways that consider population level effects [60–62], these parameters seem also important to included in risk assessment. In our study, the calculated r showed a similar tendency than the other studied endpoints, i.e, the increase of sensitivity of the animals between the F0 and the F1 generations. Interestingly, the intrinsic rate r of the F0 daphnids exposed to 6.8 µg/L of 4OHTam was not statistically different from the controls, although a significant effect on reproduction was observed. This may be due to a decrease in reproductive performance from the third brood [63], because the dynamic of r is strongly influenced by the effects on reproduction and mortality during the few first broods and less so during the rest of the exposure period. However, the plot of modelised r as a function of exposure shows well the increased effect of endoxifen on daphnids' fitness between the first (F0) and second (F1) generations (Figure 3.4). For this chemical, the calculated population growth was clearly reduced in the second generation, which is in accordance with the hypothesis of a generation weakened by maternal exposure. In the second generation exposed to 4OHTam, the estimated r was reduced at each exposure level and this result raises question about the offspring fitness in the next generations, and at lower concentrations than those used in this study. For 4OHTam indeed, the NOECs for r and for the reproduction were considered as below the lowest concentrations tested in this study, i.e., < 1.8 µg/L. The NOEC of endoxifen that was calculated for the intrinsic rate of the population growth was 0.4 µg/L, which is close to the concentrations of tamoxifen that were measured in the natural aquatic environment (i.e., up to 0.22 µg/L [23,25]). However, because only few points are available for modelling r, the tendency of population growth of both molecules should be confirmed. Also, multigenerational experiments and risk investigations of these chemicals at the population level are encouraged.

In spite of their pharmacological high potency and increased releases into the environment, before the present study, endoxifen and 4OHTam had never been tested on aquatic species. Although here we show the effects of these tamoxifen metabolites on *D. pulex*, additional chronic experiments should be conducted on daphnids and other aquatic species to compare the results and better understand these chemicals' effects in aquatic environments. In general, pharmaceutical metabolites are not well studied in ecotoxicology [64,65], and we believe that the question of prodrugs is too little considered in this field. However, the problem of active, or more active, metabolites should be fully integrated in any environmental ecotoxicology assessment of pharmaceuticals. Prodrugs are often used in chemotherapy, e.g., Miproxifene Phosphate, Capecitabine, Captopril, Cyclophosphamide, Sulfasalazine, Loperamide, Fosphenytoin, Bambuterol, etc. [20,66,67], and also in several other treatments against nausea, convulsion, anesthesia, inflammation, etc., such as valganciclovir, sulindac, prednisolone phosphate, propofol phosphate [68], etc. They improve absorption, limit side effects and increase the selectivity of target cells. Before 2008, 5-7% of world pharmaceutical sales were prodrugs [69], and now, according to Rautio [20] this percentage is close to 10%. We therefore hope that the present study will help to promote more integrated assessments of pharmaceutical ecotoxicity, to give some evidence that metabolites can impair non-target organisms, and to stimulate monitoring of the metabolites in the environment.

Supporting information (SI)

Part I

Predicted 4OHTam and endoxifen concentrations used for ecotoxicity tests

To predict concentrations of 4OHTam and endoxifen, a two steps procedure was followed: (1) we estimated the stability of 4OHTam and endoxifen in daphnia medium, (2) we measured the concentration of the two metabolites in different test solutions, and we established a relationship between the nominal (i.e., theoretical) and measured concentrations.

Stability of 4OHTam and endoxifen in incubation medium (step 1)

The stability of 4OHTam and endoxifen in the incubation medium were assessed in parallel experiments that were performed during a master thesis [70]. The design of these experiments is shown in Figure 3.5. Briefly, the nominal concentrations in final solutions were 1, 3, 100 and 300 µg/L of 4OHTam and endoxifen, with DMSO percentages of 0.1, 0.0001, 0.1 and 0.01%, respectively. These metabolites and DMSO concentrations corresponded to those that would be used if chronic ecotoxicological tests were performed on daphnids. The final concentrations contained 50 mL of incubation medium (without daphnids). Each dilution was prepared in laboratory glassware with the incubation medium (Elendt M4 [8]) that is used for daphnids in ecotoxicological experiments. Final solutions were stored for two days in a Coolstore® environmental chamber (16: 8 h light: dark photoperiods at 21 ± 1°C), which corresponds to conditions that are used in ecotoxicological experiments.

The final test solutions were sampled immediately after preparation (t0), after 12 h (t12), 24 h (t24) and 48 h (t48) of exposure to the Coolstore® conditions. The stock solutions were sampled just after preparation (i.e., the day before the test) and just before t0. Solutions were collected in PP microtubes (400 µg/L, 3 replicates per concentration, n = 60 for each chemical) and the aliquots were immediately frozen at -80°C until analyses. 4OHTam and endoxifen concentrations were analysed by liquid chromatography coupled to triple stage tandem mass spectrometry (LC-MS/MS), according to a validated method developed at the Laboratory of Clinical Pharmacology of the CHUV (University Hospital Centre of the Canton of Vaud, Switzerland [71]).

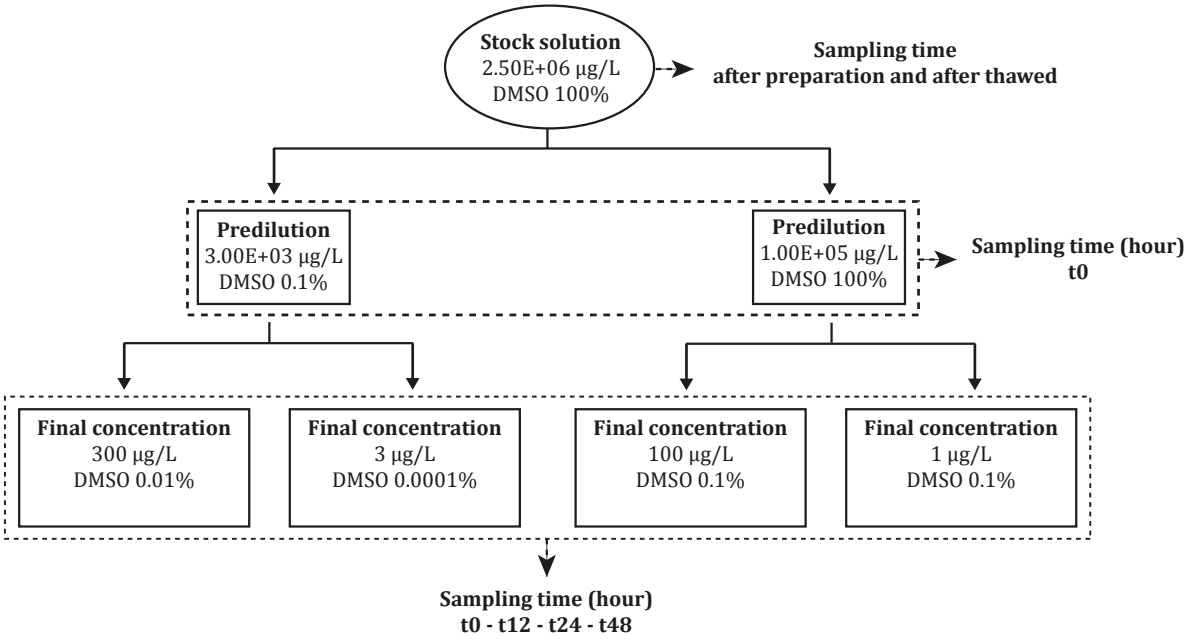

Figure 3.5: Experimental design of the analytical methods that were run in parallel with 4OHTam and endoxifen (nominal concentrations). Sampling was performed at different times (3 replicates per concentration, n = 60 for each chemical). The volume of the final concentrations was 50 ml each (without daphnia)

The means of the measured concentrations (triplicate) were compared over time (time zero compared with other times) to evaluate the stability of the molecules during 48h (Fisher's least significant difference method, 95% confidence level, Stagraphics Centurion software, version 16.07 for Windows, Virginia USA, www.statgraphics.com). The results show that main differences in concentrations occurred during the first 12 hours, and that the mean measured concentrations were stable afterward until t48 (Table 3.3), except for 300 µg/L and 100 µg/L of 4OHTam. Note that measured concentrations were lower than the nominal by more than 90% in almost every solution, and we assumed that interactions with the incubation medium and glass adsorption were the causes of chemical loss, as Chamart et al. [72] observed it for tamoxifen.

Concentrations of 4OHTam and endoxifen in ecotoxicological experiments (step 2)

Three ecotoxicological experiments were performed with 4OHTam and endoxifen: acute-, chronic pre-, and two-generational- tests. During these experiments, randomly chosen test solutions were set aside in separate glass flasks (without daphnids). These test solutions were sampled at t0 and after 48 hours (t48) of exposure to the Coolstore® environmental chamber. The stock solutions were sampled after preparation and the day of the experiment. At the end of the experiments, a total of 344 samples (206 and 138 for 4OHTam and endoxifen, respectively, including the samples from the stability experiments) were analysed by LC-MS/MS system of the CHUV [71].

Table 3.3: Stability of 4OHTam and endoxifen in daphnia medium (without daphnids). The measured concentrations are expressed as the mean of triplicate samples. Statistical significant differences between means of a concentration over times are calculated at the 95.0% confidence level. Difference between the nominal and the actual concentrations are expressed in percent

ID	Time (h)	DMSO (%)	Nominal (µg/L)	Measured (µg/L)		SD	Measured / Nominal (%)	Measured (µg/L)		SD	Measured / Nominal (%)
				4OHTam				Endoxifen			
[stock]	0	100	2.5E+06	4.2E+06		2.2E+02	-68.622	3.1E+06		1.4E+05	-22.420
[pre-diluted]	0	100	1.0E+05	1.5E+05		1.2E+04	-54.205	1.5E+05		2.3E+04	-54.041
[pre-diluted]	0	0.1	3000	764.701		310.998	74.510	2.6E+03		5.0E+02	14.141
[1]	0	0.01	300	17.340	**	6.049	94.220	43.022	♯	11.941	85.659
[1]	12	0.01	300	14.045		3.591	95.318	31.512	♯	6.745	89.496
[1]	24	0.01	300	12.549		4.459	95.817	79.783		34.765	73.406
[1]	48	0.01	300	7.851		2.363	97.383	66.522		9.957	77.826
[2]	0	0.1	100	7.430	*	1.205	92.570	16.292	*	6.301	83.708
[2]	12	0.1	100	4.416	★	1.065	95.584	8.724		2.160	91.276
[2]	24	0.1	100	4.863	**	1.395	95.137	8.916		0.750	91.084
[2]	48	0.1	100	2.104		0.342	97.896	8.467		2.125	91.533
[3]	0	0.0001	3	0.940	*	0.089	68.664	0.263		0.082	91.225
[3]	12	0.0001	3	0.122		0.058	95.920	0.352		0.146	88.255
[3]	24	0.0001	3	0.092		0.045	96.924	0.401		0.214	86.647
[3]	48	0.0001	3	0.134		0.015	95.526	0.227		0.046	92.418
[4]	0	0.1	1	0.858	*	0.116	14.171	0.274	*	0.071	72.626
[4]	12	0.1	1	0.056		0.011	94.356	0.115		0.047	88.525
[4]	24	0.1	1	0.085		0.018	91.453	0.126		0.054	87.364
[4]	48	0.1	1	0.053		0.034	94.697	0.105		0.035	89.482

** $p < 0.05$ when compared to t48
* $p < 0.05$ when compared to t12, t24, t48
★ $p < 0.05$ when compared to t24 and t48
♯ $p < 0.05$ when compared to t24

Predicted concentration of 4OHTam and endoxifen

Polynomial regression

The measured concentrations of 4OHTam and endoxifen obtained during the stability and the ecotoxicological experiments were plotted against their respective nominal concentrations. Then, they were fitted using a polynomial regression analysis (Figure 3.6). The equations of the fitted model of 4OHTam and endoxifen are:

$$\text{Ln(pC) of 4OHTam} = -2.61748 + 1.19858*\text{Ln(nC)} + 0.0244905*\text{Ln(nC)2} \quad \text{(S1)}$$

$$\text{Ln(pC) of endoxifen} = -1.59776 + 1.14688*\text{Ln(nC)} + 0.013011*\text{Ln(nC)2} \quad \text{(S2)}$$

where pC = predicted concentration, nC = nominal concentration, and the unit = µg/mL (natural log). The R^2 = 0.95 and 0.97 for 4OHTam and endoxifen, respectively (residual plots were also used to check that the assumptions for regression analysis were met, Stagraphics Centurion software, version

16.07 for Windows, Virginia USA, www.statgraphics.com). As explained in the materials & methods (part 2.1) the unmeasured and measured concentrations of the two-generational study were predicted concentrations from the equations S1 or S2.

Note that even with less analysed concentrations the equation of the fitted model gives an r^2 of 0.99 for 4OHTam and endoxifen, and the predicted concentrations are close to the nominal (Table 3.4). This is the case for the results of the stability experiment, for example.

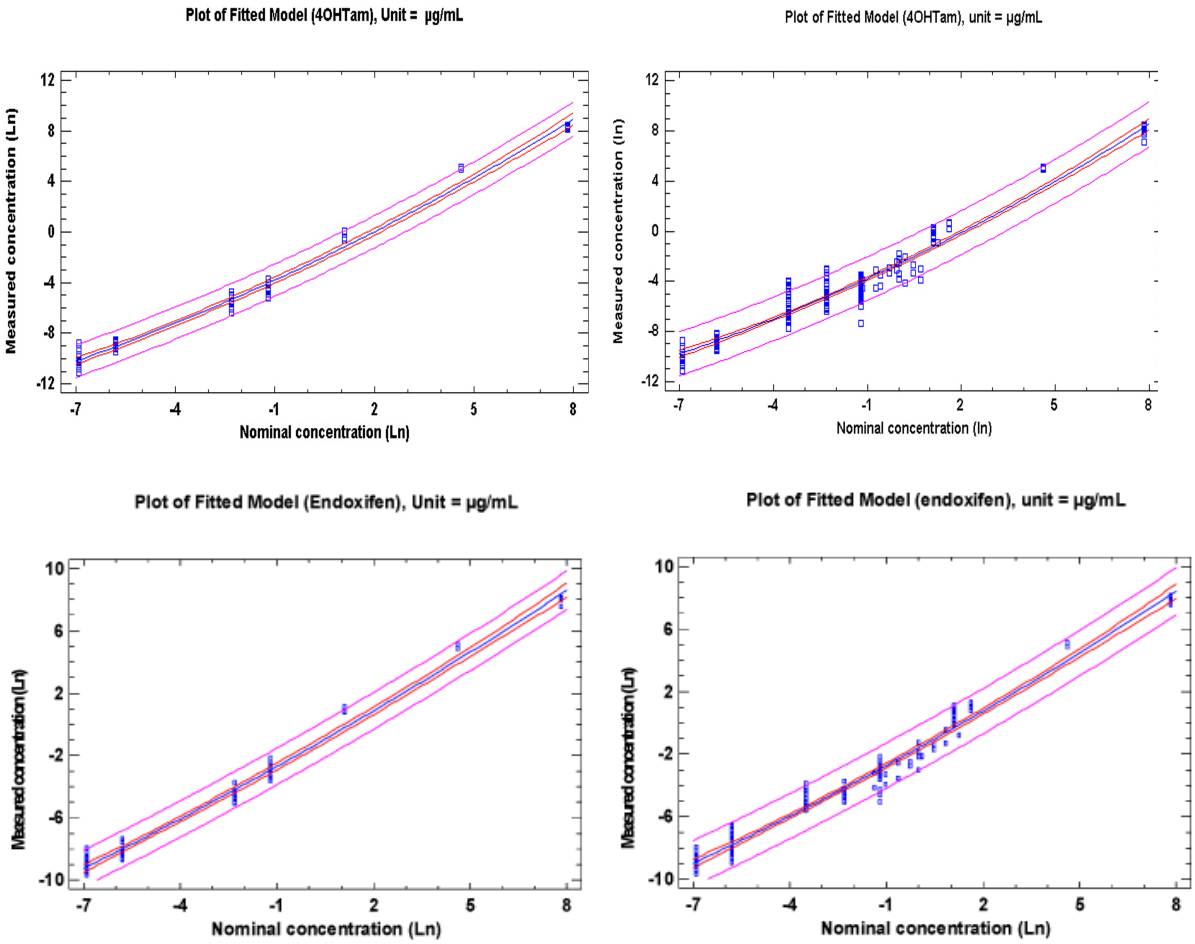

Figure 3.6: Polynomial regression of measured concentrations *vs* nominal concentrations of 4OHTam and endoxifen (natural log). The 60 measured concentrations obtained in the stability experiment were fitted against their respective nominal concentration (non-linear regression on the left graph). On the right graph, additional measured concentrations (n = 206 and 138 for 4OHTam and endoxifen, respectively) were fitted using a new non-linear regression curves (on the right of the table). Graphs show the 95.0% prediction intervals for new examination (i.e., predicted concentration) and 95.0% confidence intervals for the mean of observations. The prediction and confidence intervals correspond to the inner and outer bounds, respectively. Unit = µg/L

Table 3.4: Predicted concentrations of 4OHTam and endoxifen and their confidence limits. The measured concentrations are expressed as the mean of triplicate samples. Predictions were calculated using a polynomial regression of measured concentrations *vs* nominal concentrations from a stability experiment

	Solutions	Nominal (µg/L)	Mean measured (µg/L)	SD	Predicted [ug/L] Confidence limit 95% (lower; upper)	DMSO (%)
4OHTam	[stock]	2.5E+06	4.2E+06	2.2E+02	5.7E+06 (3.5E+06 ; 9.2E+06)	100
	[pre-diluted]	1.0E+05	1.5E+05	1.2E+04	4.0E+04 (3.0E+04 ; 5.4E+04)	100
	[pre-diluted]	3000	764.701	310.998	311.826 (238.948 ; 406.927)	0.1
	[1]	300	12.946	4.115	17.541 (13.791 ; 22.310)	0.01
	[2]	100	4.703	1.002	4.844 (3.895 ; 6.024)	0.1
	[3]	3	0.322	0.052	0.116 (0.093 ; 0.144)	0.0001
	[4]	1	0.263	0.045	0.040 (0.030 ; 0.054)	0.1
Endoxifen	[stock]	2.5E+06	3.1E+06	1.4E+05	4.3E+06 (2.7E+06 ; 6.6E+06)	100
	[pre-diluted]	1.0E+05	1.5E+05	2.3E+04	6.2E+04 (4.7E+04 ; 8.1E+04)	100
	[pre-diluted]	3000	2.6E+03	5.0E+02	795 (623 ; 1016)	0.1
	[1]	300	55.210	15.850	52.830 (42.335 ; 65.928)	0.01
	[2]	100	10.60	2.83	15.093 (12.348 ; 18.448)	0.1
	[3]	3	0.31	0.12	0.330 (0.270 ; 0.404)	0.0001
	[4]	1	0.16	0.05	0.105 (0.081 ; 0.138)	0.1

Part II

4OHTam and endoxifen concentrations in the two-generational experiment

The concentrations of 4OHTam and endoxifen that were tested on daphnids during the two-generational experiments (21 days of exposure per generation) were selected on the basis of preliminary ecotoxicological experiments. An acute toxic test (2 days of exposure) was performed first, to find the range of concentrations that induced toxic effects on daphnids. Then, a pre-chronic experiment over two generations (21 days of exposure per generation) was run at low concentrations, i.e., at concentrations that had no effect in the acute experiment. The two-generational experiments of this study were performed using concentrations that covered some acute effect and no chronic effect levels. Figure 3.7 summarises the predicted concentrations, the nominal concentrations, and the adverse effects of 4OHTam and endoxifen in the acute tests, the chronic pre-tests and the two-generational tests.

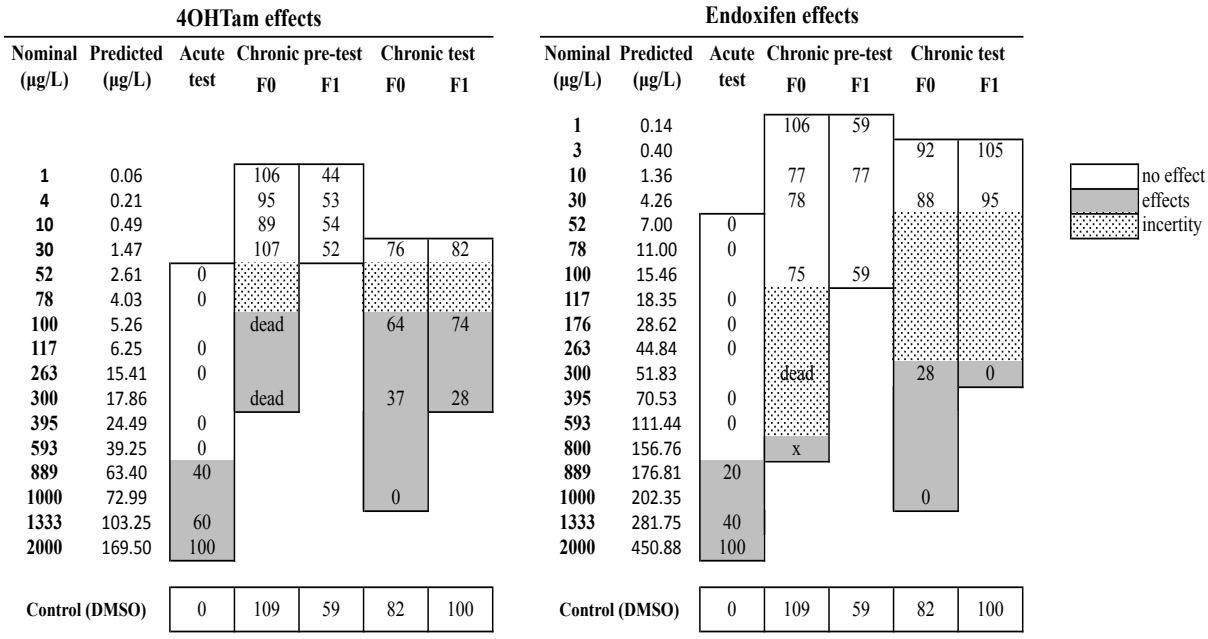

Figure 3.7: Overview of the test concentrations and the toxic effects of 4OHTam and endoxifen that were used in different ecotoxicological tests. The two-generational experiments of this study are named as chronic test on the figure (21 days of exposure per generation). In the acute test (2 days of exposure), the studied end points were death and immobilisation (n =165 and 180 for 4OHTam and endoxifen respectively), while they were death and the total neonates produced per individual (no replicate) in the chronic pre-test (n = 6 and 4 for F0 and F1 exposed to 4OHTam chronic pre-test, and n = 6 and 3 for F0 and F1 exposed to endoxifen, respectively). The exposure periods in the chronic pre-test were 21 and 13 days for F0 and F1, respectively. The dotted zones are uncertainty areas in which toxic effect may be possible.

Acute testing procedure

Acute experiments were performed on *D. pulex* neonates (< 24 h, > 3rd brood) exposed 48 hours to 4OHTam or endoxifen with a protocol adapted from the OECD procedure. Three hundred and forty-five neonates (n =165 and 180 for 4OHTam and endoxifen, respectively) were randomly pipetted and placed in 10 mL of test concentrations (5 individuals per concentrations, 3 replicates). The predicted concentrations exposure were 2.61, 4.03, 6.25, 15.41, 24.49, 39.25, 63.40, 103.25, 169.50 µg/L for 4OHTam, and 7, 11, 18.35, 28.62, 44.84, 79.53, 111.44, 176.81, 281.75, 450.88 µg/L for endoxifen. These concentrations correspond to nominal concentrations of 52, 78, 117, 263, 395, 593, 889, 1333, 2000 µg/L for 4OHTam and endoxifen, as shown in Table 3.5. Both experiment were run in parallel. They included a blank (i.e., no chemicals) and a solvent control (0.08 % for 4OHTam and endoxifen) that corresponded to the highest percentage of DMSO used in the tests. Same replicates as for chemicals were performed in controls. The individuals were not fed and they were place in a Coolstore® environmental chamber with 16: 8 h light: dark photoperiods at 21 ± 1°C. The study endpoints were death and immobilisation after 24 and 48 hours of exposure to chemicals.

Chronic pre-testing procedure

4OHTam and endoxifen chronic pre-tests were run in parallel over two generations of *D. pulex* (F0 and F1). The F0 generation was randomly selected from a single stock of mothers.

Eighteen neonates (< 24 h, > 3rd brood) were pipetted and isolated in separate glass beakers with 50 mL of medium (1 individual per concentration, no replicate). The predicted test concentrations were: 0.06, 0.21, 0.49, 1.47, 5.26, 17.86 µg/L for 4OHTam and 0.14, 1.36, 4.26, 15.46, 51.83, 156.76 µg/L for endoxifen. Theses predicted concentrations corresponded to the nominal concentrations of 1, 4, 10, 30, 100 and 300 µg/L for 4OHTam, and of 1, 10, 30, 100, 300 and 800 µg/L for endoxifen, as shown in Table 3.5. Two controls were performed in parallel: a blank (i.e., without chemical) and a solvent control (0.03 % of DMSO) that corresponded to the highest solvent percentage used in endoxifen test (1 individual per beaker, 3 replicates). On the twenty-first day of maternal exposure, thirteen neonates (< 24 h) were collected to form the generation F1. One neonate per beaker (no replicate for chemicals and 3 replicates for controls) was transferred to 50 mL of corresponding maternal concentration medium for thirteen additional days. The predicted concentrations at 5.26 and 17.86 µg/L of 4OHTam, and at 51.83 and 156.76 µg/L of endoxifen were not performed because effects in the first generation were already observed. Studied endpoints were the total neonates produced per individual and maternal death after 21 and 13 days of exposure for F0 and F1, respectively. The effects were daily recorded and the neonates produced were discarded except on the twenty-first day. Daphnids were fed daily with a suspension of Tetramin® and 0.2 mgC/daphnia of *Pseudokirchneriella subcapitata*. The test medium was renewed every two days and individuals were maintained in the Coolstore® environmental chamber with 16: 8 h light: dark photoperiods and at 21 ± 1°C.

In the acute test and the chronic pre-experiments, chemical parameters (i.e., pH, T°, water hardness, conductivity and oxygen) were measured at the beginning and end of the experiment. The results fell within standard values [73]. Potassium dichromate ($K_2Cr_2O_7$) assay were also performed to follow daphnia fitness. The results were consistent with our previous laboratory $K_2Cr_2O_7$ experiments, where the mean (±SD) was: 0.76 (± 0.27) µg/mL.

Table 3.5: Nominal and predicted concentrations of 4OHTam and endoxifen in the acute (2 days), the pre-chronic (21 and 13 days for F0 and F1, respectively) and the chronic toxicity tests (21 days for F0 and F1). Predictions were calculated using a polynomial regression of measured concentrations *vs* nominal concentrations. The highest DMSO percentage of each experiment is shown in the last rows

Test		Nominal [µg/L]	Predicted [µg/L] Confidence limite 95% (lower; upper)	Solvent [%]
4OHTam	Acute test	2000	169.50 (974.50 ; 199.45)	0.08
		1333	103.25 (593.31 ; 120.85)	
		889	63.40 (364.15 ; 73.80)	
		593	39.25 (225.31 ; 45.42)	
		395	24.49 (140.53 ; 28.18)	
		263	15.41 (88.36 ; 17.62)	
		117	6.25 (35.79 ; 7.07)	
		78	4.03 (23.06 ; 4.54)	
		52	2.61 (14.98 ; 2.94)	
	Chronic pre-test	300	17.86 (102.47 ; 20.47)	0.03
		100	5.26 (30.15 ; 5.94)	
		30	1.47 (8.45 ; 1.66)	
		10	0.49 (2.82 ; 0.57)	
		4	0.21 (1.19 ; 0.25)	
		1	0.06 (0.35 ; 0.08)	
	Chronic test	1000	98.91 (79.53 ; 123.01)	0.04
		300	23.78 (19.49 ; 29.02)	
		100	6.78 (5.63 ; 8.18)	
		30	1.80 (1.49 ; 2.19)	

Test		Nominal [µg/L]	Predicted [µg/L] Confidence limite 95% (lower; upper)	Solvent [%]
Endoxifen	Acute test	2000	450.88 (378.40 ; 537.25)	0.08
		1333	281.75 (237.22 ; 334.63)	
		889	176.81 (149.38 ; 209.28)	
		593	111.44 (94.50 ; 131.41)	
		395	70.53 (60.06 ; 82.84)	
		263	44.84 (38.34 ; 52.43)	
		176	28.62 (24.58 ; 33.33)	
		117	18.35 (15.83 ; 21.28)	
		78	11.00 (9.39 ; 13.88)	
		52	7.00 (6.01 ; 8.75)	
	Chronic pre-test	800	156.76 (132.57 ; 185.37)	0.03
		300	51.83 (44.26 ; 60.70)	
		100	15.46 (13.35 ; 17.90)	
		30	4.26 (3.70 ; 4.90)	
		10	1.36 (1.16 ; 1.58)	
		1	0.14 (0.10 ; 0.18)	
	Chronic test	1000	202.35 (170.78 ; 239.75)	0.04
		300	51.83 (44.26 ; 60.70)	
		30	4.26 (3.70 ; 4.90)	
		3	0.40 (0.33 ; 0.49)	

Acknowledgments

This project was funded by the Swiss National Science (http://p3.snf.ch/project-132344) and supported by the Faculty of Biology and Medicine Research Commission Fund, University of Lausanne. The authors would like to thank Sandra Cruchon for her meticulous and precise work at the Laboratory of Clinical Pharmacology (Lausanne), Florine Baillot for the parallel lab experiences (University of Lausanne), and the statisticians Marianne Gex-Fabry (University of Geneva) and Théophile Emmanouilidis (University of Lausanne) for their explanations and patience in statistics.

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Chapter 4

Are the toxic effects magnified over four generations of *D. pulex*?

In this chapter, the multigenerational effects of tamoxifen and 4OHTam on *D. pulex* are reported and discussed. These chemicals were chosen because they were the most toxic anticancer compounds among the pharmaceuticals tested in this thesis and because magnified effects over generations were suspected, even at low concentrations. Therefore, four generations of daphnids were exposed 14 days each to increasing concentrations of tamoxifen or 4OHTam. The aim of this study was to assess whether daphnids sensitivity towards these chemicals increased over generations. The studied end points on daphnids were the size, the reproduction, the viability and the intrinsic rate of natural increase (r). The design of the study was adapted from previous experiments that were performed on daphnids with tamoxifen and its metabolites (see chapter 2 and 3). In this chapter, additional prospective experiments are also presented about organisms' ability to recover when animals were withdrawn from chemical, and about daphnids exposed to tamoxifen and 4OHTam used in combination.

Multigenerational effects of the anticancer drug tamoxifen and its metabolite 4-hydroxy-tamoxifen on *Daphnia pulex*

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Will be submitted to *Environmental Science & Technology*

Abstract

Tamoxifen and its metabolite 4-hydroxy-tamoxifen (4OHTam) are two potent molecules that have anticancer properties on breast cancers. After consumption, patients excrete both chemicals in wastewaters and tamoxifen was measured in wastewaters and natural waters. The concentrations of 4OHTam in waters have never been reported. A single study reported 4OHTam effects on the microcrustacean *Daphnia pulex*. The effects of tamoxifen and 4OHTam over more than two generations are unknown in aquatic invertebrates. The main goal of this study was to assess the sensitivity of the microcrustacean *Daphnia pulex* over four generations, based on the size, the reproduction, the viability and the intrinsic rate of natural increase (r). Additional experiments were carried out to observe whether the effects of tamoxifen and 4OHTam were reversible in the next generation after descendants were withdrawn from chemical stress (i.e., recovery experiment), and whether the lowest test concentration of each chemical induced toxic effects when both concentrations were combined (i.e., mixture experiments). Our results showed that tamoxifen and 4OHTam induced adverse effects at environmentally relevant concentrations. Tamoxifen and 4OHTam impaired size, viability, reproduction and intrinsic rate of natural increase (r) in the four generations of treated daphnids but these effects were not magnified over generations. Tamoxifen was more potent than 4OHTam on *D. pulex*. When used in mixture, the combination of tamoxifen and 4OHTam induced effects in offspring while no effects were observed when these chemicals were tested individually. In the recovery experiment, the reproduction and the size were reduced in offspring withdrawn from chemicals. Our results suggest that tamoxifen and its metabolite may be a relevant pharmaceutical to consider in risk assessment.

Introduction

Pharmaceuticals are among thousand of other xenobiotics that invade the aquatic environment (for reviews see [1,2]). Over the last decades, thousands of tons of drugs have been produced yearly to treat human ailments [3,4], and human consumption seems to be the primary pathway for pharmaceuticals to reach the aquatic environment [5]. Once consumed, intact and/or metabolised forms of the drug are excreted via faeces and urine. Traditional sewage treatment processes remove organic matter [6], but some classes of synthetic pharmaceuticals are not efficiently eliminated. Although ozone and activated carbon treatments have been proposed to remove these chemicals more efficiently, many sewage treatment plants (STPs) continue to operate without these technologies [7]. Therefore, the excreted pharmaceuticals and their metabolites were identified in STP effluents and in natural waters [2,3,8–13].

Pharmaceuticals in the environment are of great concern because of their potentially harmful impact on ecosystem structure and functioning, including humans via water and fish consumption [14]. Anticancer compounds are an important family to consider for four main reasons among others. First, they may be toxic to living organisms, even at therapeutic concentrations. Indeed, traditional anticancer agents rarely target abnormal cells exclusively [15], and frequent side effects are observed in normal cell types, such as mutagenic, carcinogenic or teratogenic effects [16]. Second, the manufacture, consumption, and final release of anticancer pharmaceuticals may rise with time in response to an increase in cancer patients [17,18]. Third, these pharmaceuticals were measured in STP effluents and natural waters [3,9,16,19–23], proving that they reach aquatic environments. Finally, the distribution, the half-life and the long-term effects of anticancer drugs in the aquatic environment are sparsely known. As other pharmaceuticals, their continuous release in waters gives them the status of pseudo-persistent compounds [24]. This prolonged presence exposes fauna and flora to potentially long-term effects, which can adversely modulate eukaryotic organisms homeostasis [2,25].

Tamoxifen is a carcinogen and an endocrine disruptor that is used worldwide as a treatment or an adjuvant treatment of early and advanced breast cancer in males and females. In 1980, tamoxifen was approved as a hormonal agent mainly due to its similar efficiency in humans, but less toxic, as dietylstilboestrol [26,27]. Additionally, tamoxifen is a triphenylethylene drug that behaves as a selective estrogen receptor modulator with anti-estrogenic effects (inhibiting agent) and estrogenic-like effects (stimulating agent) on alpha and beta estrogen receptors (ERα, ERβ) depending on the target tissues. Its anticancer action in breast tumours may also be explained by its antagonist properties on the estrogen-related receptors (ERRs) [28] with an amino acid sequence that is over 60% identical to ERα and ERβ [29]. In addition to its own pharmacological potential, tamoxifen is considered a pseudo-prodrug [30] because active metabolites are formed by liver metabolism in vertebrates [31]. Its

metabolite, 4-hydroxy-tamoxifen (4OHTam), has a higher potency and affinity for ERs than tamoxifen and also a higher affinity for ERRα and ERRγ [32–34].

Tamoxifen and 4OHTam are mainly excreted in faeces. Tamoxifen also has a long half-life in humans [35]. For example, less than 10% of an oral tamoxifen dose of 90 mg was reported to be excreted after 10 days [36]. In patients chronically treated with tamoxifen, the concentrations in 24-h samples of faeces ranged from 230-1092 µg and 123-579 µg for tamoxifen and 4OHTam, respectively [37]. Tamoxifen can pass through STPs unchanged. It was measured in effluents [38,39,13] and natural waters, including groundwater [40,13,41], in concentrations ranging from 0.02 to 0.37 µg/L, and from 0.01 to 0.21 µg/L, respectively. No data exists for metabolites. Tamoxifen causes reproductive effects on fish [42,43], immobilises the microcrustacean *Daphnia magna* [44] and inhibits the naupliar development [45]. These adverse effects were observed in either acute or one generational experiments. Recently, a two generational study showed morphological abnormalities on *D. pulex* descendants that were exposed to environmentally tamoxifen concentrations (*Borgatta et al. [46], submitted*). Another two generational study reported a decrease in reproduction and offspring size when daphnids were treated with 4OHTam. Based on the calculated population growth (i.e., intrinsic rate of natural increase), this study also suggested that magnified effects were possible if experiments had been performed on additional generations (*Borgatta et al.[47] submitted*). To the best of our knowledge, the effects of tamoxifen or of 4OHTam on more than two generations of daphnids have not been studied. Also, several authors have risen concerns about the serious lack of chronic exposure studies of drug residues in aquatic organisms [16,25,48]. Brennan et al. [49] drew attention to the paucity of multigenerational testing and underlined the needs of multigenerational ecotoxicity experiments with estrogen-like compounds considering the shorter life cycle of animal in general with respect to human. While alarming effects were observed in humans, the case of diethylstilbestrol still remains an classic example [50], nothing is known on the possible effects on other organisms. In invertebrates, Brennan et al. [49] showed a significant decrease in daphnids fertility over consecutive generations after long-term exposure to diethylstilbestrol. The experiment performed by Kidd et al. [51] on fathead minnows was also an impressive example of the long-term effects of 17α-ethynylestradiol, a synthetic estrogen used in birth-control pills. By the second year of this study, a complete crash of fathead minnow populations and a near extinction of this species from the studied lake were observed.

The first aim of this study was to evaluate the sensitivity of the primary consumer *D. pulex* exposed to acute and chronic levels of tamoxifen and 4OHTam. The second goal of the study was to observe whether the effects were magnified over four generations exposed during 14-d to tamoxifen and 4OHTam, considering the size, the reproduction and the longevity of the daphnids, which were sensitive endpoints in our previous studies [46,47]. Fourteen days of exposure was chosen because daphnids produce about 4 to 5 clutches during this period in our laboratory conditions, which is

sufficient to highlight the reproductive tendency of treated animals. It is also assumed that daphnids rarely survive beyond the stage of the 4th adult instar under natural conditions [52]. In this study, prospective experiments were performed to observe whether the effects of tamoxifen and 4OHTam were reversible in offspring after removal of the chemical stress. Indeed, it is known that tolerance or adaptation to environmental stress exists in treated organisms, which involves physiologic acclimation or genetic modifications by natural selection, respectively [53,54]. Additionally, side effects can persist in animals that were withdrawn from chemicals, such as the anti-inflammatory drug ibuprofen [55]. Finally, we performed another prospective experiment to observe whether the lowest test concentration of each chemical, which singularly does not affect the daphnids, induces toxic effects when the animals were exposed to both chemicals because the aggregate action of tamoxifen and its metabolites is suspected to result in therapeutic beneficial effect of tamoxifen [34,56,57].

Materials & Methods

Chemicals

Tamoxifen and 4-OHTam were purchased from Sigma/Fluka (>99%, lot: 011M1682V and E8284 for tamoxifen and 4OHTam, respectively). A day before the experiments, the chemicals were dissolved in pure DMSO [58]. These stock solutions were prepared at the measured concentrations of 3.87E+06 µg/L and 1.34E+07 µg/L for 4-OHTam and tamoxifen, respectively. They were stored in glass flasks, in the dark at -80°C for no longer than the tests duration. The stock solutions were thawed at room temperature before each use.

Test organisms

In our laboratory, *D. pulex* from the *arenata* strain have been reared for three years in conditions that maintain asexual reproduction [47]. Individuals that were mass-cultured and individuals that were chronically exposed to chemicals were fed daily with both 0.2 mgC/daphnia of *Pseudokirchneriella subcapitata* algae and suspension of Tetramin®[59,60]. They were reared in glass flasks placed in a Coolstore® incubator sets to 21°C with a 16/8 hour light/dark cycle. After feeding, the location of these flasks was randomized and changed daily. Daphnids respective medium was renewed every two days in conditions that maintain parthenogenesis reproduction: total hardness 90 ± 5 mg/l as $CaCO_3$; pH 8 ± 0.2; conductivity adjusted to 25°C, 283 ± 13 µS/cm; dissolved oxygen > 5 mg/L. These chemical parameters were measured every four days. Potassium dichromate ($K_2Cr_2O_7$) assays were performed weakly and at the beginning and the end of each experiment. During the experiments, their EC50s (±SD) corresponded to the $K_2Cr_2O_7$ results in our laboratory and were 0.6 (± 0.23) µg/mL.

Acute toxicity experiment

Daphnids were exposed two days to tamoxifen and 4OHTam in acute experiments. For each chemical, these experiments were performed twice and the results were combined. Neonates (< 24 h, > 3rd brood) were randomly pipetted in glass tubes at the following concentrations: 2.78, 3.79, 5.26, 6.02, 7.31, 8.87, 10.46,12.60,14.44, 15.32, 18.37, 20.48, 22.34, 29.34, 42.40 µg/L of tamoxifen, and 18.89, 25.13, 26.28, 31.04, 36.73, 38.40, 47.59, 50.58, 59.06, 80.85, 93.18, 111.07, 126.44, 138.72 µg/L of 4OHTam (five individuals per vessel, 3 replicates). These exposure levels are predicted concentrations that were calculated from measured concentrations. Test solutions were regularly sampled and analysed, the measured concentrations were plotted against their respective nominal level using a polynomial regression. The equations of the fitted models, the number of samples and the nominal concentrations are presented in the supplementary information. Two controls, in four replicates, were performed in parallel: a blank (i.e., with no chemicals) and a solvent control that contained 0.004% and 0.063% DMSO for tamoxifen and 4OHTam, respectively. These percentages corresponded to the maximum solvent percentage that was used at the highest test concentration of each chemical. Note that DMSO levels were not analysed in the test solutions. The studied endpoint was the immobilisation of the living daphnids > 20 seconds, despite a gentle agitation of the tube, and they were observed after 24 and 48 h of exposure. The individuals were not fed during the experiment.

Multigenerational test procedure

Tamoxifen and 4OHTam toxicity tests were performed over four generations (F0, F1, F2 and F3). The concentrations used in these experiments were based on results from acute tests and from previous range-finding tests. The experimental design is shown in Figure 4.1. Neonates (< 24-h, > 3rd brood) were randomly pipetted from parthenogenetic mothers to form the first generation (F0, n = 111). Three individuals per beaker (3 replicates per concentration) were placed in 150 mL of test solutions. Based on previous experiments, the predicted test concentrations were: 0.16, 1.48, 17.86 and 72.99 µg/L of 4OHTam and 0.12, 0.67 and 5.26 µg/L of tamoxifen (see supplementary information for corresponding nominal concentrations). The solvent percentage in each test concentrations was 0.01% and 0.002% of DMSO for 4OHTam and tamoxifen, respectively. The same percentages of DMSO were used in solvent controls (DMSO level was not analysed), and a blank was performed in parallel. Solvent controls and blanks were performed in four replicates. On the fourteenth day, neonates aged < 24 h were collected to form the second generation (F1, n = 100). Whenever possible, three individuals per concentrations were placed in 150 mL of corresponding maternal concentration medium (3 replicates). Each fourteenth day of exposure, the same procedure was repeated to form the next generations F3 (n = 93) and then the F4 (n = 92).

Prospective recovery and mixture experiment procedures

Prospective recovery performances were observed on eighteen neonates aged < 24-h. Three neonates per beaker (2 replicates) were pipetted from the F1 that was exposed to 1.48 and 17.86 µg/L of 4OHTam, and to 0.67 µg/L of tamoxifen. These neonates were named as R1, R2 and R3, respectively (Figure 4.1) and they were placed in daphnia medium exempted from chemicals. The selected concentrations corresponded to toxic and non-toxic reproductive concentrations in the F0. No recovery follow-up was performed at 5.26 µg/L of tamoxifen because not enough neonates were produced at day 14. Mixture experiment was performed with twelve additional neonates. They were exposed to the lowest concentrations of each chemical, i.e., 0.12 and 0.16 µg/L of tamoxifen and 4OHTam, respectively. Three neonates (2 replicates) were pipetted from the F2 that was exposed to 0.12 µg/L of tamoxifen, and they were named M1. Three other neonates (2 replicates) were taken from the blank F2, and they were named M2. Individuals used in the recovery and the mixture toxicity experiments were reared during 14 days in similar manner as individuals that were reared in the multigenerational test.

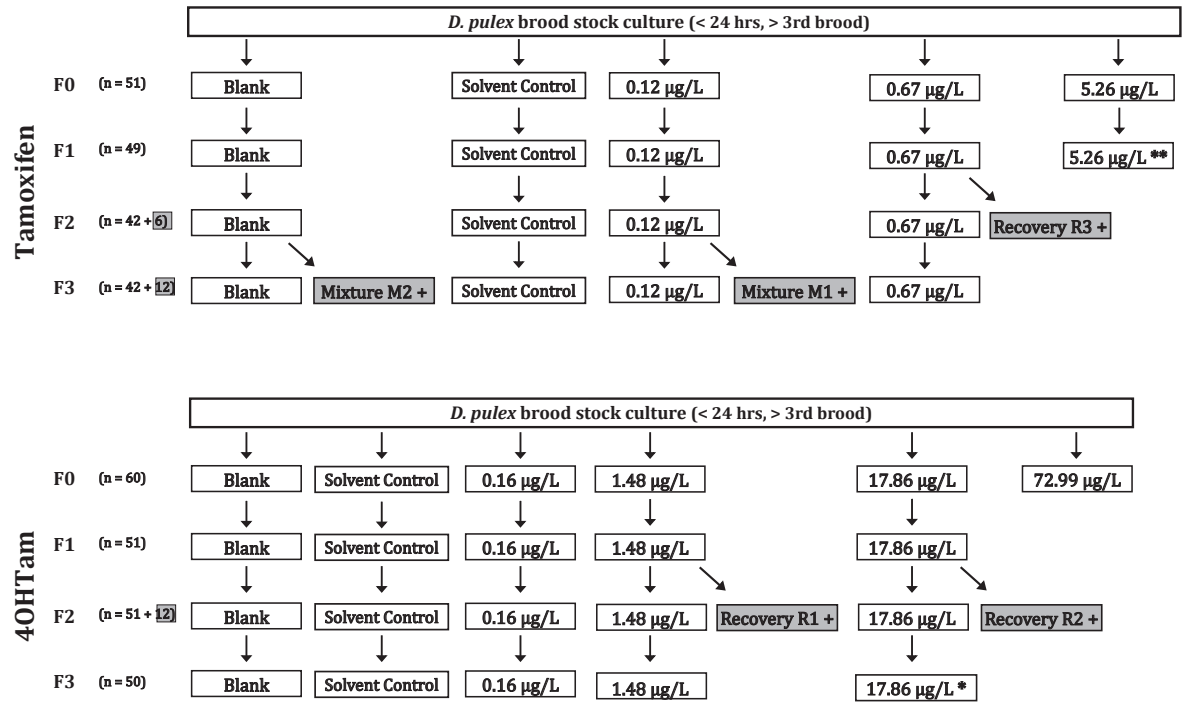

Figure 4.1: Experimental design for multigenerational tests. Four successive generations (from F0 to F3) of *D. pulex* were exposed 14 days each to tamoxifen or 4OHTam (n = 184 and 212 for tamoxifen and 4OHTam, respectively). In grey background, thirty additional individuals were placed in daphnia medium exempted from chemical (i.e., R1, R2 and R3) or in mixture medium (i.e., M1 and M2, n = 12) of 0.12 and 0.16 µg/L of tamoxifen and 4OHTam, respectively. "*" 3 organisms/beaker (3 replicates) except 1 replicate with 2 individuals, "" 3 organisms/beaker (3 replicates) except 1 replicate with 1 individuals, "+" 3 organisms/beaker (2 replicates), blank and solvent control = 3 organisms/beaker (4 replicates). The DMSO solvent percentages were 0.01 and 0.002 % for 4OHTam and tamoxifen, respectively**

D. pulex reproduction, mortality and size

In each 14 days experiment, the number of dead parents (i.e., mortality) and the number of neonates produced per beaker were recorded daily. The neonates were discarded, except on day 14 of each generation. On the fourteenth day of exposure, the size of parents was measured from the rostrum to the end of caudal spine, using an Olympus polarization microscope BX51 with digital image system (Colorview). The size differences between individuals, X %, were calculated as follow:

$$X = 1-(z / y)*100 \quad (1)$$

where z is the mean size of the control in µm, and y is the mean size of the treated organism in µm.

Intrinsic rate of natural increase (r)

The intrinsic rate of natural increase (r) was calculated using mortality and birthrate results in each beaker. The results were performed by iteration of the Euler-Lotka equation [61]:

$$\Sigma\, l_x m_x e^{-rx} = 1 \quad (2)$$

where l_x is the proportion of individuals surviving to age x, m_x is the number of neonates produced per surviving adult at age x, and x is time expressed in days.

Statistics

Acute testing results of each chemical were expressed as a sigmoidal dose-response curve, from which the acute EC50s and confidence intervals were calculated. The chemicals were tested at least two times (two independent assays). The results from the two respective assays were pooled and analysed using Prism5 (Graphpad Inc., CA, USA) to estimate the concentrations giving x% effect ECx by non-linear regression (log agonist vs. normalized response-variable slope). The dose-response curves were generated by the following nonlinear regressions:

$$Y= 100/(1+10^{((LogEC50-X)*HillSlope)}) \quad (3)$$

where x is the logarithm of concentration. Y is the response, and Y starts at 0 and goes to 100 with a sigmoid shape. The HillSlope results were 2.1 and 4.5 and for tamoxifen and 4OHTam, respectively.

EC50 values, corresponding to the 50% immobilization for *D. pulex*, were the test endpoints in acute tests, whereas no-observed effects concentrations (NOECs) were used in chronic tests. In the multigenerational experiment, the reproduction was calculated as the average of total neonates that was produced per adult over the exposure period. Reproduction and size results were first compared between blank and DMSO controls. Because no significant differences in daphnia size, reproduction and intrinsic rate were observed between these two controls, both were combined and used as unique control. Effects on daphnids exposed to mixture were compared to the combined control of 4OHTam

of the same generation. Comparisons were run using a one-way ANOVA test, which was followed by the Bonferroni post hoc test ($\alpha < 0.05$). Calculations were performed using the software GraphPad Prism (Stagraphics Centurion software, version 16.07 for Windows, Virginia USA, www.statgraphics.com).

Results

Acute toxicity experiment

The acute effects of tamoxifen and 4OHTam on *D. pulex* are shown in Figure 4.2. Tamoxifen was the most toxic compound followed by 4OHTam; the concentration that immobilized 50% of the individuals, i.e., EC50 (IC 95%), was: 9.49 (8.1 to 11.14) and 42.04 (38.29 to 46.18) µg/L for tamoxifen and 4OHTam, respectively.

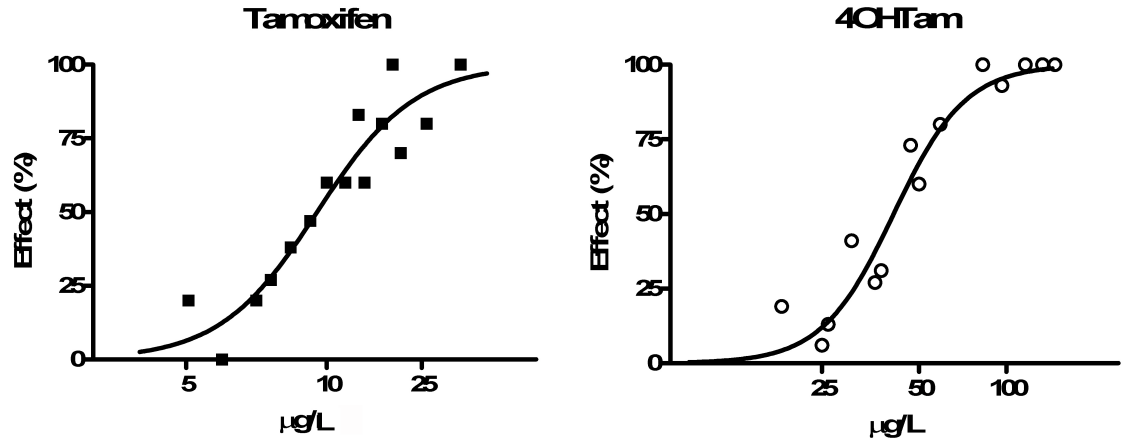

Figure 4.2: Acute toxicity effects on *D. pulex*. Immobilization was recorded after 2 days of exposure to tamoxifen or 4OHTam. For tamoxifen, the HillSlope is 2.1, r^2 is 0.89, EC50 is 9.49 µg/L, n is 115, while for 4OHTam the HillSlope is 4.52, r^2 is 0.94, EC50 is 42.04 µg/L, n is 250. The exposures are predicted concentrations from measured test solutions (µg/L)

Multigenerational testing

Daphnia reproduction, mortality and size

D. pulex were exposed 14 days to tamoxifen and 4OHTam, upon four generations (i.e., from F0 to F3). The toxic effects are summarized in Table 4.1 and Table 4.2. The reproduction and size of the F0 generation exposed to 5.26 µg/L of tamoxifen were significantly reduced ($p < 0.05$) compared to controls by about 93% and 31%, respectively. Note that size differences were already observed by visual assessment during the first week of F0 exposure since these one week-old individuals looked

like neonates < 24-h of the control. At day 14, the seven living parents that were exposed to 5.26 µg/L of tamoxifen produced a total of seven neonates to form the next generation F1. This result represents an average of one neonate per daphnids, while the average neonate per daphnia from the control was 6.5. Among these seven individuals, five died before maturity (i.e., before day 6.5 in our laboratory conditions) and their size was visually equal to control neonates. The sixth individual died on day 9, and it size was measured and compared to neonates (< 24-h) of controls (Figure 4.3). Its body-length was 844.42 µm, whilst mean neonate size of controls was 771.74 µm (± 32.18). At the end of the 14 day exposure, no neonates were produced by the F1 and only one adult survived with a size 36% smaller than controls. The size and reproduction of F1 and F2 in all other concentrations were not significantly different from control. The size of the F3 exposed to 0.12 and 0.67 µg/L of tamoxifen was statistically higher than control, by about 6 and 3%, and the reproduction was not different from controls. The no observed effect concentration (NOEC), which is the highest tested concentrations where the studied endpoint was not significantly different from the controls, was calculated for reproduction (NOECrep), size (NOECsize) and calculated growth population (NOECr). In each generation, these NOECs of tamoxifen were 0.67 µg/L, except in the F1, whose NOECr was 0.12, and in the F3 where the NOECsize and the NOECr could not be calculated because they were below the lowest test concentration. Therefore, these two NOECs were considered as < 0.12 µg/L.

In the 4OHTam experiment, 100% of the individuals died within the first 24 hours of exposure to 72.99 µg/L. The size and the reproduction of the four generations exposed to 17.86 µg/L were significantly different ($p < 0.001$) from control: the size of the F0, F1, F2 and F3 was reduced by 12%, 21%, 37% and 11%, respectively, and the reproduction of the F0, F1, F2 and F3 was reduced by 26%, 49%, 47% and 32%, respectively. The size of the F1 and the F2 that were exposed to 1.48 µg/L of 4OHTam was significantly reduced by 9% and 6%, respectively. Finally, the mean size of the F0 exposed to 0.16 µg/L was significantly higher than control, by 4%. This increase in size was considered as adverse effect. Therefore, the NOECsize was 0.16 µg/L in each generation, except in the F0, whose NOEC was below the lowest test concentration, i.e., < 0.16 µg/L. Regarding reproduction, the NOECrep was 1.48 µg/L in each generation.

Reproductive and developmental toxicities observed in daphnids treated with tamoxifen and 4OHam in this study were equivalent with side effects already described in a previous study [46]. Although morphological abnormalities were not studied in this multigenerational experiment, we randomly chose neonates to be examined under microscope to confirm our previous observation. We found premature neonates that were smaller than 24-h neonate from controls, and neonates with abnormal morphology, i.e., curved spine and large antennas; as an example, **Figure 4.4** shows one individual with such abnormalities.

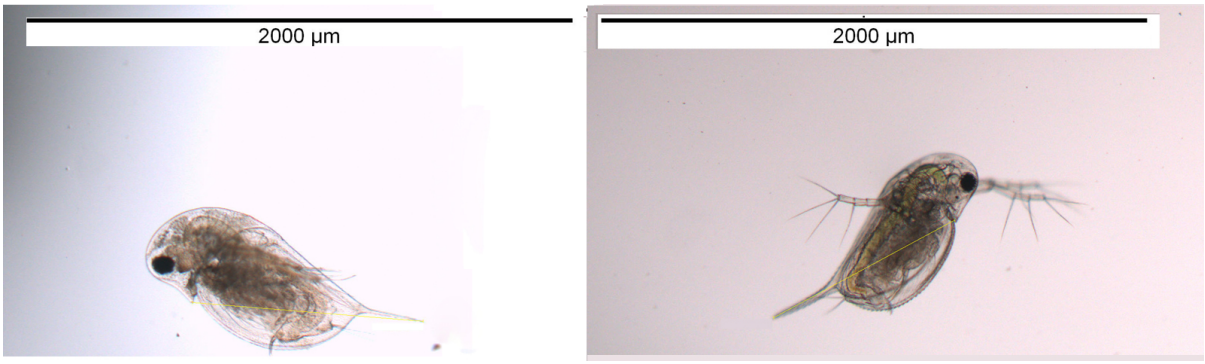

Figure 4.3: Size of *D. pulex* exposed nine days to 5.26 µg/L of tamoxifen. On the left, a 9 day-old daphnia exposed to tamoxifen. On the right, a neonate aged < 24h produced by DMSO control of the same generation.

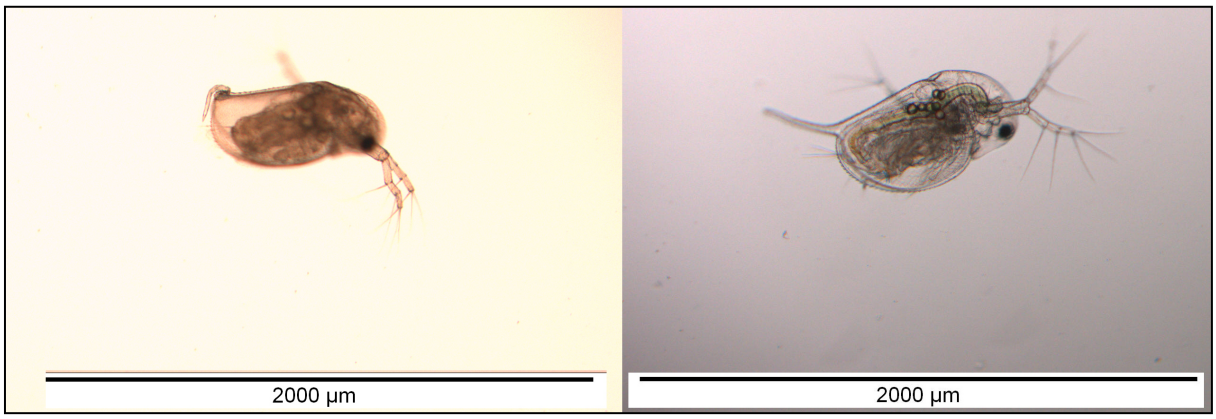

Figure 4.4: Abnormal *D. pulex* exposed to 0.16 µg/L of 4OHTam. On the left, neonate < 24-h with curved spine and thick antennas. On the right, neonate of the same aged from control.

Intrinsic rate of natural increase (r)

The intrinsic rate of natural increase (r) of F0 and F1 exposed to 5.26 and 0.67 µg/L of tamoxifen, respectively, was significantly reduced compared to control, while r was significantly increased in the F3 exposed to 0.12 and 0.67 µg/L ($p < 0.05$, Table 4.1 and Table 4.2). The calculated r was also significantly reduced in each generation exposed to 17.86 µg/L of 4OHTam. The estimated population growth was not different from controls in all other concentrations.

Recovery and mixture experiments

In the recovery experiment, the size of the R1 and the R2 were statistically smaller than control, by 5% and 7%, respectively ($p < 0.05$). The reproductive performance of R2 and R3 was significantly lower than control, by 30 and 15%, respectively. The intrinsic rate of natural increase r was also significantly reduced in R3 (Table 4.1 and Table 4.2).

The F1 neonates (< 24-h) that were born in blank (M2) or in solution of 0.12 µg/L of tamoxifen (M1), were placed in combined solution of 0.12 and 0.16 µg/L of tamoxifen and 4OHTam, respectively, during 14 days. The reproduction was significantly decreased in M1 and M2 by 20% and 21%, respectively. Pre-exposure to tamoxifen did not influence the toxicity of the mixture and these results were considered as similar in this study (Table 4.3).

Table 4.1: Multigenerational effects of tamoxifen on *D. pulex*. Four generations were exposed to tamoxifen during 14 days each. R3 are individuals that were removed at birth from solutions of 0.67 µg/L of tamoxifen and that were placed in daphnia medium exempted from chemical for 14 days. The size, reproduction and intrinsic rate of natural increase (r) are shown with mean ± standard deviation

	Treatment	Predicted (µg/L)	Mortality (%)	Longevity (days)	size (µm)	Reproduction (mean)	Intrinsic rate	NOEC size (µg/L)	NOEC reproduction (µg/L)	NOEC intrinsic rate
F0 generation	Control	-	7	14 ± 0.4	2263.64 ± 67.67	41 ± 3.4	0.38 ± 0.02	0.67	0.67	0.67
	Tamoxifen	0.12	0	14 ± 0	2289.01 ± 72.61	44 ± 6.9	0.39 ± 0.02			
		0.67	0	14 ± 0	2282.46 ± 27.53	45 ± 3.8	0.39 ± 0.01			
		5.26	22	12 ± 4.0	1559.73 ± 119.90 **	3 ± 2.6 **	0.06 ± 0.06 **			
F1 generation	Control	-	5	14 ± 0.2	2282.29 ± 141.22	43 ± 2.6	0.37 ± 0.01	0.67	0.67	0.12
	Tamoxifen	0.12	0	14 ± 0	2322.80 ± 81.65	44 ± 8.1	0.37 ± 0.02			
		0.67	22	12 ± 3.6	2346.58 ± 74.14	43 ± 1.5	0.33 ± 0.01 **			
		5.26	89	7 ± 3.5	1462.15 ♯	0 ♯	0 ♯			
F2 generation	Control	-	0	14 ± 0	2341.69 ± 147.68	53 ± 3.6	0.41 ± 0.01	0.67	0.67	0.67
	Tamoxifen	0.12	0	14 ± 0	2405.95 ± 97.09	51 ± 3.2	0.42 ± 0.01			
		0.67	0	14 ± 0	2347.86 ± 71.98	51 ± 3.0	0.40 ± 0.01			
		5.26								
		R3	0	14 ± 0	2350.52 ± 60.14	45 ± 3.5 *	0.36 ± 0.01 *			
F3 generation	Control	-	0	14 ± 0	2322.02 ± 97.13	57 ± 5.2	0.41 ± 0.01	< 0.12	0.67	< 0.12
	Tamoxifen	0.12	0	14 ± 0	2478.35 ± 54.40 **	52 ± 1.1	0.44 ± 0.01 **			
		0.67	11	14 ± 1.0	2395 ± 102.08 *	57 ± 9.8	0.44 ± 0.01 **			
		5.26								

* $p < 0.05$ (Bonferroni correct ♯ 1 individual at the end of the test

** $p < 0.001$ (Bonferroni correction)

Table 4.2: Multigenerational effects of 4OHTam on *D. pulex*. Four generations were exposed during 14 days each to chemical. R1 and R2 are individuals that were removed at birth from solutions of 1.48 and 17.86 µg/L of 4OHTam, and that were placed in daphnia medium exempted from chemical for 14 days. The size, reproduction and intrinsic rate of natural increase (r) are shown with mean ± standard deviation

	Treatment	Predicted (µg/L)	Mortality (%)	Longevity (days)	size (µm)	Reproduction (mean)	Intrinsic rate	NOEC size (µg/L)	NOEC reproduction (µg/L)	NOEC intrinsic rate
F0 generation	Control	-	7	14 ± 0.4	2283.64 ± 49.55	48 ± 4.8	0.4 ± 0.02			
	4OHTam	0.16	11	13 ± 2.0	2379.10 ± 10.63 **	49 ± 3.9	0.38 ± 0.04			
		1.48	0	14 ± 0	2293.44 ± 21.02	53 ± 0.8	0.41 ± 0	< 0.16	1.48	1.48
		17.86	11	13 ± 2.3	2020.77 ± 77.10 **	35 ± 8.4 **	0.37 ± 0.05 **			
		72.99	100	1 ± 0	-	-	0 **			
F1 generation	Control	-	5	14 ± 0.2	2325.16 ± 71.08	43 ± 4.8	0.35 ± 0.01			
	4OHTam	0.16	0	14 ± 0	2246.64 ± 139.11	40 ± 9.6	0.35 ± 0.03			
		1.48	11	13 ± 1.7	2108.25 ± 140.19 **	36 ± 2.2	0.33 ± 0.01	0.16	1.48	1.48
		17.86	44	10 ± 4.5	1827.67 ± 120.78 **	22 ± 3.6 **	0.21 ± 0.07 **			
		72.99								
F2 generation	Control	-	0		2390.93 ± 80.4	49 ± 3.7	0.39 ± 0.01			
	4OHTam	0.16	0	14 ± 0	2320.73 ± 117.11	43 ± 2.2	0.38 ± 0.01			
		1.48	0	14 ± 0	2237.51 ± 55.96 *	52 ± 1.7	0.40 ± 0.01			
		17.86	33	11 ± 4.5	1519.23 ± 320.55 **	26 ± 12.5 **	0.27 ± 0.03 **	0.16	1.48	1.48
		72.99								
		R1	0	14 ± 0	2273.92 ± 103.42 *	43 ± 0.2	0.36 ± 0.01			
		R2	0	14 ± 0	2229.44 ± 138.03 **	34 ± 3.5 **	0.36 ± 0.02			
F3 generation	Control	-	0	14 ± 0	2405.84 ± 69.28	59 ± 3.6	0.42 ± 0.01			
	4OHTam	0.16	0	14 ± 0	2479.55 ± 103.17	61 ± 8.0	0.42 ± 0.03			
		1.48	0	14 ± 0	2396.77 ± 152.65	58 ± 14.7	0.41 ± 0.02	1.48	1.48	1.48
		17.86	0	14 ± 0	2154.45 ± 133.62 **	40 ± 9.4 *	0.37 ± 0.04 **			
		72.99								

* p < 0.05 (Bonferroni correction)
** p < 0.001 (Bonferroni correction)
R1: individuals from the F1 parents exposed to 1.48 µg/L
R2: individuals from the F1 parents exposed to 17.86 µg/L

Table 4.3: Mixture effects of tamoxifen and 4OHTam on *D. pulex*. Neonates of the F2 exposed to blank (M1), or neonates of the F2 exposed to 0.12 µg/L of tamoxifen were reared during 14 days in mixed solution of 0.12 µg/L and 0.16 µg/L of tamoxifen and 4OHTam, respectively. The size, reproduction and intrinsic rate of natural increase (r) are shown with mean ± standard deviation

	Treatment	Predicted (µg/L)	Mortality (%)	Longevity (days)	size (µm)	Reproduction (mean)	Intrinsic rate
	Control	0	0	14 ± 0	2405.84 ± 69.28	59 ± 3.6	0.42 ± 0.01
M1	Tamoxifen + 4OHTam	0.12 + 0.16	0	14 ± 0	2435.54 ± 90.36	47 ± 3.5 **	0.43 ± 0.01
M2	Tamoxifen + 4OHTam	0.12 + 0.16	0	14 ± 0	2400.53 ± 60.57	46 ± 2.4 **	0.41 ± 0.01

** p < 0.001 (Bonferroni correction)
M1: individuals from the F2 exposed to 0.12 µg/L of tamoxifen
M2 : individuals from the F2 blank

Discussion and conclusion

In the acute and multigenerational experiments, *D. pulex* was sensitive to tamoxifen and its metabolite 4OHTam. These chemicals were able to disrupt physiological processes of treated animals, such as the size, the viability or the reproduction, at concentrations of the same magnitude of order as the tamoxifen concentrations measured in natural waters, i.e., up to 0.21 µg/L [40,13,41]. Indeed, the NOECs of tamoxifen were between 0.12 µg/L and 0.67 µg/L, while the NOECsize and the NOECr were below 0.12 µg/L in the fourth generation. The NOECrep and the NOECr of 4OHTam were similar over the fourth generations, but the NOECsize increased with generations until 1.48 µg/L in F3. In our previous studies [46,47], the NOECrep of tamoxifen and 4OHTam were 0.72 and 1.48 µg/L, respectively, which are similar values than those obtained here, but the NOECrep and NOECr of the second generation exposed to 4OHTam suggested magnified effects (i.e, NOECs < 1.8 µg/L), which is not confirmed in this study. Further investigations would be needed to therefore confirm or infirm this observation.

In both acute and multigenerational experiments, tamoxifen displayed a higher toxicity than 4OHTam, particularly when comparing mortality and reproductive success. For examples, the second generation (F1) did not survive at 5.26 µg/L of tamoxifen, while the same generation was still living at 17.86 µg/L of 4OHTam, which is about 3-times the tamoxifen concentration. Furthermore, the lethal concentration was about 14-time higher than tamoxifen lethal concentration (i.e., 5.26 µg/L). In vertebrates conversely, tamoxifen is less potent than 4OHTam [56,62]. In addition, tamoxifen therapeutic concentrations in human are higher than the effect concentrations in daphnids. Although the internal exposure levels of tamoxifen and its metabolite are unknown in daphnids, the external concentrations (i.e., the test concentrations) were compared to the effective concentrations in treated patients, which are also considered as safe concentrations. In humans, tamoxifen plasma concentration ranges between 140 and 160 µg/L at steady-state, after chronic administration of 20 and 30 mg of tamoxifen citrate daily [37,63], which is higher than the toxic tamoxifen concentrations in daphnids. After same daily doses of tamoxifen, the plasma concentrations of 4OHTam are lower than tamoxifen, with values between 2.4 to 3.7 µg/L [37,64]. Our results show that daphnids reduced their size when exposed to 1.48 µg/L of 4OHTam already. Therefore, daphnia sensitivity to both chemicals seems to be higher than human, and this is not the case with all pharmaceuticals. For instance, in a 21 days study that was performed with daphnids exposed to diclofenac, a nonsteroidal anti-inflammatory drug, the NOEC was 25,000 µg/L, while the maximal plasma concentration in human was 588 µg/L of dicolfenac after an oral dose 25 mg [65]. Similarly, the reproduction decreased in *D. magna* exposed to 110 µg/L (nominal concentration) of the β-blocker propranolol, while the effective plasma concentrations of the drug is considered as 40 µg/L, with a maximum effect at 100 µg/L after chronic treatments [66,67].

In the recovery experiment, daphnids that were placed in medium exempted from chemical were not able to recuperate totally. For instance, the reproduction decreased in R1 and R2, and R2 size was also reduced. The same effects were observed in their treated parents (F1) and sisters (F2) exposed to 4OHTam. In R3, the decrease in reproduction and population growth was not fully understood because no effect was observed in their treated parents exposed to tamoxifen. For R1 and R2, we hypothesize that repair processes failed, despite the absence of chemicals in medium. Such repair processes occur at different levels of the biological organizations, such as the molecular, cellular and tissue levels. Molecular damages involve protein, lipids or/and DNA damages that can be reversed by a return to normal state or direct/indirect repairs, while cellular recovery is usually possible by cell replacement. Tissue damages are repaired by cells elimination and regeneration, and also by extracellular matrix production, such as scar tissue, or by apoptosis [68,69]. Epigenetic regulations, which causes changes in phenotype or gene expression without changes in DNA sequence [70,71] may also be responsible of the observed effects. Indeed, epigenetic regulations were discovered in cells exposed to tamoxifen [72–74] and recently, Vandegehuchte et al. [75] shown that epigenetic dysregulations occurred in daphnids that were exposed to various compounds. However, based on our results it is only possible to say that 4OHTam induced persistent adverse effects in daphnids withdrawn from chemical solution, without evident explanations on repair processes failure or epigenetic inheritance. Further investigations may also be required to clarify whether the adverse effects were induced when daphnids grew in maternal brood chamber or whether changes were inherited directly from treated mothers. In toxicology, embryogenesis is known to be a critical window during which chemical can induce deleterious effects in embryos [76,77]. For instance, *ex vivo* assays could be performed in which eggs are removed from maternal brood chamber, individually deposited in separate wells and exposed at different time to tamoxifen or 4OHTam. Such experiments would determine the developmental stage at which embryos are the most susceptible to chemicals. Besides, our results show that these chemicals induced effect in developing organisms because morphological abnormalities in offspring were observed with tamoxifen and 4OHTam treatments, and also because reproduction was reduced in individuals that were either pre-exposed or not to chemical during the mixture experiment.

In the mixture experiment indeed, the reproduction was impaired in daphnids when they were exposed to the lowest test concentration of both chemicals, while daphnids exposed to each substance separately at the same concentration were not affected. Moreover, the sum of both concentrations tested in mixture was 7.36E-10 g/mol (i.e., 3.23E-10 g/mol + 4.13E-10 g/mol for 0.12 µg/L of tamoxifen and 0.16 µg/L of 4OHTam, respectively), which is below the NOECs that were calculated for reproduction of each generation exposed either to 4OHTam or tamoxifen. A synergic effect of tamoxifen and 4OHTam in daphnids [78] may be suspected, but the combined potential of these chemicals needs to be verify in complete mixture experiments (i.e., with complete individual dose

response curves) to confirm whether the mixture would not followed the concept of concentration addition. The latters are recommended without the presence of solvent, because this chemical may also have contributed to the reproductive effect found in our experiment. Our results however are in accordance with authors who claimed that tamoxifen clinical effect is the result from the aggregate effect of tamoxifen and its metabolite [34,56,57].

Regarding the calculated population growth, the intrinsic rate of natural increase (r) mainly decreased when reproduction was low, which is in accordance to Meyer et al. [79] who stated that the dynamic of r is primary influenced by the effects on reproduction and mortality during the few first broods. In the 4OHTam experiment, the smallest daphnids produced the least neonates, but they were able to survive until the next generation was formed. Low concentrations of tamoxifen and 4OHTam were able to increase daphnia size, and to our knowledge, increased size in daphnids was observed in response to stressors such as predator [80], but not in response to chemical exposure. This increase was considered as an adverse effect because it would not have been induced in absence of chemical. Although not significant, the body-sizes of the F0, F1 and F2 that were exposed to each tamoxifen concentration were higher than controls, except F3 that displayed a statistical significance. Actually, these little differences may have been magnified through generations until significant differences were observed in F3. This hypothesis would be in line with the statements that mother sizes are positively correlated to the size of neonates [52,81,82], and that females born larger mature to larger sizes than those born smaller [83]. Another hypothesis that may explain why size increased in daphnids exposed to low concentrations could be related to endocrine system, which may be modulated differently depending on exposure levels. For instance, it is known that the dose-related pattern of tamoxifen in certain biomarkers is different [84]. In liver cancer cells indeed, high concentrations of tamoxifen are agonistic, while low concentrations are predominantly antagonistic to the sex hormone-binding globulin (SHBG) gene expression [85]. In daphnids, the mechanisms of action of tamoxifen and its metabolites are unknown, but in vertebrates, tamoxifen and 4OHTam interact with ERs and ERRs. Although daphnids lack of ER, we hypothesized that these chemical may act in their ERR because a copy of the gene ERR (dappu-ERR, NR3 subfamily) was discovered in *D. pulex* [86]. This gene is close structurally to their human homologs ERRs and ERs [86]. Therefore, the sensitivity of *D. pulex* may be explained by the presence of this receptor.

In summary, our results showed that tamoxifen and 4OHTam induced adverse effects at environmental relevant concentrations and that daphnids were not able to recover after have been removed from chemical exposure. The effects on size and reproduction were not magnified over generations but morphological abnormalities were observed on offspring and the mixture of these chemicals may have a synergic action on daphnids. These results and the effects of tamoxifen in daphnia neonates or in other aquatic species, such as masculinization on fish [87,88] and developmental effects in sea urchin embryos [46,89], suggest that tamoxifen may be a relevant

pharmaceutical to consider in risk assessment. More general, anticancer drugs are overall an important family of chemicals to consider in ecotoxicology because of the expected growth in demand for these compounds, with consequent increase of their presence in the environment, and because of the release of new molecules on the market. Indeed, the global cancer rate is estimated to increase by 50% by 2020 [17] and the number of new cancer cases is expected to grow to 23.6 million each year by 2030. Such growth represent about 68% more cases than in 2012 [18]. As a consequence, new oncological molecules will be released, but probably without too much consideration on their end-life in the environment. Indeed, pre-clinical studies assess the beneficial impact of pharmaceuticals on a target population [90], but they are mainly biomedically oriented. They are performed on human cells and vertebrates organisms, primary on short-term and at high concentrations [91], while their chronic and multigenerational effects on non target species, such as the fauna and flora, are still set aside. We therefore hope that this study will help to develop awareness of the scientific community to this increasing threat for the environment.

Supporting information

Predicted concentrations

During the assay, the test concentrations were regularly sampled and analyzed, following the procedure proposed in (see chapter 2). A total of 318 and 259 were analyzed for 4OHTam and tamoxifen, respectively Table 4.4. The data were used to fit a non-linear regression that explains the relation between the measured and nominal concentrations of the respective molecule. The equations of the fitted models are:

$$\text{Ln(pC) of tamoxifen} = -3.60211 + 1.07666*\text{Ln(nC)} + 0.0333645*\text{Ln(pC)}^2 \quad \text{(S1)}$$

$$\text{Ln(pC) of 4OHTam} = -2.61748 + 1.19858*\text{Ln(nC)} + 0.0244905*\text{Ln(nC)}^2 \quad \text{(S2)}$$

where pC = predicted concentration, nC = nominal concentration, and the unit = µg/L. The $r^2 = 0.94$, and 0.95 for tamoxifen and 4OHTam, respectively (the assumptions for regression analysis were checked using residual plots). In this study, the exposure concentrations of each chemical were predicted concentrations that were calculated from the equation (S1) or (S2).

Table 4.4: Tamoxifen and 4OHTam predicted concentrations. Predictions were calculated using a polynomial regression of measured concentrations *vs* nominal concentrations (n = 318 and 259 for 4OHTam and tamoxifen, respectively). The measured concentrations are expressed as the mean of triplicate samples

	4OHTam				Tamoxifen			
	Nominal (µg/L)	Measured mean (µg/L)	SD	Predicted (µg/L)	Nominal (µg/L)	Measured mean (µg/L)	SD	Predicted (µg/L)
Chronic	3	0.16	0.06	0.16	2	0.11	0.04	0.12
	30	1.45	0.59	1.48	20	0.61	0.40	0.67
	300	11.49	6.15	17.86	200	7.78	3.36	5.25
	1000	51.37	26.28	72.99				
Acute	315			18.89	220			5.77
	404	11.55	6.46	25.13	230	1.00	0.28	6.02
	420			26.28	264			6.90
	485			31.04	280	1.05	0.21	7.31
	560			36.73	317			8.27
	582	15.53	1.71	38.40	340	2.55	2.62	8.87
	698			47.59	381			9.95
	735			50.58	400	0.90	0.14	10.46
	838	36.56	6.68	59.06	457			11.98
	945			68.21	480	1.55	0.92	12.60
	1089			80.85	548			14.44
	1225			93.18	580	2.55	0.92	15.32
	1416	75.82	26.71	111.07	658			17.48
	1575			126.44	690	3.85	2.19	18.37
	1699			138.72	830	3.20	1.27	22.34
					947			25.71
					1000	3.50	1.84	27.27
	n = 318				n = 259			

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Chapter 5

Is *D. pulex* sensitive to imatinib?

In this chapter, the anticancer drug imatinib was tested on the aquatic invertebrate species *D. pulex*. Imatinib has revolutionized the treatment and the survival of patients suffering from chronic myeloid leukaemia or gastrointestinal stromal tumours because it has a very specific mode of action in these tumour cells. The aim of this study was to observe the sensitivity of two generations of *D. pulex* towards imatinib. The range of test concentration was chosen using a preliminary chronic experiment on a single generation exposed 21 days to this chemical. The viability, the size, the reproduction and the intrinsic rate of natural increase were not impaired in *D. pulex* at the concentrations that were chosen in this experiment, except at the highest concentrations where the reproductive performance was decreased in the first treated generation.

Ecotoxicological experiment of imatinib on two generations of *D. pulex*

Introduction

Among the pharmaceuticals that are continuously released in the aquatic system, anticancer drugs were measured in sewage treatment plant (STP) effluents and in natural waters [1–4]. Consumers are considered as the primary source of pharmaceutical release in the aquatic system by sewage treatment plant (STP) effluents [5]. After consumption, pharmaceuticals are metabolised and excreted *via* urine and faeces. A general hypothesis may therefore be made such as: the more people consume pharmaceuticals, the more pharmaceuticals residues would be released in sewage waters by excreta. Because the incidence of cancers is increasing worldwide, the consumption of anticancer drugs may follow this trend. Indeed, the number of new cancer cases is expected to grow to 23.6 million each year by 2030, which would be 68% more cases than in 2012 [6]. Besides, oncology represents one of the most active fields of pharmaceutical innovation and progress [7]. The use of signal transduction inhibitors provides a typical example that illustrates the recent evolution of cancer treatments [8,9]. For instance, imatinib (Gleevec®) and the next generations of tyrosine kinase inhibitors (TKIs) are recent family of anticancer drugs that targets specific mechanisms of tumour cell biology. Imatinib was the first TKI that reached the market and this molecule has revolutionized the treatment and the survival of patients suffering from chronic myeloid leukaemia or gastrointestinal stromal tumour [10,11]. Indeed, TKIs prolong life of patients with certain types of cancers compared to other traditional treatment [12–15]. These anticancer drugs are able to disrupt specific signalling pathways that maintain cellular proliferation and this specificity is considered as higher than in the traditional anticancer therapies [16].

As other molecules, TKIs are eliminated in urines and/or faeces [17]. For instance, 68% and 13% of the imatinib dose is eliminated in faeces and in urine, respectively, within seven days [18]. About 25% of the recovered dose is unchanged imatinib and 20% is the active metabolite CGP 74588. The remaining portion is oxidised derivatives of both compounds. Considering that this treatment has a lifesaving efficacy, provided that it is taken indefinitely, its consumption should increase and thus its arrival in sewage treatment plants (STPs). Traditional and new sewage treatment processes remove efficiently organic matter [19] but not some class of chemicals such as synthetic pharmaceuticals [3,20–22]. To the best of authors' knowledge, imatinib has never been monitored in sewage and natural waters. Therefore, this molecule is considered here as a possible unremoved pharmaceutical that may reach continuously the natural water with STP effluents. The aquatic flora and fauna may be chronically exposed to the tyrosine kinase action of imatinib because a variety of related tyrosine kinases are present in eukaryotic cells [23], such as in cells of aquatic or terrestrial invertebrates [24–26]. The tyrosine protein kinases whose signal transduction pathways have been preserved throughout

the evolution are found in a variety of living organisms from yeast to mammal, including aquatic invertebrate such as mussels [27,28]. Seiler [28] specified that "although in mammalian cells there is a redundancy in signal transduction pathways that reduces the cytotoxic effects of imatinib on normal cells, thus resulting in it is relative specificity of action towards targeted neoplastic cells, this redundancy might not be as developed in non-mammalian organisms, making them potentially more vulnerable against the inhibitory actions of drugs like Gleevec®". This author also underlined that new antineoplastic drugs such as imatinib and others from the same family, which targets and inhibits the abl-kinase constitutively expressed through the recombinant form of BCRabl in chronic myelocytic leukaemia for example, could influence cellular events on orthologs of the c-abl (or c-kit) mediated signal transduction pathway in non-target organisms. In the water fleas *Daphnia pulex* for instance, insulin-like peptides and tyrosine kinase receptor family, such as insulin receptor and complex neurotrophin signalling system that includes three paralogous Trks (a neurotrophin receptor) were recently found [23,25,26,29], making it potential sensitive to the tyrosine kinase inhibitors imatinib.

Daphnids have short parthenogenic life-cycle and they are easy to maintain under laboratory conditions. This microcrustacean family is widely used in ecotoxicology experiments, and their acute and chronic sensitivity to toxicants were correlated to other species [30,31]. The aim of the study was to assess the sensitivity of *D. pulex* towards imatinib and to observe whether the effects were magnified in the descendants. The size, the reproduction and the longevity of treated animals were followed in a two-generational experiment in which each generation was exposed 14 days to imatinib.

Materials and Methods

Chemicals

Imatinib Mesylate was purchased from LC Laboratoires (>99%, lot: 10127223). The day before the experiment, the chemical was dissolved in ultrapure water (LaboStar™ 7-TWF-DI and UV systems). This stock solution was prepared at the mean measured concentrations of 790 ± 204 mg/L (mean ± standard deviation). It was stored in glass bottle, in the dark at -80°C, and thawed at room temperature before each use. Each 14-d, the stock solution was sampled and the concentration was analysed by liquid chromatography that was coupled to a triple stage tandem mass spectrometry system (LC-MS/MS). The limit of quantification (LOQ) of the LC-MS/MS assay for imatinib was 1 µg/L.

Test organisms

D. pulex from the arenata strain have been mass-cultured for three years in our laboratory (Borgatta et al., submitted). They are reared in conditions that maintain parthenogenetic reproduction (total hardness 85 ± 5 mg/l as CaCO3; pH 8 ± 0.2; conductivity adjusted to 25°C, 280 ± 14 µS/cm; dissolved oxygen > 5 mg/L). Stock daphnids as well as individuals exposed to chemicals were reared in glass beakers placed in a Coolstore® environmental chamber (16: 8 h light: dark photoperiods at 21 ± 1°C). They were fed daily with 0.2 mg C/daphnia of *Pseudokirchneriella subcapitata* algae and a suspension of Tetramin® tropical fish food (Gouvernement du Canada and Environnement Canada, 1996; OECD Guidelines for the Testing of Chemicals, 2008).

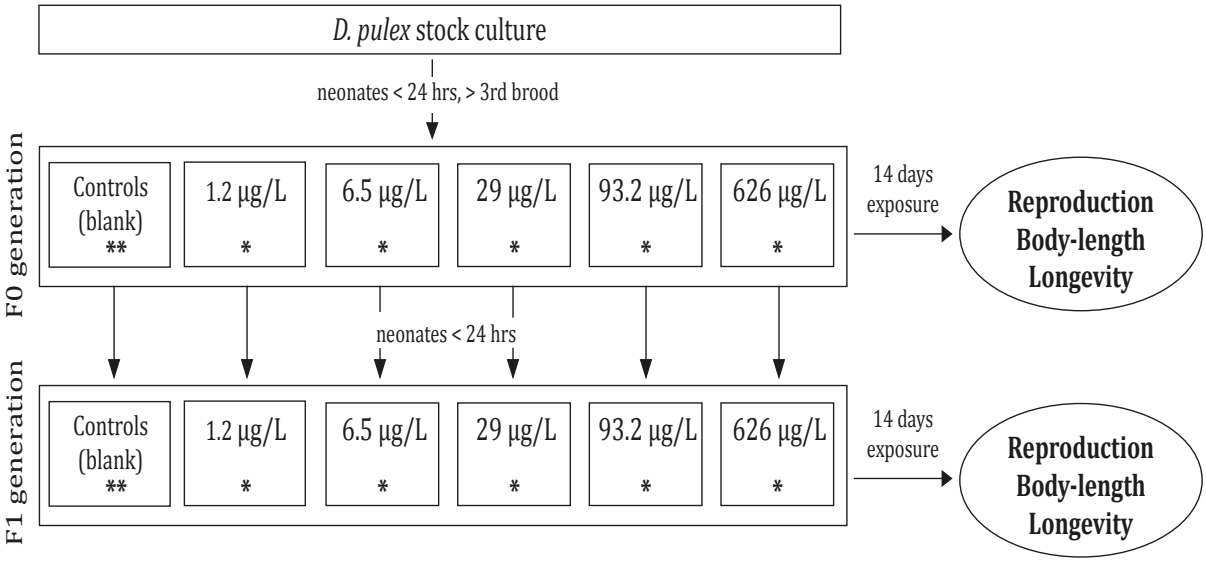

Figure 5.1: Imatinib experimental design for the two-generation toxicity test on *D. pulex*. On day 14, neonates were kept to form the second generation (F1). They were exposed to corresponding maternal concentration medium (measured concentrations). * 3 organism/beaker (3 replicates per concentration), ** 3 organisms/beaker (4 replicates per concentration)

Two-generation testing procedure

Imatinib long-term assay was run over two generations of *D. pulex* (F0 and F1). This two-generational study was adapted from previous multigenerational experiments (see chapter 4), and the framework of the experimental design is illustrated in Figure 5.1. Briefly, the parental generation (F0) was randomly selected from a single stock of mothers. Fifty-seven neonates (< 24 h, > 3rd brood) were pipetted and placed in separate glass beakers with 150 mL of medium. Each beaker contained three daphnids in triplicate. The mean measured exposure levels were: 1.2, 6.5, 29, 93.2, 626 µg/L. On the fifteenth day of maternal exposure, fifty-seven neonates (< 24-h) were collected to form the second generation,

named F1. Three neonates per replicate were transferred to 150 mL of corresponding maternal concentration medium (3 replicates). A water medium control (blank) was included in four replicates and daphnids from the controls were reared in the same environmental conditions as the test organisms. The exposure medium was renewed every two day. Imatinib test solutions were sampled and measured every two medium renewal (i.e., at renewal and just before, after 2-d of exposure in the environmental chamber, without daphnia). The nominal and measured concentrations are summarised in Table 5.1.

Table 5.1: Nominal and measured concentrations of imatinib. LOQ is 1 µg/L

Nominal (µg/L)	Mean measured (µg/L)	SD
850000	790000	203647
3	1	0
16	6	2
80	29	16
400	93	71
2000	627	135

At the beginning of the test, and each week until the end of the experiment, a potassium dichromate ($K_2Cr_2O_7$) assay was performed to assess daphnia sensitivity. The $K_2Cr_2O_7$ results fell within the values of our laboratory, i.e., 0.6 µg/mL ± 0.23 (mean EC50s ± standard deviation). Chemical parameters in test medium were measured every two days, at medium renewed, and the conditions allowed parthenogenetic reproduction during the whole experiment.

Statistics

Daphnia mortality, reproduction and size

The mortality (i.e., number of dead parents) and the number of offspring per replicate were recorded daily. The neonates were then discarded, except on day 14. On the fourteenth day of exposure, the size of parents was measured from the rostrum to the end of caudal spine, using an Olympus polarization microscope BX51 with digital image system (Colorview). Then, the mothers were discarded. The size differences X between individuals were calculated as follow:

$$X = (y - z / y)*100 \quad (1)$$

where y is the the mean size of the control in µm, and z is the mean size of the treated organism in µm. Unit = %

Intrinsic rate of natural increase (r)

The intrinsic rate of natural increase r was calculated using mortality and birthrate data and by iteration of the Euler-Lotka equation (Lotka, 1913):

$$\Sigma\, l_x m_x e^{-rx} = 1 \qquad (2)$$

where l_x is the proportion of individuals surviving to age x, m_x is the number of neonates produced per surviving adult at age x, and x is time expressed in days. Intrinsic rate of natural increase (r) was calculated for each replicate and the results are presented as the mean per replicate.

The no-observed effects concentration (NOEC), which is the highest test concentration at which the studied endpoint was not significantly different from controls, was calculated for reproduction (NOECrep), for size (NOECsize) and for r (NOECr). The reproduction was calculated as the average of total neonates that was produced per adult during the exposure period. Reproduction and size results were compared with the control. Comparisons were run using a one-way ANOVA test, which was followed by the Bonferroni post hoc test ($\alpha < 0.05$). Calculations were performed using the software GraphPad Prism (Stagraphics Centurion software, version 16.07 for Windows, Virginia USA, www.statgraphics.com).

Results and discussion

The results of the two-generational experiment in *D. pulex* exposed to imatinib are summarised in Figure 5.2. The reproduction, the size and the calculated population growth of the F0 were not different at any concentration when compared with control, except at the exposure level of 626 µg/L. At this high concentration, daphnids' reproduction was reduced by 15.9 % ($p < 0.001$). In cancer patients treated with imatinib, the effective plasma concentration required to induce an effect is also high. In patients treated with 400 mg/day of imatinib for instance, a steady-state of approximately 979 µg/L is reached in plasma after 29 days [11], which is higher than the exposure level that induced an effect on daphnids of the first generation.

However, in the second generation of daphnids exposed to imatinib, no effect was observed at any concentration. Our results cannot explain the reason of this lack of toxicity, which can either result from the physical-chemical properties of the molecule itself or the organism tolerance or adaptation to the chemical stress [32,33].

The no-observed effect concentrations (NOECs) could not be calculated because they were above the test concentrations, except for F0 whose NOECrep was 93.2 µg/L. Therefore, the NOECs were 93.2 and > 626 µg/L for the F0 and F1, respectively. A single study reported the effects of imatinib on

daphnids and the toxic concentrations were lower than those in our study [34]. After 7 and 21 days of exposure, NOECrep were 0.3 µg/L and 3 µg/L in *C. dubia* and *D. magna*, respectively, which is lower by an order of magnitude of 300 to 30 when compared with the NOEC found in this study. Although no difference were generally observed in the overall sensitivity of the *D. pulex* and the *D. magna* when animals were exposed to several chemicals [35], a difference in sensitivity to imatinib seems here to exist between *D. pulex, D. magna* and *C. dubia*.

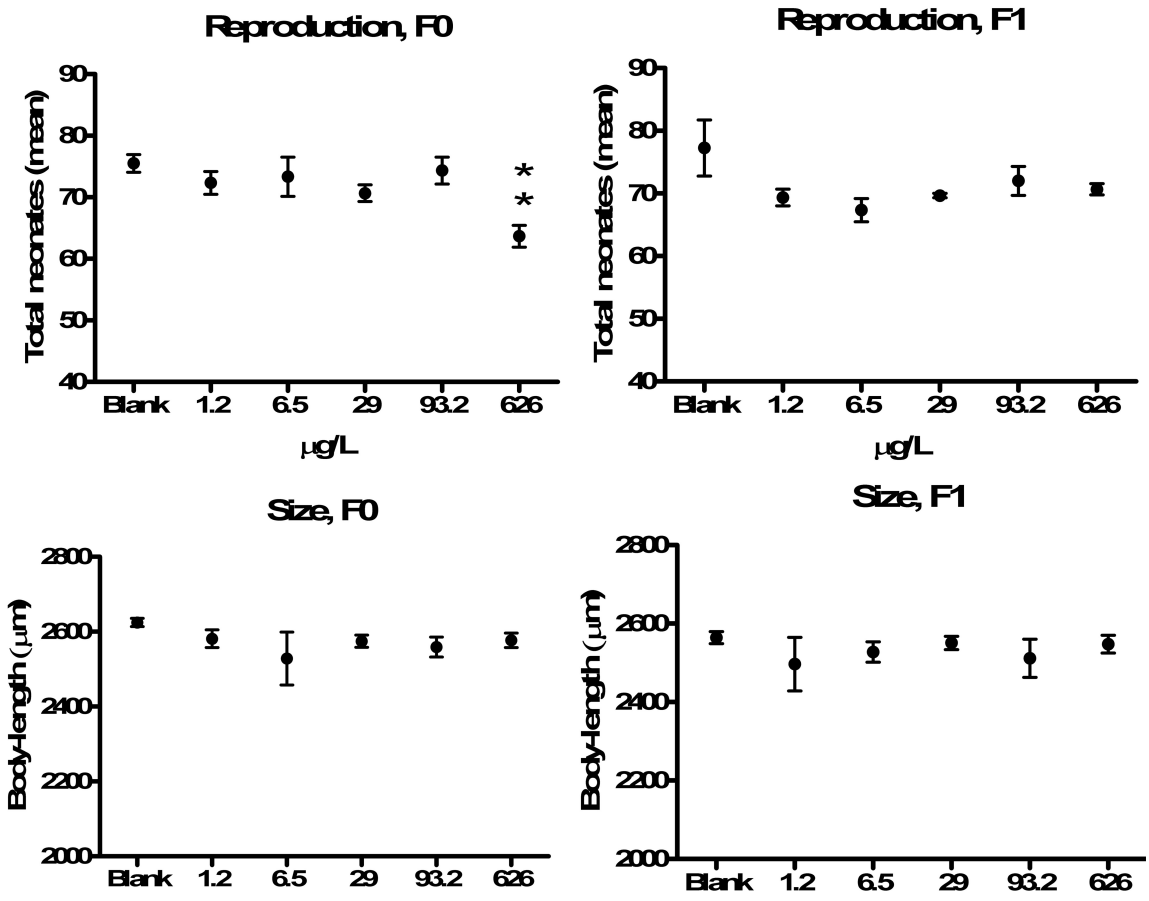

Figure 5.2: Reproduction and size of two generations (F0 and F1) of D. pulex exposed 14 days to imatinib. Reproduction is the mean number of neonates per concentration that was produced during 14-d of exposure. The size is the mean body-length of individual aged 14 days (mean ± SD, n = 57 for F0 and F1). ** p < 0.01

In this study, imatinib is not considered as toxic to *D. pulex*. However, other ecotoxicological experiments should be performed on different aquatic species such as algae and vertebrates to confirm that imatinib is a non-toxic molecule for the aquatic fauna and flora. Also, other TKIs have been marketed after imatinib, such as gefitinib, sunitinib, nilotinib, dasatinib, sorafenib and lapatinib. These new generations of molecules are used against various cancers but still with a broad prevalence for chronic myelogenous leukemia and gastrointestinal stromal tumours. Because these molecules demonstrated a definite efficacy on survival of advanced cancer patients - however less impressive than for imatinib in its specific indications - a progressive increase in their use and in their release in the environment is expected to occur. Therefore, it would be interesting to monitor these compounds in the environment and to determine their potential effects in aquatic vertebrates and invertebrates.

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Chapter 6

Does tamoxifen induce effects at the protein level?

The aim of this chapter was to identify and quantify proteins in daphnids exposed to tamoxifen using a so-called ecotoxicoproteomic experiment. Ecotoxicoproteomics aims to understand changes that occur at the protein level in cells or an organism in response to stress, such as *D. pulex* exposed to the anticancer drug tamoxifen. First, preliminary steps about extraction of daphnia proteins are described, because little is known on this procedure with aquatic invertebrates. The objective of this preliminary part was to determine an easy and efficient technic procedure to obtain sufficient biological material. The second part of the chapter contains the protein analysis results of the ecotoxicoproteomic experiment that was performed with *D. pulex* exposed for 2 and 7 days to tamoxifen.

Ecotoxicoproteomics: preliminary steps (Addendum)

Introduction

The aim of ecotoxicoproteomics is to find protein changes that occur in cells or in organisms that were exposed to toxins, such as pollutants or drugs. In a classical workflow, proteins extracted from organisms, tissues or cells are fractionated, digested into peptides with an enzyme (usually trypsin), and peptides are analysed by liquid chromatography coupled to mass spectrometry (LC-MS/MS). Identification of peptides and proteins is carried out by search of mass spectrometry data against databases containing protein sequences of the organism investigated [1]. In this way, several thousands of protein can typically be identified and quantified in biological samples if a comprehensive protein sequence database of the test organism is available. Unfortunately, little has been published on the extraction of daphnia proteins [2] for proteomic analyses. For an efficient application of proteomics to daphnids, three following steps were assessed and optimized:

1. preparation of daphnia samples
2. daphnia homogenisation and cell lysis
3. protein extraction and fractionation

The aims of this preliminary experiment were twofold. First, the quantity of daphnids needed for protein extraction was assessed. Second, the best procedure for a relevant and reproducible protein extraction was assessed and chosen.

Procedure

Sample preparation

Two different life steps were used because the protein expression profile may be different depending on the age of the daphnids: neonates 2-days old and adults 7-days old (i.e., daphnids that have just laid). Therefore, 75 and 20 individuals were reared in daphnia medium exempted from chemical during 2 and up to 7 days, respectively.

After 2 and 7 days, individuals were pipetted from the daphnia medium into 15 ml falcon tubes. Then, water was rapidly pulled out through a homemade plastic vacuum tube until the volume drops down to < 0.5 ml. Samples were immediately immersed in liquid nitrogen to freeze the contents. Each falcon tube was stored at – 80°C until homogenization and cell lysis. Protease inhibitor and urea 8M was added into frozen samples just before homogenization process. No replicate was performed.

Daphnia homogenization and cell lysis

Cell lysis and protein extraction were optimized to obtain sufficient biological material and reproducible results. The cell lysis and homogenization of the daphnids was performed with the FastPrep® homogenizer instruments or ultrasonic probe.

The FastPrep® homogenizer instrument crushed daphnids by multidirectional jerky movements of 1.4 mm glass beads. The homogenizing intensity was 5.5, for 10 seconds (5x). Eight samples could be run at the same time. The ultrasonic probe homogenized daphnids using amplitude of 30%, for 10 seconds (3x). With the available instrument samples could be only treated one by one.

LC-MS/MS analyses

Proteins were extracted and fractionated by SDS-PAGE 1D gel (i.e., sodium dodecyl sulfate polyacrylamide gel electrophoresis) according to their molecular weight, and quantified in-gel by densitometry. Gel lanes were cut into 10 pieces and proteins were in-gel digested with trypsin. Resulting peptides were analysed by LCMS/MS with a hybrid linear trap LTQ-Orbitrap XL mass spectrometer for protein identification and quantification based on spectral counting.

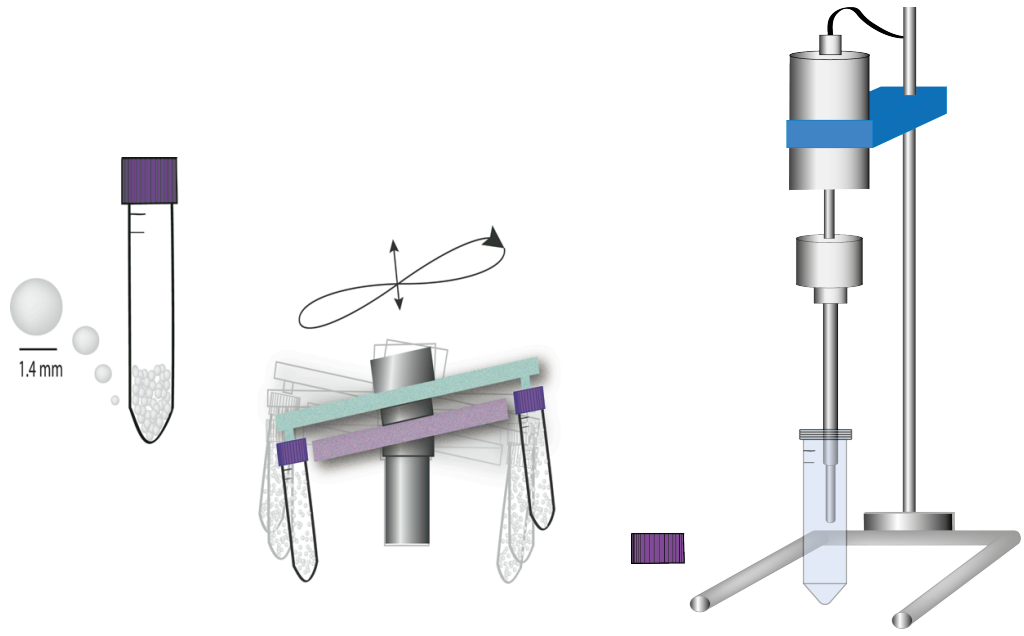

Figure 6.1: FastPrep® homogenizer (on the left) and ultrasonic probe (on the right) instruments

Results

The yield per adult daphnia (7 days old) was 8.3 and 19.6 µg of proteins for FastPrep® homogenizer and ultrasonic instrument, respectively, while the number of identified proteins was similar for both methods, with 880 and 908 identified proteins respectively (Figure 6.2). Both protocols provided sufficient material for LC-MS/MS analyses, although the homogenizer instrument was less efficient compared to ultrasonication. But the FastPrep® instrument was finally chosen, as it allows an easy, fast and parallel extraction of proteins (8 samples treated in similar manner and at the same time). Nevertheless, the bead size had to be changed for an efficient protein extraction of 2-days old daphnids, as the beads were bigger than daphnia size, decreasing their ability to crush young daphnia body. We decided therefore to use smaller beads, of 0.5-0.75 mm instead of 1.4 mm, in further ecotoxicoproteomics experiments. The number of 2-days old daphnids could thus be reduced from 100 to 70.

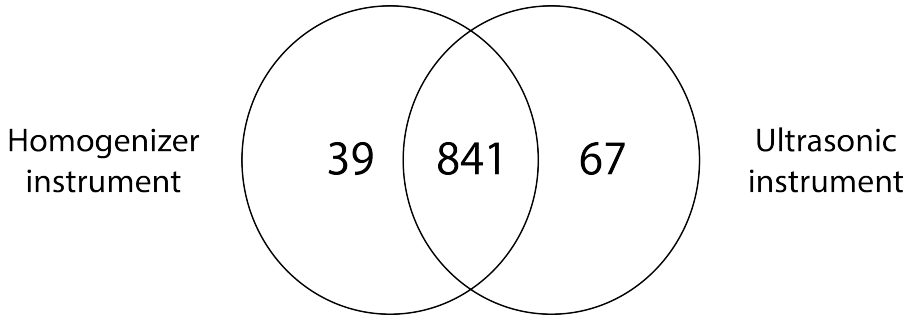

Figure 6.2: Number of identified proteins in *D. pulex* of 7 days old

Shotgun ecotoxicoproteomics of *Daphnia pulex*: biochemical effects of the anticancer drug tamoxifen

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Submitted to *Journal of Proteome Research*

Abstract

Among pollutants released into the environment by human activities, residues of pharmaceuticals are an increasing matter of concern because of their potential impact on ecosystems. The aim of this study was to analyse differences of protein expression resulting from acute (2 days) and middle-term (7 days) exposure of aquatic microcrustacean *Daphnia pulex* to the anticancer drug tamoxifen. Using a liquid chromatography - mass spectrometry shotgun approach, about 4000 proteins could be identified, providing the largest proteomics dataset of *D. pulex* published up to now. Considering both time points and tested concentrations, 189 proteins showed a significant fold change. The identity of regulated proteins suggested a decrease in translation, an increase in protein degradation and changes in carbohydrate and lipid metabolism as the major effects of the drug. Besides these impacted processes, which reflect a general stress response of the organism, some other regulated proteins play a role in *Daphnia* reproduction. These latter results are in accordance with our previous observations of the impact of tamoxifen on *D. pulex* reproduction, and illustrate the potential of ecotoxicoproteomics to unravel links between xenobiotic effects at the biochemical and at the organismal level. Data are available via ProteomeXchange with identifier PXD001257.

Introduction

For years, residues of pharmaceutical agents have been identified worldwide in the aquatic environment (for review see [3]. Apart from industrial releases and veterinary usages, human consumption is the primary origin of pharmaceutical discharged into natural waters [4]. Once consumed, pharmaceuticals are body-excreted into sewage waters in either intact and/or metabolised forms. The processes of sewage treatment plants (STPs) are not efficient for all chemicals [5], and several pharmaceutical residues have been identified in STP effluents and natural waters, such as anticancer agents [4,6–14].

Ecotoxicology studies the impact of chemicals on ecosystem health and function at various levels of the biological organisation, such as organism, population and ecosystem [15]. With the development of "omics" technologies, such as proteomics, it is possible now to unveil links between xenobiotic effects at the organism level and changes at the molecular level. Proteins in particular reflect the biochemical functions that may be directly or indirectly affected by chemical stress, and proteomics analyses may detect responses that are unforeseen and unobserved at the organism level during exposure to xenobiotics [15]. These sub-organism responses may be early signs of toxicity that would eventually impact organism health and functions. Until recently, most of ecotoxicoproteomics studies have focused on few vertebrate species because of a lack of complete protein sequence databases in invertebrate species relevant for ecotoxicology [15]. Nevertheless, the progress of genomic sequencing is steadily increasing the number of available protein sequence databases for invertebrates. A complete genomic sequence of *Daphnia pulex*, an aquatic micro-crustacean, was for example recently released (wfleabase.org). Froehlich et al. [2] showed that it could be used for LC-MS/MS proteomic analyses of this organism and another close species, *Daphnia longicephala*. Their data demonstrated proteomics to be very promising for ecotoxicological investigations of *Daphnia* species, although only a few publications have investigated the proteome of daphnids until now [15]. Indeed, daphnids are commonly used in ecotoxicology [16] because they are key organisms in the food chain and they produce clones by parthenogenesis.

Most of ecotoxiproteomics studies used two-dimensional gel electrophoresis (2D-GE) coupled to mass spectrometry analysis, which is a low throughput technique, difficult to scale and with some bias against identification of membrane or basic proteins [17]. A more interesting alternative is the shotgun method, where proteins are extracted and digested into peptides, which are then analysed by liquid chromatography (LC) coupled with tandem mass spectrometry (MS/MS). With the possibility of adding fractioning steps, several thousands of proteins can potentially be identified and quantified in one experiment. As a proof-of-principle, Ralston-Hooper et al. [18] demonstrated the efficiency of a shotgun approach to investigate the effects of drug fadrozole, a known endocrine disruptor, in fish.

This study focuses on the protein expression of *D. pulex* exposed to the anticancer drug tamoxifen. This pharmaceutical was measured in STP effluents and natural waters in concentrations up to 0.37

and 0.21 µg/L, respectively [12,19]. Tamoxifen is prescribed for the prevention and treatment of hormone receptor-positive breast cancers in humans [20–22]. It is a selective modulator of alpha and beta estrogen receptors (ERα and ERβ), which also bounds estrogen-related receptors (ERRs, [23]). Although *D. pulex* lacks homologs of estrogen receptors, it does contain a gene for an estrogen-related receptor (ERR,[24]), which may explain why *D. pulex* was sensitive to tamoxifen in a previous study that was performed in our laboratory [chapter 2]. Indeed, tamoxifen decreased reproduction and induced miscarriages and/or morphologically abnormal neonates. The aim of this study was to further investigate the impact of tamoxifen on daphnids at the biochemical level, and to analyse protein expression after acute (i.e., 2 days) and middle-term (i.e., about 7 days) exposure of *D. pulex* to tamoxifen. A gel-free, label-free quantitative proteomics approach was used, including a peptide fractionation step for maximal proteome coverage.

Materials and Methods

Chemicals

Tamoxifen was purchased from Sigma/Fluka and used without further purification (>99%, lot: 011M1682V). A stock solution was prepared in pure DMSO at the measured concentration of 25 mg/L and was stored in a glass flask at -80°C. The solution was thawed at room temperature before each use.

Test organisms

D. pulex individuals from the *arenata* strain were mass-cultured in conditions that maintain parthenogenetic reproduction. Stock and treated daphnids were fed daily with 0.2 mgC/daphnia of *Pseudokirchneriella subcapitata* algae and a suspension of tropical fish food (Tetramin®) [25,26]. The medium Elendt (M4) and tamoxifen test solutions were renewed every two days. Daphnids were reared in glass beakers placed in a Coolstore® environmental chamber with 16-h light and 8-h dark photoperiods, at 21 ± 1°C. At the beginning of the experiments, a potassium dichromate ($K_2Cr_2O_7$) assay was performed to assess daphnia sensitivity to chemicals and the results were conformed to the classical response given by the organisms. Dissolved oxygen, pH and conductivity were also controlled at the beginning and end of each medium change. The position of the beakers in the environmental chamber was randomized in order to minimize variability.

Ecotoxicological test procedure

Two and seven days experiments were carried out according to the experimental design shown in Figure 6.3. Seven hundred and sixty neonates, aged less than 24-h (3rd brood), were randomly

selected, placed in 900 mL of medium (70 individuals per glass beaker, 2 replicates) and exposed to tamoxifen at predicted concentrations of 0.1 (C1) and 2.4 (C2) µg/L, which correspond to nominal concentrations of 1.8 (C1) and 88.0 (C2) µg/L. Prediction of tamoxifen concentrations were based on a non-linear regression between nominal and measured concentrations (*Daphnia* medium without food and organisms) to take in account loss of the drug by adsorption, as described in chapter 2. After 2 days of exposure, five hundred and sixty daphnids were removed and frozen in liquid nitrogen for proteomic analyses. The two hundred individuals left were placed in 450 mL of the corresponding medium with tamoxifen (25 individuals per glass beaker, 2 replicates) until first egg laying (4 to 6 additional days), before being frozen for proteomics analyses. Two controls were carried out for both period exposures (2 replicates): a blank (i.e. Elendt M4 medium, without solvent and chemicals) and a solvent control with DMSO 0.0008 %, which corresponds to the highest concentration used in the tests. For both controls, 60 daphnids (2 replicates) were also selected for proteomics analyses after 4 days of test, as additional time point to follow evolution of proteome during *D. pulex* development.

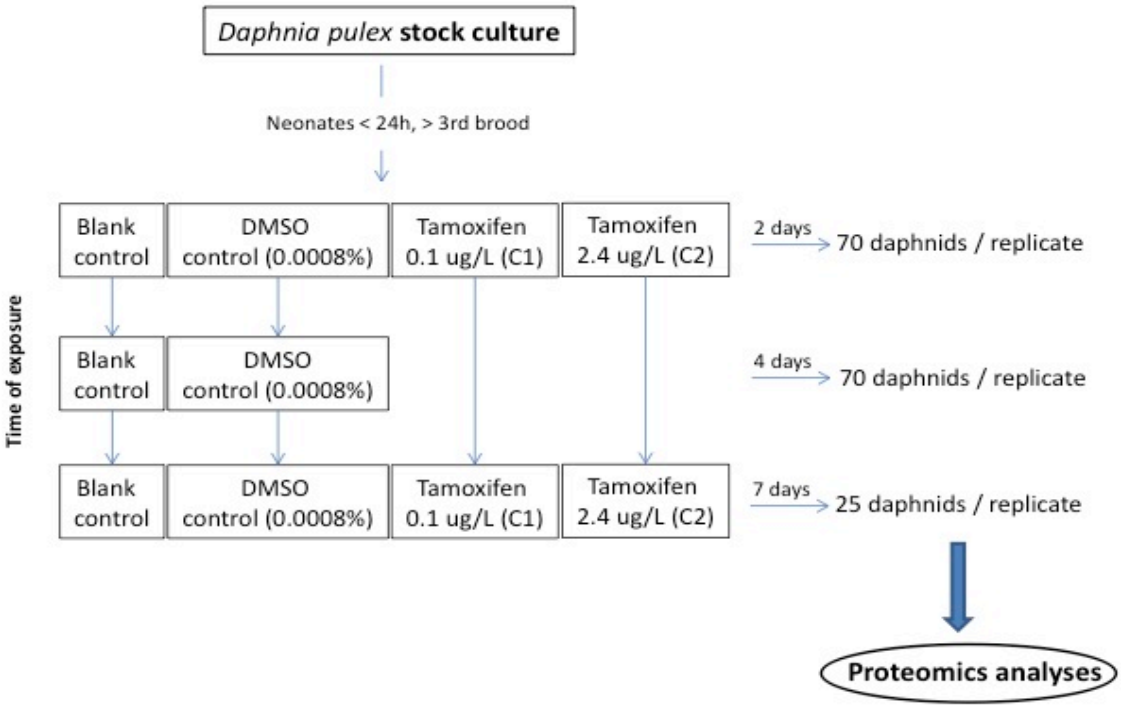


Figure 6.3: Experimental design of the *D. pulex* ecotoxicity test. Tamoxifen concentrations are predicted concentrations based on a non-linear regression between theoretical (nominal) concentrations and measured concentrations in solution (see Material and Methods for more explanations). Nominal concentrations were 1.8 (C1) and 88.0 (C2) µg/L. All tests were performed in duplicates.

Proteomics analyses

For cell lysis, 1 ml of urea 8M containing protease inhibitors (complete Protease Inhibitor Cocktail Tablet, Roche Applied Science) was added to the samples, which were then homogenized by

FastPrep©-24 (www.mpbio.com) using 0.5-0.75 mm glass beads (intensity 6.5, 4 x 20 sec). After quantification by SDS-PAGE and densitometry, a volume corresponding to 90 ug of proteins was used for further sample preparation. Proteins were reduced with dithiothreitol (5 mM) and alkylated with iodoacetamide (20 mM). After a trichloroacetate-deoxycholate precipitation, they were resuspended in 250 mM triethylammonium bicarbonate pH 8.0 containing 4M urea and digested overnight at 37°C with 2 µg of trypsin. The obtained peptide mixtures were desalted on SepPak C18 cartridges (Waters Corp., Milford, MA), dried, dissolved in 4M Urea with 0.1% Ampholytes pH 3-10 (GE Healthcare) and fractionated by off-gel focusing as described [27], using a 13 cm pH 3-10 strip. The 12 fractions obtained were desalted on a microC18 96-well plate (Waters Corp., Milford, MA), dried and resuspended in 20 µl 0.1% formic acid, 3% (v/v) acetonitrile for LC-MS/MS analysis. Fractions 10 and 11 were pooled in the same volume.

Mass spectrometry analyses were performed by the Protein Analysis Facility (University of Lausanne). The tryptic peptide samples were analyzed by LC-MS/MS on an Ultimate 3000 RSLCnano HPLC system coupled to a hybrid linear trap LTQ-Orbitrap XL mass spectrometer (Thermo Scientific). Mobile phases used were (A) H_2O:MeCN 97:3 (v/v) + 0.1 % formic acid and (B) H_2O:MeCN 20:80 (v/v) + 0.1 % formic acid. Five µL of sample were loaded onto a trapping microcolumn Acclaim PepMap 100 C18 (2 cm x 100 µm, Dionex) in H_2O:MeCN 97:3 (v/v) + 0.1 formic acid at a flow rate of 3.5 µL/min. After 12 min, they were eluted and separated on a reversed-phase nanocolumn Acclaim PepMap RSLC C18 column (75 µm ID x 15 cm, 2 µm, Dionex) at a flow rate of 300 nl/min. The gradient used lasted 125 min, starting at 12 min with 2 % of solvent B, from 2 to 11 % of B in 5 min, 11 to 40 % B in 73 min, 40 to 55 % in 9 min, 55 to 95 % in 2 min, 95 % B during 3 min, 95 to 2 % in 6 min and 15 min at 2 %. In data-dependent acquisition controlled by Xcalibur 2.0 software (Thermo Scientific), the ten most intense precursor ions detected in the full MS survey performed in the Orbitrap (range 350 - 1700 m/z, resolution 60 000 at m/z 400) were selected for fragmentation, and fragment ions were analyzed in the ion trap. MS^2 was triggered by a minimum signal threshold of 10'000 counts and carried out at a relative collision energy of 35 % (CID), with an isolation width of 4.0 amu. Only precursors with a charge higher than one were selected for fragmentation and the m/z of fragmented precursors was then dynamically excluded from any selection during 60 s.

LC-MS/MS data were analyzed and quantified with MaxQuant version 1.3.0.5 [28], using Andromeda as search software [29] against UniProt (release 2012_06) database restricted to *Daphnia pulex* taxonomy (30'137 sequences). Cleavage specificity was trypsin/P (cleavage after K, R, including KP and RP) with two missed cleavages. Mass tolerances were of 6 ppm for the precursor and 0.5 Da for CID tandem mass spectra. The iodoacetamide derivative of cysteine was specified as a fixed modification, and oxidation of methionine, protein N-terminal acetylation, deamidation of asparagine and glutamine were specified as variable modifications. Protein and peptide identifications were filtered at 1% FDR established by MaxQuant against a reversed sequence database. A minimum of

one unique peptide was necessary to discriminate sequences which shared peptides. Sets of protein sequences which could not be discriminated based on identified peptides were listed together as protein groups. Label-free quantification (LFQ) was performed by MaxQuant, as described in Cox et al. [30], using intensity maximum over the retention time profile and summing intensities of the different isotopic peaks. All proteins with quantified values were retained at first but filtered in subsequent steps. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository with the dataset identifier PXD001257 [31].

LFQ data were further analyzed using R software and packages (R Core Team, 2013). Data were normalized using the variance stabilizing method (vsn), applying a generalized log2 transformation (glog2) on intensities [32]. For each tested concentration, fold change (in log2 scale) was calculated as the median of duplicate normalized protein intensities compared to corresponding DMSO controls (2 or 7 days). For each comparison, only proteins appearing in all 4 replicates and quantified by a minimum of 1 peptide were considered. Statistical analyses were performed with local-pooled-error (LPE) estimates, allowing significance testing for a small number of replicates [33,34]. The Benjamini-Hochberg method was used for multiple testing correction [35], and a LPE-based test was carried out for outlier detection, as described by Jain et al. [33] , in order to compensate the decrease of LPE robustness for duplicate experiments. For each tested concentration and time point, significant proteins were further filtered, only keeping proteins with i) a p-value < 0.05 after Benjamini-Hochberg correction, ii) identified with at least 3 peptides, iii) not tagged as outliers by the LPE test at the concentration tested or for the DMSO control. For each time point (2 or 7 days), proteins showing also some significant effect of DMSO alone compared to control medium (M4) were discarded.

Protein annotations (Gene Ontology, GO, PANTHER protein family) and functional analysis were done with PANTHER (www.pantherdb.org, [36]). Additional annotations were retrieved from UniProt knowledgebase (www.uniprot.org). Statistical overrepresentation tests of significant proteins were carried out for GO categories against the whole *D. pulex* genome using the PANTHER tool, and applying Bonferroni correction for multiple testing with a P-value threshold of 0.05. Test results and the subsequent graphs of GO terms were visualized using R (version 3.0.2, R Core Team, 2013) and the Cytoscape software package (version 2.8.3) [37]. The corresponding scripts are publicly available (https://github.com/PAFGit/PAF-Ecotoxicoproteomics-DPulex). Additional information about putative function of studied proteins was obtained using homology search with NCBI-BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) against the UniProtKB/Swiss-Prot database, and further processing BLAST results with Blast2GO (http://www.blast2go.com, [38]).

Results

Experimental set-up, coverage and quality of data

The impact of tamoxifen on *D. pulex* at the biochemical level was assessed by exposing neonates to two different concentrations of the drug (C1: 0.1, C2: 2.4 µg/L) during 2 days or until the first laying of eggs (6-8 days), before being processed for proteomic analyses.

Considering the whole dataset, 3940 proteins were identified with a minimum of one unique peptide and quantified in at least one replicate sample (suppl. Table S1). Most of them (3662) obtained some biological annotations through the Panther database (suppl. Table S2). As a trade-off between on the one hand proteome coverage and number of experimental conditions, and on the other hand time of sample analyses, the number of biological replicates had to be reduced to 2 for each experimental condition. The Pearson correlation factor of log2 of intensities was nevertheless superior to 0.9 for all duplicates (r = 0.922-0.984), showing a good reproducibility of replicate experiments (suppl. Figure S1A and S1B). After filtering out proteins identified with less than 3 peptides and, for each comparison of treatment and DMSO control, those having one missing quantitative value, we obtained 2711, 2685, 2474 and 2513 quantified proteins for C1 – 2 days, C2 – 2 days, C1 – 7 days and C2 - 7 days treatments respectively (suppl. Table S3).

Considering both time points and tested concentrations, 189 proteins showed a significant fold change with LPE test, after multiple-testing correction and removal of outliers (suppl. Table S4). Most of the regulated proteins were observed in the C2 – 7 days (170) treatment, i.e. at the highest concentration tested and for the longer exposure time, while the numbers of significant proteins were 32, 12 and 5 in C2 – 7 days, C2 – 2 days and C1 – 2 days treatments respectively. Noteworthily, we observed consistent results of increased or decreased expression of those 189 proteins between some of the different experimental conditions. In particular, the Pearson correlation factor of log2 of protein ratios was equal to 0.88 between C1 and C2 tamoxifen treatments at 7 days, and of 0.79 between 2 and 7 days for the C2 treatment (suppl. Figure S2). For the following result presentation and discussion, all significant proteins of both time points and tested concentrations were considered together. About one third of hits were positively regulated, whereas the remaining two thirds showed a decreased expression after tamoxifen treatment. While a higher exposure (time and concentration) resulted in more significantly regulated proteins, a higher variance of DMSO control replicates in 2 days experiments probably decreased statistical testing sensitivity and the number of significant proteins with 2 days treatments.

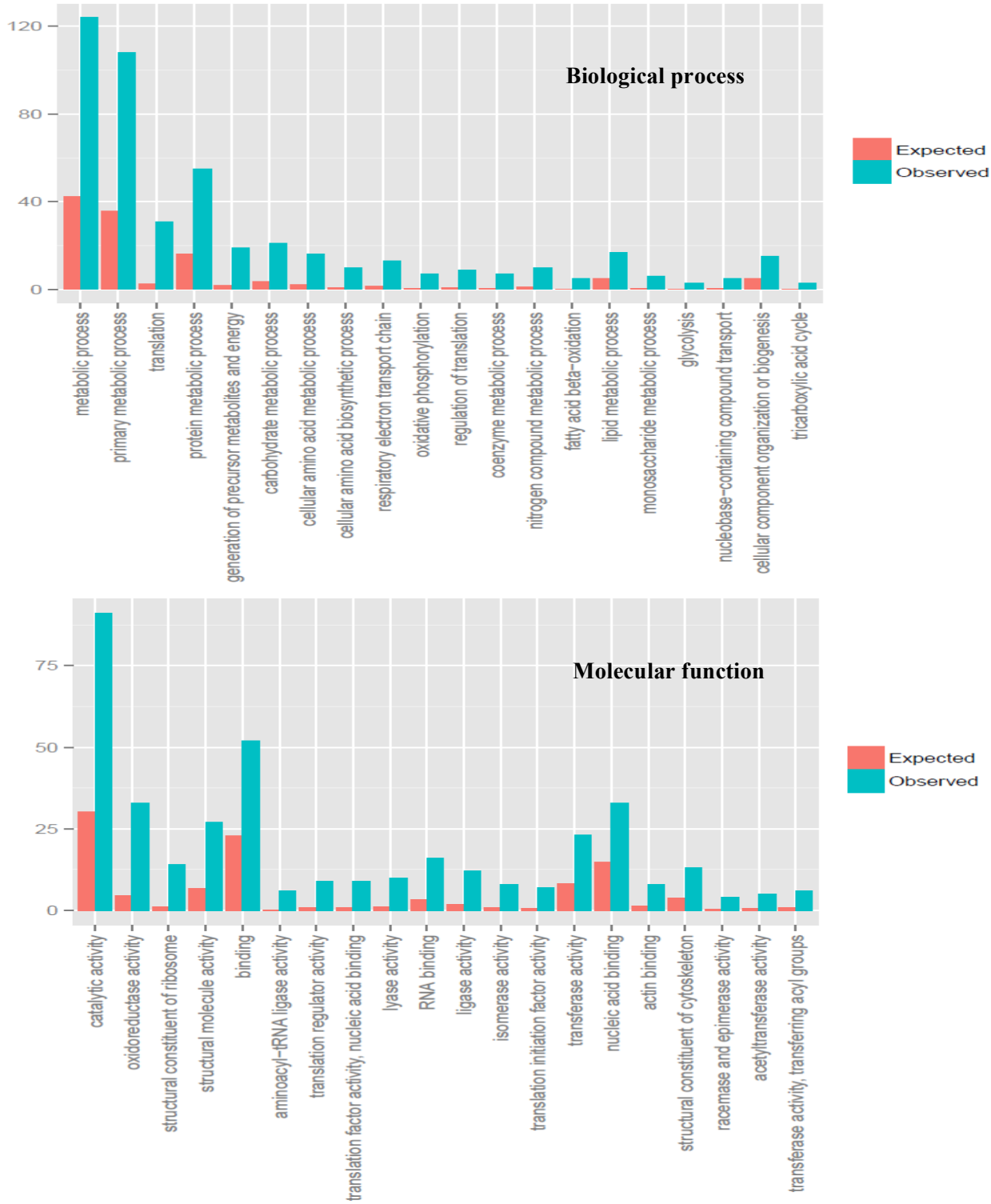


Figure 6.4: Statistical overrepresentation tests of significantly regulated proteins (180) were carried out against the whole *D. pulex* genome using the PANTHER tool (www.pantherdb.org). When a GO category had a p-value < 0.05, after Bonferroni correction for multiple testing, there was overrepresentation of proteins with this particular annotation compared to the reference list (whole genome/proteome). The histogram plot shows the number of regulated proteins observed in our dataset with a particular annotation (green columns) and the number of regulated proteins expected to have this annotation based on the whole proteome list (red columns). Only significant GO categories are shown: biological process and molecular function

Among those 189 proteins, 180 were annotated with PANTHER database and 3 with UniProt, while 6 proteins could not be annotated at all, even using BLAST homology search (suppl. Table S5). Statistical overrepresentation tests of the annotated proteins were carried out for Gene Ontology (GO) categories in Biological Process (BP) and Molecular Function (MF) classes. In each class, 20 GO categories showed significant enrichment of tested proteins compared to the whole *D. pulex* genome (Figure 6.4), altogether including 118 proteins with 97 and 109 proteins for BP and MF class respectively (suppl. Table S6). For a better understanding of relationships between proteins and significant GO categories, a graphical representation was used with proteins as nodes and shared annotations as edges. The edge width between 2 proteins was proportional to the minimum percentage of shared annotations and node color scaled according to log of fold change for the different concentrations and time points. In order to clarify the results, some annotations that were considered as too general, such as metabolic process, primary metabolic process, protein metabolic process (GO-BP) or catalytic activity, structural molecule activity, binding (GO-MF), were not included in the figures (Figure 6.5 and Figure 6.6, and suppl. Figure S2 & S3).

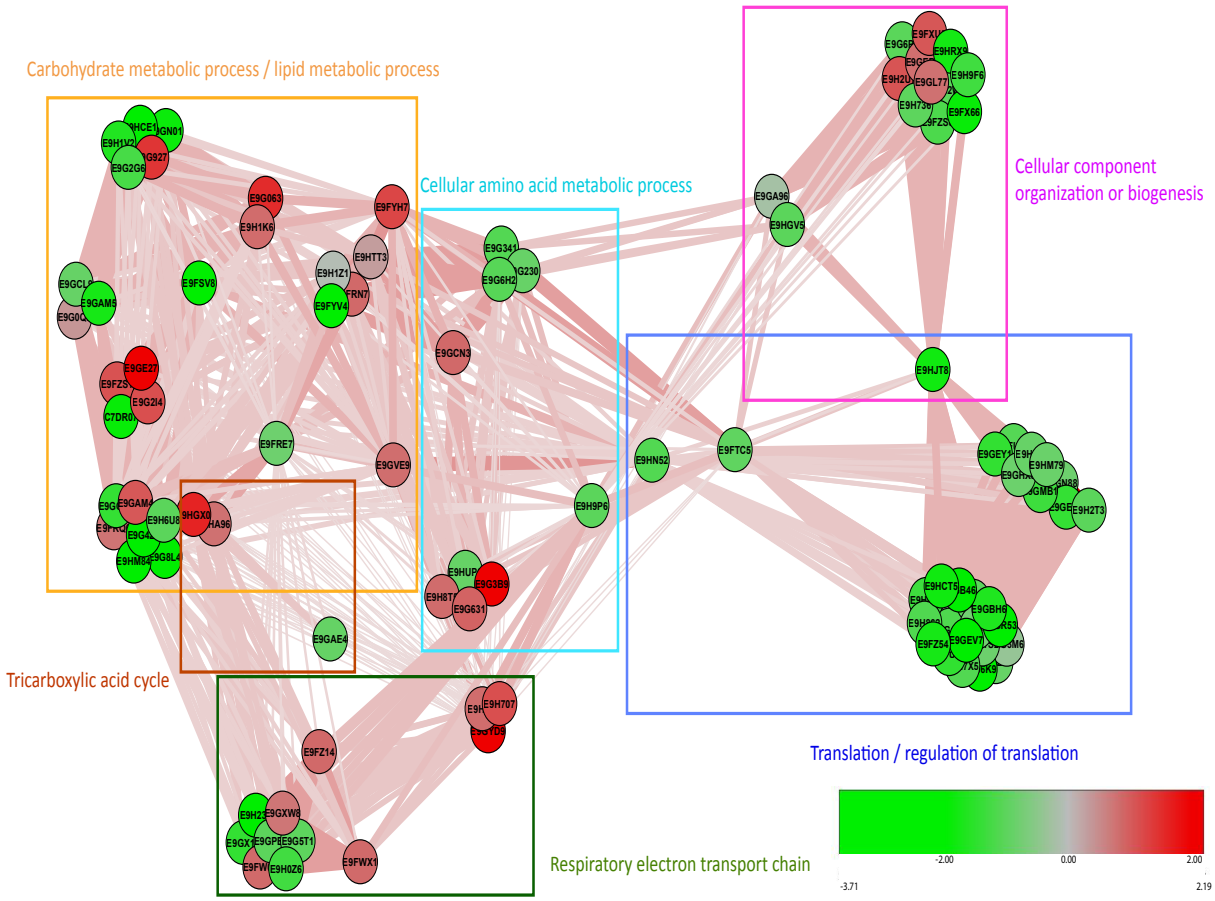


Figure 6.5: Graphical representation of relations between proteins and significant GO categories with proteins as nodes and shared annotations as edges (biological process). Edge width between 2 proteins was proportional to the minimum percentage of shared annotations and node colour scaled according to log2 of protein fold change at 7 days for the highest tamoxifen concentration tested (C2). Results for other time points and concentrations tested are shown in supplementary figure S2 and S3. Only significant GO categories were used, but too general annotations, such as metabolic process, primary metabolic process, protein metabolic process (BP) or catalytic activity, structural molecule activity, binding (MF) were not included for a better clarity of the graphs. Proteins sharing the same annotation are included in colored boxes for the most relevant GO categories

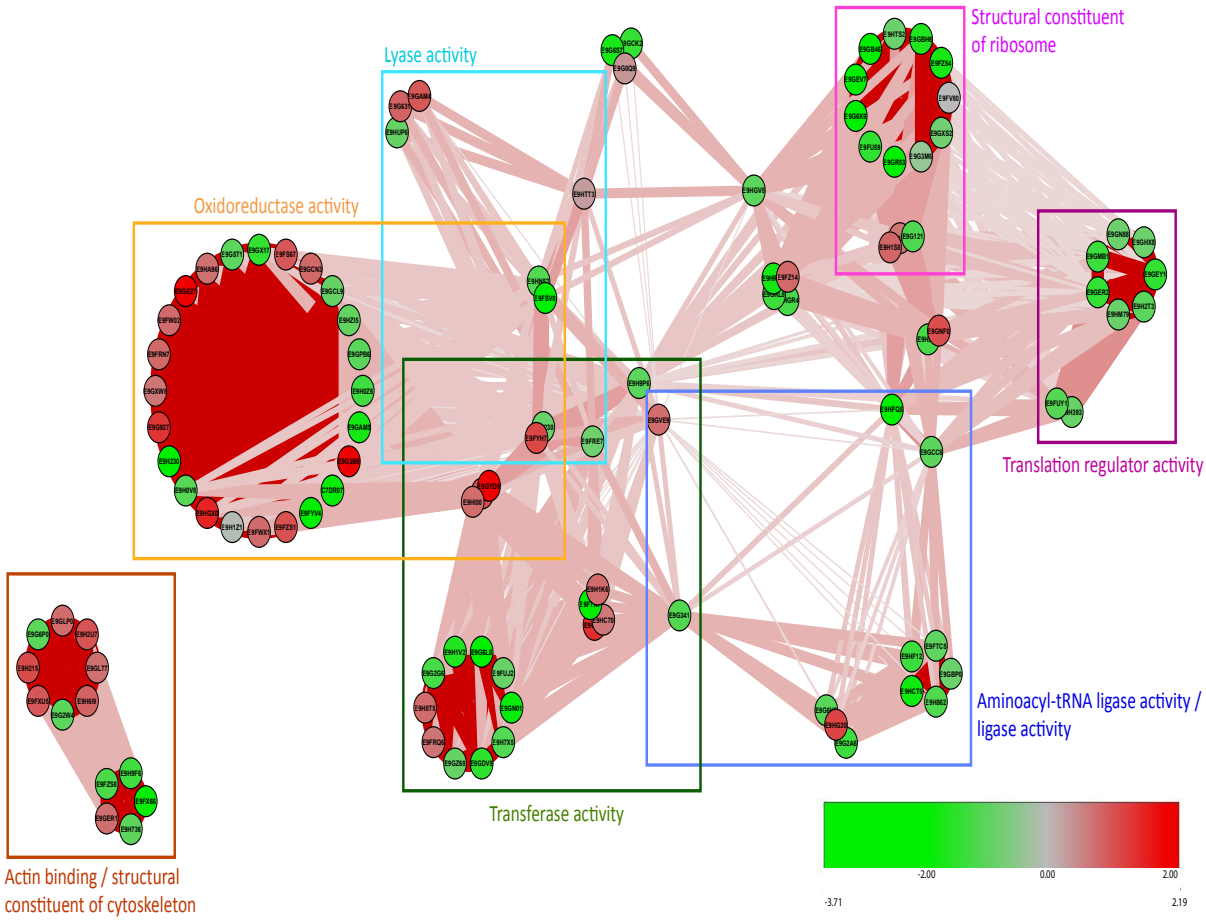


Figure 6.6: Graphical representation of relations between proteins and significant GO categories with proteins as nodes and shared annotations as edges (molecular function). Edge width between 2 proteins was proportional to the minimum percentage of shared annotations and node colour scaled according to log2 of protein fold change at 7 days for the highest tamoxifen concentration tested (C2). Results for other time points and concentrations tested are shown in supplementary figure S2 and S3. Only significant GO categories were used, but too general annotations, such as metabolic process, primary metabolic process, protein metabolic process (BP) or catalytic activity, structural molecule activity, binding (MF) were not included for a better clarity of the graphs. Proteins sharing the same annotation are included in colored boxes for the most relevant GO categories

Disruption of protein homeostasis

Among the 118 proteins with significant GO enrichment terms, about 30 proteins were related to translation or regulation of translation (Figure 6.5 & suppl. Table S4), and they were all negatively regulated. We could identify in particular ribosomal proteins (16), aminoacyl tRNA-ligases (7), translation initiation factors (5) and a translation activation factor. We additionally observed the decrease of a nucleolar protein (E9H9X9) involved in ribosome biogenesis and of a Mago nashi-like protein (E9GA58), which is part of an mRNA splicing complex. Conversely, a protein (E9GNF0) involved in RNA catabolic processes and translation repression was clearly up-regulated with a fold

change higher than 2. In parallel, a protein (E9I5Q2) belonging to the "translocation protein SEC62" family, a signal peptidase complex subunit (E9G1G4) and two protein glycosyl transferases (E9GZ69, E9G6L8), involved in co-translational protein targeting to membranes, were also strongly down-regulated (suppl. Table S4). The associated dolichol-phosphate mannosyl transferase (E9GN01), essential as mannosyl donor in pathways leading to protein glycosylation, was similarly decreased, as well as a calnexin isoform, potentially working as chaperone assisting glycoprotein synthesis (E9GMP2). In addition, expression of some proteins related to intracellular protein transport, such as Ras-related protein Rab-1B (E9G8G7), clathrin heavy chain (E9GTP3), a coatomer subunit (E9GA08) and an adaptin (E9GKM8), was reduced, with an exception represented by the increase of a translocon associated protein (E9GL54).

In parallel with the decrease of protein translation, we observed an increase of proteasome components (E9GIX9, E9HAP5, E9GZ78, E9G7M2) involved in protein degradation, while some proteasome regulatory subunits (E9FTY4, E9H6N4) and Cand1 (E9GRL8), a negative regulator of ubiquitin ligases, were down-regulated (suppl. Table S4). In addition, a proteolytic protein such as ATP-dependent Clp protease proteolytic subunit was increased after tamoxifen treatment. However, it should be noted we also observed a decrease of an ubiquitin-specific protease (E9HFQ5).

Finally, we observed the up-regulation of a 10 kDa heat shock protein (E9G787), a chaperone induced by stress, which is essential for mitochondrial protein folding and is involved in apoptosis.

Changes in carbohydrate/lipid metabolism and metabolic energy generation

Twenty proteins were associated with the significant enriched GO term "carbohydrate metabolic process", 12 with "lipid metabolic process", 17 with metabolic energy production (GO: tricarboxylic acid cycle or respiratory electron transport chain process, Figure 6.5 & suppl. Table S4), and they included either up- or down-regulated hits.

The proteins related to carbohydrate metabolism, in particular glycolysis or gluconeogenesis, included up-regulated phosphoglycerate kinase (E9FRQ6) and enolase (E9GAM4), while glucose-6-phosphate isomerase (E9GCK2) expression decreased. Linking the glycolytic pathway to the tricarboxylic cycle, one component of the pyruvate dehydrogenase complex (E9H1K6) also showed significant increase. In the tricarboxylic acid cycle, levels of citrate synthase (E9FRE7), isocitrate dehydrogenase (E9GAE4) and of an isoform of 2-oxoglutarate dehydrogenase (E9GLV0) were reduced, but expression of malate dehydrogenases (E9HGX0, E9HA96) and fumarate hydratase (E9G631) was augmented. Notably a mitochondrial 2-oxoglutarate/malate carrier protein (E9HM84), playing an important role in gluconeogenesis, was decreased. Among other impacted carbohydrate metabolic processes, two proteins of pentose-phosphate pathway, glucose-6-phosphate 1-dehydrogenase (E9GCL9) and 6-phosphogluconate dehydrogenase (E9GAM5), were down-regulated. Additionally a

putative glycogen phosphorylase (E9G2G6) and a glycogen synthase (E9H1V2) were also decreased after tamoxifen treatment.

Oxidative phosphorylation was clearly impacted, with one cytochrome oxidase (E9GI31) and various NADH dehydrogenases (E9GX17, E9G5T1, E9FV79, E9H230, E9GPB6, E9H0Z6) down-regulated after treatment, whereas a cytochrome oxidase (E9GXW8) and a cytochrome b-c1 complex subunit (E9FW02) were increased.

Regarding lipid metabolism, expression of two electron transfer flavoproteins (E9GYD9, E9FWX1), three acyl CoA dehydrogenases (E9H707, E9HI00, E9FRN7), an enoyl CoA hydratase (E9GVE9), an acetyl CoA acetyltransferase (E9HC70), and a 2-hydroxyacyl-CoA lyase (E9FYH7), which are all part of fatty acid oxidation pathways, was up-regulated after treatment. Conversely a mitochondrial tricarboxylate transport protein (E9G4Z8), playing an important role in fatty acid biosynthesis, was strongly down-regulated. An exception to this general trend was represented by the decrease of trifunctional enzyme subunit alpha (E9HN52), which is part of fatty acid beta-oxidation pathway.

Related to the metabolic processes discussed above, we also observed an increase in several enzymes involved in the catabolism of cellular amino-acids in various metabolic intermediates, such as 3-hydroxyisobutyrate dehydrogenase (E9GCN3) for valine degradation, dihydropteridine reductase (E9G3B9) for phenylalanine conversion to tyrosine, aspartate aminotransferase (E9H8T5) for aspartate degradation and lipoamide acyltransferase component of branched-chain alpha-keto acid dehydrogenase complex (E9G063) for branched amino-acids degradation.

Constituents of cytoskeleton are impacted by tamoxifen treatment

Thirteen proteins were associated with the significant enriched GO term “structural constituent of cytoskeleton” or “actin binding” (Figure 6.6 & suppl. Table S4). Among down-regulated proteins, we found several muscle proteins, such as alpha-actinin (E9G6P0) and myosin (E9FZS8), and two proteins playing a role in oogenesis: filamin-A (E9G2W4) [39] and moesin (E9H9F6) [40]. Additionally decreased proteins included a dynein heavy chain (E9FX66) and another myosin (E9H736), which could be important for the specification of the anterior/posterior axis of the embryo [41].

Tamoxifen treatment stimulated the expression of beta-tubulin (E9GER1), a F-actin-capping protein subunit (E9GL77), a calponin-like protein (E9H6I9), of which a *Drosophila melanogaster* homolog is known to be regulated by juvenile hormone [39], and two muscle-specific proteins (E9H215, E9GLP0) belonging to calponin family. Interestingly, profilin (E9H2U7) and villin-like protein quail (E9FXU5), involved in oogenesis [42,43] were also up-regulated.

Other proteins related to muscle, a calcium-transporting ATPase (sarcoplasmic/endoplasmic reticulum type) (E9HR84), a ryanodine receptor (E9FTU9), a LIM-9 isoform (E9FSI1) and a PDZ and LIM

domain protein (E9HGQ3), known to interact with alpha-actinin, were decreased, as well as a glycosyl transferase (E9H0V8), which plays a role in collagen formation. It should also be noted that in *Drosophila melanogaster* a homolog of the ryanodine receptor was shown to be essential for larval development [44].

Effects of tamoxifen on proteins involved in reproduction

Besides proteins with significant GO enrichment terms, we were also interested by regulated proteins related to reproduction (Table 6.1), as our previous results showed an effect of tamoxifen on daphnids in chronic tests [45]. In addition to the proteins involved in oogenesis that we mentioned in the previous chapter (filamin, moesin, profilin, villin, myosin), we identified two negatively regulated proteins (E9GDK0, E9GJA1), which are homologs of *Drosophila melanogaster* Rab6 and Rab11 of Rab GTPases family (suppl. Table S4). These proteins have many functions related to vesicle and membrane trafficking, and in particular have been shown to be involved, with another down-regulated protein of our dataset (maternal protein exuperantia, E9HLH8), in oocyte polarization, an essential step in embryo development [46–48]. Two other proteins playing an important role in oocyte development, E9H8M2 and E9G6B6, homologs to *Drosophila* aubergine [49] and baiser [50] proteins respectively, were also clearly decreased after tamoxifen treatment.

In our dataset we also observed the decrease of four proteins (E9FVG7, E9H082, E9H8K5, E9HJQ1) classified in vitellogenin-2 family by PANTHER database, although they share only low homology between them and with vitellogenins from other invertebrate species. Two other vitellogenins fused with a superoxide dismutase domain were also impacted by tamoxifen, being either overexpressed (E9GVW7) or down-regulated (E9HZI5) after treatment. However, it should be noted that we also identified two other fused vitellogenins (E9GVW1, E9HZI6) and six other proteins from vitellogenin-2 family which did not change their expression after treatment (suppl. Table S2).

Potential effects of tamoxifen on nuclear hormone receptors

Because of potential binding of tamoxifen on *D. pulex* estrogen-related receptor ERR, we were also interested in proteins potentially involved in regulation of nuclear hormone receptors (Table 6.1). Indeed, we observed an increase of a homolog of prohibitin-2 (E9GAM6), which is an estrogen receptor (ER)-selective co-regulator in vertebrates [51]. Additionally, an up-regulated protein (E9G0Y9) could be related to nuclear receptor co-activator 5, which has been shown to interact with nuclear estrogen receptors in humans [52] (suppl. Table S4).

Impact of DMSO solvent on protein expression

As tamoxifen is a lipophilic molecule, DMSO was used as solubilization solvent. In order to assess potential impact of DMSO on *D. pulex* protein expression, comparisons were also carried out between blanks (i.e. only medium M4, without solvent and chemicals) and DMSO (0.0008%) control samples at all time points. After multiple-testing correction and removal of outliers, 13 and 45 proteins showed a significant fold change for 2 and 7 days of treatment respectively (suppl. Table S7). Altogether, about 20% of hits were negatively regulated, whereas the others showed an increased expression with DMSO. Among positively regulated proteins, about one third was related to protein translation (ribosomal proteins, translation initiation factors).

Table 6.1: . Selection of proteins potentially involved in *D. pulex* reproduction or development and showing a significant fold change with LPE test, after multiple-testing correction and removal of outliers. All tamoxifen treatment ratios are displayed (C1 – 2 days, C2 – 2 days, C1 – 7 days, C2 - 7 days), and significant ones are highlighted in grey. PANTHER family names (www.pantherdb.org) are shown as annotations, and UniProt ID of *Drosophila* homologs are included when they are referenced in the main text.

UniProt ID	2 days log C1/DMSO	2 days log C2/DMSO	7 days log C1/DMSO	7 days log C2/DMSO	PANTHER Family/Subfamily	UniProt ID of *Drosophila* homolog
E9FVG7	-0.53	-0.98	-0.4	**-0.81**	Vitellogenin-2 (PTHR23345:SF1)	
E9FTU9	-1.29	**-1.74**	-0.84	**-1.14**	Ryanodine receptor 44F (PTHR13715:SF74)	RY44_DROME
E9FXU5	0.42	0.47	0.66	**1.07**	Villin-like protein quail (PTHR11977:SF37)	QUAI_DROME
E9G0Y9	0.49	0.8	0.47	**0.76**	Neosin (PTHR23295:SF2)	
E9G2W4	0.12	-0.49	0.03	**-1.03**	Filamin-A (PTHR11915:SF251)	FLNA_DROME
E9G6B6	0.31	-1.11	-1.61	**-1.72**	Transmembrane EMP24 domain-containing protein 10 (PTHR22811:SF58)	TMEDA_DROME
E9GAM6	0.11	0.39	0.45	**1.05**	Prohibitin-2 (PTHR23222:SF1)	
E9GDK0	-0.89	-1.2	-0.83	**-0.91**	GH09086P (PTHR24073:SF352)	RAB6_DROME
E9GJA1	-0.98	-1.39	-1.26	**-1.25**	DRAB11 (PTHR24073:SF87)	O18335_DROME
E9GVW7	-0.76	-0.42	**0.99**	0.08		
E9H082			0.2	**-0.96**	Vitellogenin-2 (PTHR23345:SF1)	
E9H2U7	0.44	0.38	0.15	**1.1**	Profilin (PTHR11604:SF0)	PROF_DROME
E9H736	**-2.21**	-0.74	-0.76	-1.01	Dilute class unconventional myosin, isoform C (PTHR13140:SF191)	A1Z6Z8_DROME
E9H8K5	-1.14	**-1.79**	**-0.68**	-0.65	Vitellogenin-2 (PTHR23345:SF1)	
E9H8M2	-0.54	-0.58	-1.58	**-1.58**	PIWI-like protein 1 (PTHR22892:SF32)	AUB_DROME
E9H9F6	0.19	-0.84	-0.93	**-1.32**	Moesin/ezrin/radixin homolog 1 (PTHR23281:SF18)	MOEH_DROME
E9HJQ1	-0.69	-1.18	-1.06	**-1.24**	Vitellogenini-2 (PTHR23345:SF1)	
E9HLH8	0.67	0.25	-1.14	**-0.84**	Maternal protein exuperantia (PTHR12384:SF1)	EXU_DROME
E9HZI5	1.28	-0.32	0.41	**-0.95**	Superoxide dismutase [CU-ZN] (PTHR10003:SF32)	

Discussion

With a total of 3940 identified and quantified proteins, we report what is to our knowledge the largest proteomic dataset from *D. pulex* published until now. This high number was the result of multiple proteomics analyses at several stages of *D. pulex* life cycle, and of extensive sample fractionation by off-gel focusing prior to LC-MS/MS analyses. It should be noted that since the seminal paper by Froehlich et al. [2] showing the potential of LC-MS/MS for high-throughput gel-free proteomics of *D. pulex*, we only found one article using this technique, but it was restricted to the study of *D. pulex* phosphoproteome [53]. We did not discuss all our data here because of the paper focus on tamoxifen effects on *D. pulex*. In particular, the results of proteomics analyses after 4 days of test (without tamoxifen treatment) were not considered, but they are available for the scientific community as additional time point, which allows following proteome evolution during *D. pulex* development.

One of our concerns for data interpretation was the incomplete annotation of *D. pulex* proteome, as many proteins are still labeled as "putative uncharacterized protein" in UniProt database. Indeed, Colbourne et al. [54] showed that more than a third of Daphnia's genes had no detectable homologs in any other available proteome. This issue could be partly alleviated in our study using the PANTHER database, completed by BLAST homology search, which allowed getting some annotation for about 93% of our identified proteins, and 96% of the 189 significantly regulated proteins. Nevertheless, while most conserved proteins from primary metabolism and translation machinery could be readily characterized, many other proteins were either scarcely annotated or had only low homology with more annotated homologous proteins, which complicated functional interpretation of our results. In this context, most useful information came from the well characterized and complete proteome of *D. melanogaster*, as it belongs to the same Pancrustacea taxon as *D. pulex.*

The identity and annotations of the 189 significantly regulated proteins showed a decrease in translation, an increase in protein degradation, changes in carbohydrate/lipid metabolism, and an impact on reproduction as the major effects of tamoxifen. These impacted processes reflect most likely a general stress response of the organisms, shifting their energy allocation from growth, energy storage, and/or reproduction to survival adaptations. Indeed, Rowe et al. [55] [55] suggested that upon stress, such as environmental pollution, organisms may temporarily reduce other energy costly functions in order to maintain their basic metabolic rate (metabolic cost hypothesis). It should be noted that tamoxifen had similarly been shown on the one hand to induces oxidative stress and mitochondrial apoptosis in rat liver [56], and on the other hand to stimulate glycolysis and inhibit gluconeogenesis in rats as well [57]. In his review, Tomanek [58] also underlined the link between shift of energy metabolism (from aerobic metabolism to alternative pathways of ATP production, such as glycolysis and b-oxidation) and response to oxidative stress. He also suggested that cytoskeleton could be a common cellular target of various environmental stresses.

Jansen et al. [59] previously used the metabolic cost hypothesis to explain the decreased levels of vitellogenin-1 in *Daphnia magna* exposed to natural and anthropogenic stressors. Vitellogenins are the major precursors of the egg-yolk proteins, vitellins, which are sources of nutrients during embryonic development, and they are used as biomarkers of exposure to estrogenic chemicals in aquatic invertebrates [60,61]. In our study indeed, some proteins related to the vitellogenin-2 family were also down-regulated, although some other proteins from the same family did not change their expression after treatment. These contrasting results probably reflect the different, yet unknown, roles of the various members of this protein family. Interestingly, we also observed either the over-expression of one vitellogenin fused with superoxide dismutase domain or the decrease of another such fused vitellogenin, depending on tamoxifen concentration. Such fusion proteins have only been found in some crustaceans [62,63] and their exact function is not yet fully elucidated. Nevertheless, it can be hypothesised that stress situations are associated with oxidative stress at the cellular level, which could result in increased levels of anti-oxidative enzymes such as copper/zinc superoxide dismutases to prevent organisms from cell and tissue damages.

The decrease of various proteins potentially involved in reproduction, that we observed in this study, can be related to previous results (chapter 2), where two generation of *D. pulex* were exposed to tamoxifen at concentrations from 0.15 to 5.15 µg/L. In those experiments, the reproduction rate of the first generation dropped drastically, with about 60% less neonates in the treated animals than in controls. This effect on reproduction was observed after 12 days of exposure to 5.15 µg/L of tamoxifen, while the highest concentration tested here was 2.4 µg/L (C2). Furthermore, teratogenic effects, such as abnormal neonates and aborted eggs, were found at concentrations from 0.15 to 0.72 µg/L. As a comparison, tamoxifen was shown to impair reproduction in fish at a nominal concentration of 625 mg/L, while tamoxifen concentrations equal or higher than 25 mg/L induced various trans-generational effects in progeny and altered vitellogenin levels in adults [64].

Tamoxifen was found in the aquatic environment, including groundwater, at concentrations up to 0.21 µg/L [65,12,66], and the no observed effect concentration (NOEC) previously calculated in *D. pulex* for reproduction was 0.72 µg/L, whereas this NOEC was < 0.15 µg/L when based on observed morphological abnormalities [45]. Similarly, the lower concentration tested here (C1: 0.1 µg/L) is of the same order of magnitude as tamoxifen environmental concentrations. Because some effects of the drug on *D. pulex* protein expression were already observed at this concentration, we determined a proteomics NOEC < 0.1 µg/L. Morphological abnormalities as ecotoxicological endpoint seem therefore to have the same sensitivity as proteomics analyses in this particular case.

It is not clear if tamoxifen has an effect on *D. pulex* reproduction because of the general stress response (metabolic cost hypothesis), or through some more specific mechanism, for example involving the homolog of the estrogen-related receptor known to be present in daphnids [24]. In our results, the up-regulation of some proteins potentially involved in regulation of nuclear hormone receptors seems however to support the second hypothesis.

One interest of this study was to highlight potential biomarkers for early detection of tamoxifen harmful effects on *D. pulex*. As most significantly regulated proteins were observed at the highest exposure in time and concentration, only a few candidates could be suitable for early detection of the drug impact on daphnids, based on our results at 2 days. It is actually expected that the most interesting effects of the drug on *D. pulex* reproduction cannot be observed before the daphnids have reached their sexual maturity. Then, among the significant hits observed with 2 days treatment, we should ideally select proteins with: i) high abundance, ii) good annotation and clear function, iii) specific response to treatment, avoiding for example ribosomal proteins regulated by the general stress response of the organisms. The latest point is of course quite difficult as the exact mechanism of action of tamoxifen in daphnids is not known. Furthermore some significant effects observed at 2 days were not confirmed at longer exposures, either because of higher variance (and consequently lower statistical testing sensitivity) of some protein quantification or because of *D. pulex* adaptation to tamoxifen effects after some time. At this stage the most promising potential biomarkers seem therefore to be one protein of the vitellogenin-2 family (E9H8K5) and the ryanodine receptor (E9FTU9), which both were identified with a high number of peptides and were already down-regulated after 2 days of treatment at the highest concentration tested. Of course, further experiments will be necessary to confirm this very preliminary choice or to select some other more promising candidates.

Finally, an important question for future ecotoxicoproteomics experiments is the effect of DMSO on protein expression in *D. pulex*. Although DMSO is considered as non-toxic in daphnids [67] and is often used in ecotoxcological experiments to dissolve chemicals poorly soluble in water, our data seem to show that at all time points this solvent can have an impact on organisms, at least at the biochemical level.

Conclusions

We could show the effects of tamoxifen on *D. pulex* at the protein level, and unravel potential links between drug impact on daphnid reproduction and observations at the biochemical level. In agreement with our previous results, some tamoxifen effects were already observed at concentrations close to those detected in the aquatic environment. Nevertheless, further experiments would be needed to confirm and determine the robustness of our observations. New experiments would also benefit from constant advances in mass spectrometer performance. Our data were acquired on an Orbitrap mass spectrometer of the first generation, and much progress has since been done in instrument speed and sensitivity, as demonstrated by the Fusion Tribrid™, the last generation of Orbitrap mass spectrometer [68]. Using our sample preparation method, such instrumental improvements could allow either increasing proteome coverage, or having the same number of proteins identified with a smaller

number of sample fractions and/or lower instrumental analysis time. Ultimately, it will also be possible to study protein expression of daphnids at the individual level with fair proteome coverage.

Further ecotoxicoproteomics studies should also ideally include quantification of protein post-translational modifications (PTM), as it is an essential level of protein activity regulation in eukaryotes. As far as we know, there was however only one study of PTMs in daphnids by Kwon et al. [53]. After phosphopeptide enrichment by TiO_2, they identified 103 phosphorylation sites in 91 *D. pulex* proteins, of which 21 sites in 20 proteins were conserved between *D. pulex* and humans. These are very useful data, as phosphorylation is the most studied PTM in proteomics, but post-translational modifications remain yet a largely unexplored area in daphnids

Furthermore, as tamoxifen can be metabolized in more or equally toxic compounds, effects of most relevant metabolites on protein expression in daphnids also deserve to be tested. Indeed, it has been recently shown in a two-generation study that metabolites such as 4-hydroxy-tamoxifen and endoxifen had an effect on *D. pulex* reproduction at concentrations of the same order of magnitude as tamoxifen concentrations [69]. Further ecotoxicoproteomics experiments with tamoxifen and its metabolites should also be carried out in other organisms for a better assessment of tamoxifen impact on aquatic ecosystems.

In conclusion, ecotoxicoproteomics studies cannot replace classical ecotoxicological tests, which can provide, relatively quickly and with little instrumentation, a view of chemical compound effects on organisms at the individual and population levels. But proteomics analyses allow understanding the xenobiotic effects at the biochemical level and highlighting their potential mechanisms of toxicity. It is therefore an essential complementary tool, which will be increasingly used in ecotoxicology in the near future to improve risk assessment of critical pollutants in the environment.

Acknowledgments

This project was funded by the Swiss National Science Foundation (http://p3.snf.ch/project-132344) and supported by the Research Commission of the Faculty of Biology and Medicine (University of Lausanne). The authors would like to thank Sandra Cruchon for her meticulous work at the Laboratory of Clinical Pharmacology (CHUV), Alexandra Potts-Xenarios at the Protein Analysis Facility for the preparation of proteomics samples and Dr. Manfredo Quadroni for his comments on the manuscript. We also acknowledge the PRIDE team for the deposition of our data to the ProteomeXchange Consortium.

Supporting information

Legends

Supplementary table 1 (see original manuscript) MaxQuant output table (= proteinGroups) after removal of contaminants, reverse proteins and proteins only identified by site. Only the 3940 proteins identified with a minimum of one unique peptide and quantified in at least one replicate sample were then kept. Relative standard deviation of LFQ peak intensity was calculated for proteins quantified in both replicates of each experiment.

Supplementary table 2. (see original manuscript) List of all proteins (3662) getting some annotations through PANTHER (www.pantherdb.org).

Supplementary table 3. (see original manuscript) Quantitation results of comparison between samples and DMSO controls after, for each comparison, filtering out of proteins identified with less than three peptides and those having one missing quantitative value. Data were normalized using the variance stabilizing method (vsn), applying a generalized log2 transformation (glog2) on intensities. For each tested concentration and time point, fold change (in log2 scale) was calculated as the median of duplicate normalized protein intensities compared to corresponding DMSO controls, or to M4 blanks for DMSO samples. For each comparison, only proteins appearing in all 4 replicates and quantified by a minimum of 1 peptide were considered. Statistical analyses were performed with local-pooled-error (LPE) estimates. Benjamini-Hochberg method was used for multiple testing corrections and a LPE-based test was carried out for outlier detection.

Supplementary table 4. List of the 189 proteins showing a significant fold change with LPE test, after multiple-testing correction and removal of outliers. PANTHER annotations are included and all tamoxifen treatment ratios are shown (C1 – 2 days, C2 – 2 days, C1 – 7 days, C2 - 7 days), with significant ones highlighted in grey.

UniProt ID	Peptides	Log ratio C1/DMSO 2d	Log ratio C2/DMSO 2d	Log ratio C1/DMSO 7d	Log ratio C2/DMSO 7d	Protein name	PANTHER Family/Subfamily
C7DR07	11	-0.20	-0.66	-2.23	**-1.93**	Putative uncharacterized protein	PROTEIN Y7A5A.1 (PTHR10801:SF2)
E9FQU4	8	0.68	0.82	0.92	**0.85**	Putative uncharacterized protein	LETHAL (3) 03670 (PTHR21588:SF1)
E9FRE7	20	-0.02	-0.48	-0.10	**-0.82**	Citrate synthase	CITRATE SYNTHASE, MITOCHONDRIAL (PTHR11739:SF8)
E9FRN7	12	0.83	1.03	0.89	**0.88**	Putative uncharacterized protein	3-HYDROXYACYL-COA DEHYDROGENASE TYPE-2 (PTHR24316:SF251)
E9FRQ6	22	0.51	1.05	**0.90**	0.76	Phosphoglycerate kinase	PHOSPHOGLYCERATE KINASE (PTHR11406:SF6)
E9FS67	11	0.43	1.20	0.16	**1.07**	Putative uncharacterized protein	PROTEIN C07D8.6 (PTHR11732:SF144)
E9FSI1	14	-0.15	-0.45	-1.39	**-1.07**	Putative uncharacterized protein	LIM-9 ISOFORM (PTHR24205:SF4)
E9FSQ9	4	0.46	-0.33	-2.05	**-1.37**	Putative uncharacterized protein	40S RIBOSOMAL PROTEIN S25 (PTHR12850:SF5)
E9FSV8	11	-0.28	-1.65	-1.26	**-2.00**	Putative uncharacterized protein	GDP-L-FUCOSE SYNTHASE (PTHR10366:SF259)
E9FTB0	8	-0.37	-1.40	-1.82	**-2.34**	Putative uncharacterized protein	GM23292P (PTHR21106:SF2)
E9FTC5	20	-0.32	-0.80	-1.67	**-0.97**	Putative uncharacterized protein	CYSTEINE--TRNA LIGASE, CYTOPLASMIC (PTHR10890:SF3)
E9FTU9	63	-1.29	**-1.74**	-0.84	**-1.14**	Putative uncharacterized protein	RYANODINE RECEPTOR 44F (PTHR13715:SF74)
E9FTX7	11	-0.43	-1.85	-3.65	**-3.26**	Putative uncharacterized protein	NAT1 (PTHR22767:SF2)
E9FTY4	9	-0.47	-1.42	-1.53	**-1.35**	Putative uncharacterized protein	26S PROTEASOME NON-ATPASE REGULATORY SUBUNIT 13 (PTHR10539:SF0)
E9FU59	23	0.15	-0.96	**-1.44**	**-1.48**	Putative uncharacterized protein	40S RIBOSOMAL PROTEIN S3A (PTHR11830:SF0)
E9FUJ2	11	-0.76	-1.12	-0.55	**-0.89**	Putative uncharacterized protein	SUBFAMILY NOT NAMED (PTHR11590:SF16)
E9FUY1	13	-0.01	-0.76	-0.84	**-1.09**	ABC protein, subfamily ABCF	ATP-BINDING CASSETTE SUB-FAMILY F MEMBER 2 (PTHR19211:SF15)
E9FV57	32	0.45	0.55	0.68	**0.70**	Putative uncharacterized protein	ARGININE KINASE (PTHR11547:SF18)
E9FV79	9	-0.28	-0.76	-0.25	**-0.85**	Putative uncharacterized protein	NADH DEHYDROGENASE [UBIQUINONE] 1 BETA SUBCOMPLEX SUBUNIT 10 (PTHR13094:SF1)
E9FV80	13	-0.62	-1.75	**-3.92**		Putative uncharacterized protein	60S RIBOSOMAL PROTEIN L3 (PTHR11363:SF5)
E9FVG7	201	-0.53	-0.98	-0.40	**-0.81**	Putative uncharacterized protein	VITELLOGENIN-2 (PTHR23345:SF1)
E9FVV4	5	1.20	0.26	-0.56	**-0.86**	Putative uncharacterized protein	
E9FW02	10	0.16	0.28	0.20	**0.87**	Cytochrome b-c1 complex subunit Rieske, mitochondrial	CYTOCHROME B-C1 COMPLEX SUBUNIT RIESKE, MITOCHONDRIAL (PTHR10134:SF4)
E9FWW3	6	0.36	-0.54	**0.85**	0.47	Putative uncharacterized protein DpGSTM1	SUBFAMILY NOT NAMED (PTHR11571:SF108)
E9FWW9	21	-0.38	-1.32	-1.96	**-1.96**	Alpha subunit of putative Na+/K+ ATPase	SUBFAMILY NOT NAMED (PTHR24093:SF244)
E9FWX1	11	0.18	0.92	0.39	**0.87**	Putative uncharacterized protein	ELECTRON TRANSFER FLAVOPROTEIN SUBUNIT BETA (PTHR21294:SF0)
E9FX66	64	-1.28	-1.53	**-2.89**	**-1.91**	Putative uncharacterized protein	CYTOPLASMIC DYNEIN 1 HEAVY CHAIN 1 (PTHR10676:SF28)
E9FXJ7	16	-0.09	-1.09	-0.09	**-1.36**	Hemoglobin	GLOBIN 1, ISOFORM A (PTHR11442:SF36)
E9FXU5	40	0.42	0.47	0.66	**1.07**	Putative uncharacterized protein	VILLIN-LIKE PROTEIN QUAIL (PTHR11977:SF37)
E9FYH7	13	0.46	0.48	0.49	**1.25**	Putative uncharacterized protein	2-HYDROXYACYL-COA LYASE 1 (PTHR18968:SF6)
E9FYV4	7	-1.00	-1.22	-1.99	**-2.03**	Putative uncharacterized protein	PROTEIN DHS-20 (PTHR24316:SF215)
E9FZ14	29	0.28	0.53	0.64	**0.88**	Putative uncharacterized protein	ATPASE, H+ TRANSPORTING, LYSOSOMAL V1 SUBUNIT B2-RELATED (PTHR15184:SF11)
E9FZ54	3	0.53	-0.44	**-2.96**	**-1.77**	Putative uncharacterized protein	40S RIBOSOMAL PROTEIN S26-RELATED (PTHR12538:SF0)
E9FZS1	8	0.46	1.21	1.21	**1.12**	Putative uncharacterized protein	CARBONYL REDUCTASE 1-RELATED (PTHR24322:SF58)
E9FZS8	189	0.54	-0.44	-0.53	**-1.19**	Myosin heavy chain isoform 3	MYOSIN HEAVY CHAIN, ISOFORM O-RELATED (PTHR13140:SF364)
E9G063	13	0.20	0.80	0.65	**1.54**	Putative uncharacterized protein	LIPOAMIDE ACYLTRANSFERASE COMPONENT OF BRANCHED-CHAIN ALPHA-KETO ACID DEHYDROGENASE COMPLEX, MITOCHONDRIAL (PTHR23151:SF46)
E9G064	15	-0.67	-1.45	-2.60	**-1.64**	Putative uncharacterized protein	PUMILIO DOMAIN-CONTAINING PROTEIN KIAA0020 (PTHR13389:SF0)
E9G0H6	3	0.82	1.76	-1.07	**-3.17**	Putative uncharacterized protein	
E9G0P4	33	-0.22	-0.67	-0.61	**-0.83**	Putative uncharacterized protein	SUBFAMILY NOT NAMED (PTHR11590:SF40)
E9G0Q9	14	-0.14	-0.39	**0.96**	0.40	Putative uncharacterized protein	GLUCOSAMINE-6-PHOSPHATE ISOMERASE (PTHR11280:SF5)
E9G0Y9	12	0.49	0.80	0.47	**0.76**	Putative uncharacterized protein	NEOSIN (PTHR23295:SF2)
E9G119	9	0.00	-0.27	0.04	**-0.85**	Prostaglandin D2 synthase-like protein	HEMATOPOIETIC PROSTAGLANDIN D SYNTHASE (PTHR11571:SF118)
E9G121	14	0.01	-0.74	**-1.57**	**-1.15**	Putative uncharacterized protein	40S RIBOSOMAL PROTEIN S16 (PTHR21569:SF4)
E9G153	11	-1.08	-1.52	-1.17	**-1.62**	Beta subunit of putative Na+/K+ ATPase	AT04468P-RELATED (PTHR11523:SF22)
E9G1G4	3	-0.16	-1.16	-1.08	**-1.37**	Putative uncharacterized protein	SIGNAL PEPTIDASE COMPLEX SUBUNIT 3 (PTHR12804:SF0)
E9G1N6	4	0.59	0.34	**-4.12**		Putative uncharacterized protein	PROTEIN C13B4.1, ISOFORM A (PTHR23130:SF66)
E9G230	12	-0.01	-0.21	-0.45	**-0.90**	Uroporphyrinogen decarboxylase	UROPORPHYRINOGEN DECARBOXYLASE (PTHR21091:SF2)

Supplementary table 4 (continued)

UniProt ID	Peptides	Log ratio C1/DMSO 2d	Log ratio C2/DMSO 2d	Log ratio C1/DMSO 7d	Log ratio C2/DMSO 7d	Protein name	PANTHER Family/Subfamily
E9G2A6	33	-0.03	-0.74	-1.05	**-1.35**	Putative uncharacterized protein	FI05224P (PTHR11451:SF22)
E9G2G6	54	-0.49	-0.97	**-1.12**	**-1.24**	Phosphorylase	GLYCOGEN PHOSPHORYLASE, MUSCLE FORM (PTHR11468:SF5)
E9G2I4	9	0.58	1.24	0.75	**1.16**	Putative uncharacterized protein	PROTEIN ATH-1 (PTHR10655:SF17)
E9G2W4	100	0.12	-0.49	0.03	**-1.03**	Putative uncharacterized protein	FILAMIN-A (PTHR11915:SF251)
E9G341	29	-0.37	-0.53	-0.70	**-1.11**	Putative uncharacterized protein	CAD PROTEIN (PTHR11405:SF5)
E9G3B9	13	0.40	1.26	**1.41**	**2.00**	Putative uncharacterized protein	DIHYDROPTERIDINE REDUCTASE (PTHR15104:SF0)
E9G3M6	11	0.57	-0.15	**-1.00**	-0.40	Putative uncharacterized protein	40S RIBOSOMAL PROTEIN S14 (PTHR11759:SF1)
E9G4Z8	5	-1.48	-1.98	-3.00	**-3.60**	Putative uncharacterized protein	TRICARBOXYLATE TRANSPORT PROTEIN, MITOCHONDRIAL (PTHR24089:SF102)
E9G5T1	18	-0.16	-0.79	-0.55	**-1.00**	Putative uncharacterized protein	NADH DEHYDROGENASE [UBIQUINONE] 1 ALPHA SUBCOMPLEX SUBUNIT 9, MITOCHONDRIAL (PTHR12126:SF1)
E9G631	16	0.54	0.93	0.73	**0.94**	Putative uncharacterized protein	FUMARATE HYDRATASE, MITOCHONDRIAL (PTHR11444:SF1)
E9G6B6	5	0.31	-1.11	-1.61	**-1.72**	Putative uncharacterized protein	TRANSMEMBRANE EMP24 DOMAIN-CONTAINING PROTEIN 10 (PTHR22811:SF58)
E9G6C5	4	1.46	0.26	-1.49	**-1.39**	Putative uncharacterized protein	
E9G6H2	11	-0.09	-0.63	-0.37	**-1.07**	Putative uncharacterized protein	GLUTAMATE--CYSTEINE LIGASE CATALYTIC SUBUNIT (PTHR11164:SF0)
E9G6K9	10	0.22	**-1.82**	**-3.04**	**-2.62**	Putative uncharacterized protein (Fragment)	60S RIBOSOMAL PROTEIN L13 (PTHR11722:SF0)
E9G6L8	7	-0.72	**-2.34**	-2.51	**-2.54**	Putative uncharacterized protein	DOLICHYL-DIPHOSPHOOLIGOSACCHARIDE--PROTEIN GLYCOSYLTRANSFERASE SUBUNIT STT3A (PTHR13872:SF21)
E9G6P0	57	-0.24	-1.08	**-1.04**	**-1.04**	Putative uncharacterized protein	ALPHA-ACTININ, SARCOMERIC (PTHR11915:SF270)
E9G6S7	3	-1.08	-1.32	-1.70	**-1.72**	Putative uncharacterized protein	PROTEIN DPY-11 (PTHR18929:SF81)
E9G787	5	1.04	1.54	0.51	**0.98**	Putative uncharacterized protein	10 KDA HEAT SHOCK PROTEIN, MITOCHONDRIAL (PTHR10772:SF5)
E9G7M2	5	-0.13	0.28	0.52	**0.69**	Proteasome subunit beta type	PROTEASOME SUBUNIT BETA TYPE-4 (PTHR11599:SF5)
E9G8G7	7	-0.67	-0.97	-1.19	**-1.32**	Putative uncharacterized protein	RAS-RELATED PROTEIN RAB-1B (PTHR24073:SF212)
E9G8L4	12	-0.19	-1.48	**-3.05**	**-3.06**	Putative uncharacterized protein	MIP08013P1 (PTHR24089:SF50)
E9G8S6	4	**2.13**	0.44	-1.68	-1.11	Putative uncharacterized protein	
E9G927	16	0.33	1.17	1.12	**1.43**	Putative uncharacterized protein	ALCOHOL DEHYDROGENASE CLASS-3 (PTHR11695:SF269)
E9GA02	6	-0.05	-0.81	**-3.61**	**-2.47**	40S ribosomal protein S24	40S RIBOSOMAL PROTEIN S24 (PTHR10496:SF0)
E9GA08	35	-0.50	-0.81	-1.32	**-1.07**	Putative uncharacterized protein	COATOMER SUBUNIT ALPHA (PTHR19876:SF1)
E9GA58	8	0.13	-0.91	-1.19	**-1.44**	Mago nashi-like protein	PROTEIN MAGO NASHI HOMOLOG (PTHR12638:SF0)
E9GA96	12	0.36	-0.40	**-1.29**	-0.23	Putative uncharacterized protein	40S RIBOSOMAL PROTEIN S7 (PTHR11278:SF0)
E9GAE4	28	0.00	-0.36	-0.13	**-0.92**	Isocitrate dehydrogenase [NADP]	
E9GAI5	14	-0.29	-0.39	0.29	**0.85**	Putative uncharacterized protein	PROTEIN Y59C2A.1 (PTHR11705:SF61)
E9GAM4	26	-0.41	-0.09	**0.83**	**1.06**	Enolase	ENOLASE (PTHR11902:SF7)
E9GAM5	13	-0.74	-1.14	-1.61	**-1.77**	6-phosphogluconate dehydrogenase, decarboxylating	6-PHOSPHOGLUCONATE DEHYDROGENASE, DECARBOXYLATING (PTHR11811:SF25)
E9GAM6	11	0.11	0.39	0.45	**1.05**	Putative uncharacterized protein	PROHIBITIN-2 (PTHR23222:SF1)
E9GB46	5	-0.14	-1.44	-2.86	**-2.57**	Putative uncharacterized protein	60S RIBOSOMAL PROTEIN L28 (PTHR10544:SF0)
E9GBH6	11	-0.17	-1.18	-2.28	**-1.67**	Putative uncharacterized protein	40S RIBOSOMAL PROTEIN S11 (PTHR10744:SF9)
E9GBP0	27	-0.40	-1.11	-1.04	**-0.84**	Putative uncharacterized protein	ISOLEUCINE--TRNA LIGASE, CYTOPLASMIC (PTHR11946:SF11)
E9GCC8	11	-0.20	-0.97	-0.09	**-1.02**	Putative uncharacterized protein	SERINE--TRNA LIGASE, CYTOPLASMIC (PTHR11778:SF0)
E9GCI5	7	-0.89	-1.38	-0.99	**-1.79**	Hemoglobin	NEUROGLOBIN (PTHR22924:SF30)
E9GCK2	20	-0.35	-1.50	-1.48	**-1.44**	Glucose-6-phosphate isomerase	GLUCOSE-6-PHOSPHATE ISOMERASE (PTHR11469:SF4)
E9GCL9	13	-0.83	-1.19	-0.03	**-0.91**	Glucose-6-phosphate 1-dehydrogenase	GLUCOSE-6-PHOSPHATE 1-DEHYDROGENASE (PTHR23429:SF0)
E9GCN3	12	0.11	1.04	0.66	**0.88**	Putative uncharacterized protein	3-HYDROXYISOBUTYRATE DEHYDROGENASE, MITOCHONDRIAL (PTHR22981:SF7)
E9GD42	6	-0.37	-0.80	-0.10	**-1.38**	Putative uncharacterized protein	HEMATOPOIETIC PROSTAGLANDIN D SYNTHASE (PTHR11571:SF118)
E9GDK0	10	-0.89	-1.20	-0.83	**-0.91**	Putative uncharacterized protein	GH09086P (PTHR24073:SF352)
E9GDV9	11	0.08	-0.88	-0.49	**-1.51**	Putative uncharacterized protein	CYTOSOLIC SULFOTRANSFERASE 1-RELATED (PTHR11783:SF15)
E9GE27	5	0.96	**2.16**	1.19	2.19	Putative uncharacterized protein	PROTEIN DHS-13 (PTHR24322:SF239)
E9GER1	27	0.23	0.56	0.28	**0.81**	Putative uncharacterized protein	TUBULIN BETA-1 CHAIN (PTHR11588:SF9)
E9GER2	19	-0.86	-1.32	-2.06	**-1.44**	Putative uncharacterized protein	EUKARYOTIC TRANSLATION INITIATION FACTOR 3 SUBUNIT L (PTHR13242:SF0)
E9GEV7	11	-0.42	-1.00	**-1.99**	**-2.03**	Putative uncharacterized protein	RIBOSOMAL PROTEIN S15A (PTHR11758:SF4)
E9GEY1	12	0.30	-0.98	-1.56	**-1.46**	Eukaryotic translation initiation factor 3 subunit E	EUKARYOTIC TRANSLATION INITIATION FACTOR 3 SUBUNIT E (PTHR10317:SF0)
E9GHX8	30	0.02	-0.78	-1.00	**-0.85**	Putative uncharacterized protein	CLUSTERED MITOCHONDRIA PROTEIN HOMOLOG (PTHR12601:SF6)
E9GI31	10	-0.26	-1.29	-1.47	**-2.69**	Putative uncharacterized protein	CYTOCHROME C OXIDASE SUBUNIT IV ISOFORM 1 (PTHR10707:SF10)
E9GIX9	11	0.34	0.85	0.95	**1.89**	Proteasome subunit alpha type	PROTEASOME SUBUNIT ALPHA TYPE-2 (PTHR11599:SF16)
E9GJA1	10	-0.98	-1.39	-1.26	**-1.25**	Putative uncharacterized protein	DRAB11 (PTHR24073:SF87)
E9GKM8	15	-0.59	-1.33	-1.00	**-1.23**	Putative uncharacterized protein	AP-2 COMPLEX SUBUNIT ALPHA (PTHR22780:SF4)
E9GL54	3	-1.31	0.72	0.28	**1.53**	Putative uncharacterized protein	TRANSLOCON-ASSOCIATED PROTEIN SUBUNIT BETA (PTHR12861:SF3)
E9GL77	7	0.64	0.87	0.58	**0.79**	Putative uncharacterized protein	F-ACTIN-CAPPING PROTEIN SUBUNIT BETA (PTHR10619:SF0)

Supplementary table 4 (continued)

UniProt ID	Peptides	Log ratio C1/DMSO 2d	Log ratio C2/DMSO 2d	Log ratio C1/DMSO 7d	Log ratio C2/DMSO 7d	Protein name	PANTHER Family/Subfamily
E9GLP0	6	-0.01	0.98	0.59	**0.85**	Putative uncharacterized protein	SUBFAMILY NOT NAMED (PTHR18959:SF51)
E9GLV0	32	0.07	-0.43	-0.58	**-0.87**	Putative uncharacterized protein	NEURAL CONSERVED AT 73EF, ISOFORM I (PTHR23152:SF6)
E9GMB1	15	-0.66	-1.37	-1.22	**-1.35**	Putative uncharacterized protein	ATP-DEPENDENT RNA HELICASE DDX6-RELATED (PTHR24031:SF76)
E9GMP2	12	0.64	0.10	-0.95	**-1.32**	Putative uncharacterized protein	CALNEXIN 99A, ISOFORM C (PTHR11073:SF1)
E9GN01	7	-0.46	-1.66	-2.04	**-1.88**	Putative uncharacterized protein	DOLICHOL-PHOSPHATE MANNOSYLTRANSFERASE (PTHR10859:SF37)
E9GN74	11	0.79	**1.87**	**0.93**	**0.95**	Putative uncharacterized protein	
E9GN81	4	0.23	0.55	0.87	**1.27**	ATP-dependent Clp protease proteolytic subunit	ATP-DEPENDENT CLP PROTEASE PROTEOLYTIC SUBUNIT, MITOCHONDRIAL-RELATED (PTHR10381:SF11)
E9GN88	14	-0.16	-0.45	-0.78	**-0.83**	Putative uncharacterized protein	EUKARYOTIC TRANSLATION INITIATION FACTOR 2 SUBUNIT 3 (PTHR23115:SF9)
E9GNF0	9	0.36	1.06	0.79	**1.17**	Putative uncharacterized protein	GH08269P (PTHR13586:SF0)
E9GPB6	15	-0.57	-1.12	-0.56	**-1.02**	Putative uncharacterized protein	NADH DEHYDROGENASE [UBIQUINONE] FLAVOPROTEIN 1, MITOCHONDRIAL (PTHR11780:SF0)
E9GR53	11	-0.32	-1.46	-2.38	**-2.38**	Putative uncharacterized protein	40S RIBOSOMAL PROTEIN S2 (PTHR13718:SF4)
E9GRL8	18	-1.27	-1.34	-1.23	**-1.34**	Putative uncharacterized protein	PROTEIN CAND-1 (PTHR12696:SF0)
E9GTP3	67	-0.67	-1.26	**-1.19**	**-1.15**	Putative uncharacterized protein	CLATHRIN HEAVY CHAIN (PTHR10292:SF1)
E9GVE9	9	0.43	0.43	0.53	**0.82**	Putative uncharacterized protein	ENOYL-COA HYDRATASE, MITOCHONDRIAL (PTHR11941:SF24)
E9GVW7	150	-0.76	-0.42	**0.99**	0.08	Vitellogenin fused with superoxide dismutase (fragment)	
E9GX17	7	0.12	-0.90	-1.32	**-1.45**	Putative uncharacterized protein	NADH DEHYDROGENASE [UBIQUINONE] 1 ALPHA SUBCOMPLEX SUBUNIT 6 (PTHR12964:SF0)
E9GXS2	18	0.25	-0.70	**-0.97**	**-0.78**	Putative uncharacterized protein	40S RIBOSOMAL PROTEIN S3 (PTHR11760:SF9)
E9GXW8	12	0.73	0.73	**0.89**	0.74	Putative uncharacterized protein	CYTOCHROME C OXIDASE SUBUNIT 5A, MITOCHONDRIAL (PTHR14200:SF11)
E9GXZ9	8	0.12	-0.40	-2.02	**-2.38**	Putative uncharacterized protein	SULFIDE:QUINONE OXIDOREDUCTASE, MITOCHONDRIAL (PTHR10632:SF2)
E9GYD9	11	0.29	1.07	1.21	**2.08**	Putative uncharacterized protein	ELECTRON TRANSFER FLAVOPROTEIN SUBUNIT ALPHA, MITOCHONDRIAL (PTHR10909:SF91)
E9GZ69	12	-0.27	-0.65	-0.96	**-0.91**	Putative uncharacterized protein	DOLICHYL-DIPHOSPHOOLIGOSACCHARIDE--PROTEIN GLYCOSYLTRANSFERASE SUBUNIT 1 (PTHR21049:SF0)
E9GZ78	8	0.58	0.40	0.33	**1.05**	Proteasome subunit beta type	PROTEASOME SUBUNIT BETA TYPE (PTHR11599:SF4)
E9H082	34			0.20	**-0.96**	Putative uncharacterized protein	VITELLOGENIN-2 (PTHR23345:SF1)
E9H0V8	12	0.11	-0.57	-0.76	**-1.07**	Putative uncharacterized protein	GLYCOSYLTRANSFERASE 25 FAMILY MEMBER (PTHR10730:SF2)
E9H0Z6	13	-0.57	-1.17	-1.55	**-1.30**	Putative uncharacterized protein	NADH DEHYDROGENASE [UBIQUINONE] IRON-SULFUR PROTEIN 2, MITOCHONDRIAL (PTHR11993:SF10)
E9H1K6	19	0.22	0.71	0.66	**0.85**	Putative uncharacterized protein	DIHYDROLIPOYLLYSINE-RESIDUE ACETYLTRANSFERASE COMPONENT OF PYRUVATE DEHYDROGENASE COMPLEX, MITOCHONDRIAL (PTHR23151:SF9)
E9H1S8	12	0.75	1.29	-0.02	**0.92**	Putative uncharacterized protein	HETEROGENEOUS NUCLEAR RIBONUCLEOPROTEIN A0 (PTHR24012:SF339)
E9H1V2	12	-0.76	-1.01	-1.92	**-1.65**	Putative glycogen synthase	GLYCOGEN [STARCH] SYNTHASE-RELATED (PTHR10176:SF0)
E9H1Y1	9	-0.57	-0.93	-0.96	**-1.07**	Putative uncharacterized protein	AT04468P-RELATED (PTHR11523:SF22)
E9H1Z1	4	-1.11	**-2.32**	-1.18	-0.08	Putative uncharacterized protein	VERY-LONG-CHAIN 3-OXOOACYL-COA REDUCTASE LET-767-RELATED (PTHR24316:SF68)
E9H215	9	0.12	0.86	**0.91**	**1.19**	Putative uncharacterized protein	MUSCLE-SPECIFIC PROTEIN 20 (PTHR18959:SF23)
E9H230	3	-0.65	**-2.29**		**-3.71**	Putative uncharacterized protein	NADH DEHYDROGENASE [UBIQUINONE] 1 BETA SUBCOMPLEX SUBUNIT 5, MITOCHONDRIAL (PTHR13178:SF0)
E9H2T2	20	0.05	0.73	**0.79**	**0.81**	Putative uncharacterized protein	SUBFAMILY NOT NAMED (PTHR11851:SF105)
E9H2T3	18	0.22	-0.02	-1.19	**-1.03**	Putative uncharacterized protein	EUKARYOTIC TRANSLATION INITIATION FACTOR 3 SUBUNIT D (PTHR12399:SF0)
E9H2U7	6	0.44	0.38	0.15	**1.10**	Profilin	PROFILIN (PTHR11604:SF0)
E9H393	11	0.40	0.10	-0.85	**-0.90**	ABC protein, subfamily ABCF	ATP-BINDING CASSETTE SUB-FAMILY F MEMBER 1 (PTHR19211:SF14)
E9H3T0	11	**2.04**	0.42	-1.29	0.75	Putative uncharacterized protein	NUCLEOLIN 1-RELATED (PTHR24012:SF330)
E9H4B8	14	0.11	-0.27	-0.31	**-0.85**	Putative uncharacterized protein	FRAGILE X MENTAL RETARDATION SYNDROME-RELATED PROTEIN 1 (PTHR10603:SF7)
E9H4K0	5	-1.32	**-2.40**	-0.49	-0.60	Putative uncharacterized protein	FI18627P1 (PTHR11782:SF30)
E9H6I9	13	0.64	1.23	**0.82**	**0.93**	Putative uncharacterized protein	CALPONIN-LIKE PROTEIN CHD64 (PTHR18959:SF39)
E9H6N4	15	-0.49	**-1.79**	-2.25	**-1.78**	Putative uncharacterized protein	26S PROTEASOME NON-ATPASE REGULATORY SUBUNIT 3 (PTHR10758:SF2)
E9H6U8	21	-0.65	-0.74	-0.89	**-1.03**	Putative uncharacterized protein	CALCIUM-BINDING MITOCHONDRIAL CARRIER PROTEIN ARALAR1 (PTHR24089:SF13)
E9H707	15	-0.05	-0.03	0.78	**1.16**	Putative uncharacterized protein	LONG-CHAIN SPECIFIC ACYL-COA DEHYDROGENASE, MITOCHONDRIAL (PTHR10909:SF196)
E9H736	3	**-2.21**	-0.74	-0.76	-1.01	Putative uncharacterized protein	DILUTE CLASS UNCONVENTIONAL MYOSIN, ISOFORM C (PTHR13140:SF191)
E9H7R2	14	0.01	-0.38	-0.99	**-1.15**	Putative uncharacterized protein	AT24389P (PTHR11153:SF8)
E9H7X5	62	-0.65	-0.99	-1.35	**-1.12**	Putative uncharacterized protein	TRANSLATIONAL ACTIVATOR GCN1 (PTHR23346:SF7)
E9H862	50	-0.26	-1.23	-1.07	**-1.14**	Putative uncharacterized protein	BIFUNCTIONAL GLUTAMATE/PROLINE--TRNA LIGASE (PTHR10119:SF15)
E9H8K5	99	-1.14	**-1.79**	**-0.68**	-0.65	Putative uncharacterized protein	VITELLOGENIN-2 (PTHR23345:SF1)
E9H8M2	26	-0.54	-0.58	-1.58	**-1.58**	Piwi/Aubergine-like protein variant 1	PIWI-LIKE PROTEIN 1 (PTHR22892:SF32)
E9H8T5	23	0.47	0.38	0.19	**0.84**	Aspartate aminotransferase	GLUTAMATE OXALOACETATE TRANSAMINASE 1, ISOFORM B (PTHR11879:SF5)
E9H9F6	23	0.19	-0.84	-0.93	**-1.32**	Putative uncharacterized protein	MOESIN/EZRIN/RADIXIN HOMOLOG 1 (PTHR23281:SF18)
E9H9P6	14	0.04	-0.63	-1.20	**-1.03**	Putative uncharacterized protein	PROTEIN ANON-37CS (PTHR10742:SF250)

Supplementary table 4 (continued)

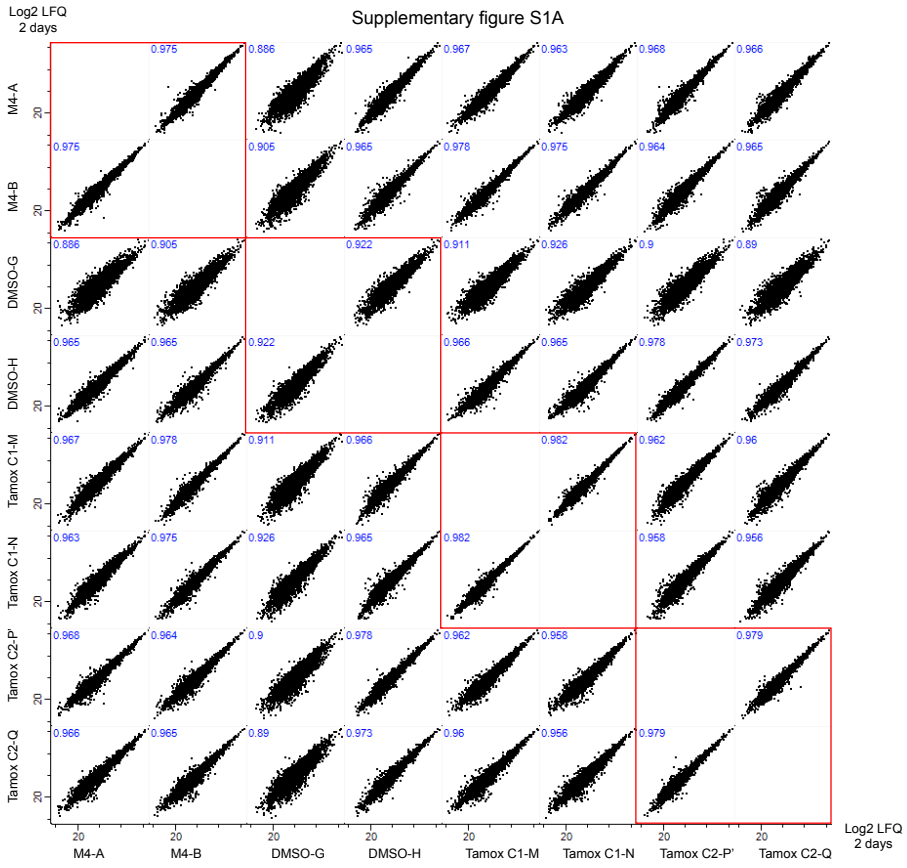
UniProt ID	Peptides	Log ratio C1/DMSO 2d	Log ratio C2/DMSO 2d	Log ratio C1/DMSO 7d	Log ratio C2/DMSO 7d	Protein name	PANTHER Family/Subfamily
E9H9X9	19	-0.08	-0.77	-1.73	**-1.25**	Nucleolar protein-like protein 5A	NUCLEOLAR PROTEIN 56 (PTHR10894:SF0)
E9HA01	21	0.27	-0.43	**-1.55**	**-1.27**	Putative uncharacterized protein	SUBFAMILY NOT NAMED (PTHR11590:SF40)
E9HA96	14	0.61	1.38	0.65	**0.79**	Malate dehydrogenase	MALATE DEHYDROGENASE, CYTOPLASMIC (PTHR23382:SF3)
E9HAP5	9	0.16	0.29	0.68	**1.37**	Proteasome subunit alpha type	PROTEASOME SUBUNIT ALPHA TYPE-6 (PTHR11599:SF11)
E9HC70	7	0.42	0.57	0.79	**0.71**	Putative uncharacterized protein	ACETYL-COA ACETYLTRANSFERASE, MITOCHONDRIAL (PTHR18919:SF79)
E9HCE1	3	-2.09	**-3.25**	-2.80	**-3.11**	Putative uncharacterized protein	PROTEIN F56B3.6 (PTHR12300:SF22)
E9HCT5	19	-0.54	-1.16	-2.13	**-1.83**	Putative uncharacterized protein	VALYL-TRNA SYNTHETASE, ISOFORM A (PTHR11946:SF5)
E9HD09	5	-0.26	0.04	-0.02	**1.50**	Putative uncharacterized protein	FERRITIN (PTHR11431:SF4)
E9HF12	23	-0.48	-1.38	-1.69	**-1.34**	Putative uncharacterized protein	LEUCINE--TRNA LIGASE, CYTOPLASMIC (PTHR11946:SF51)
E9HFQ5	5		-1.93	-1.29	**-2.31**	Ubiquitin carboxyl-terminal hydrolase	SUBFAMILY NOT NAMED (PTHR24006:SF421)
E9HG20	18	0.26	0.51	0.71	**1.26**	Adenylosuccinate synthetase	ADENYLOSUCCINATE SYNTHETASE (PTHR11846:SF0)
E9HGQ3	6	0.53	-0.12	-1.06	**-1.19**	Putative uncharacterized protein	PDZ AND LIM DOMAIN PROTEIN ZASP (PTHR24214:SF29)
E9HGR4	3	1.23	1.06	-0.95	**-1.25**	Putative uncharacterized protein	LYSINE-SPECIFIC DEMETHYLASE LID (PTHR10694:SF8)
E9HGT3	15	-0.63	-0.76	-1.59	**-0.75**	Putative uncharacterized protein	EPOXIDE HYDROLASE 1 (PTHR21661:SF10)
E9HGV5	21	-0.21	-0.74	-0.70	**-1.03**	DNA topoisomerase 2	DNA TOPOISOMERASE 2 (PTHR10169:SF38)
E9HGX0	20	0.93	0.91	**0.95**	**1.63**	Malate dehydrogenase	MALATE DEHYDROGENASE, MITOCHONDRIAL (PTHR11540:SF16)
E9HI00	20	-0.02	-0.17	0.51	**0.83**	Putative uncharacterized protein (Fragment)	MEDIUM-CHAIN SPECIFIC ACYL-COA DEHYDROGENASE, MITOCHONDRIAL (PTHR10909:SF240)
E9HIB3	21	0.05	0.40	0.65	**1.37**	Putative uncharacterized protein	DIPEPTIDASE B, ISOFORM A (PTHR11963:SF9)
E9HJQ1	15	-0.69	-1.18	-1.06	**-1.24**	Putative uncharacterized protein	VITELLOGENIN-2 (PTHR23345:SF1)
E9HJT8	5	**2.50**	-0.29	-2.39	**-1.83**	Putative uncharacterized protein	40S RIBOSOMAL PROTEIN S17-RELATED (PTHR10732:SF0)
E9HLH8	13	0.67	0.25	-1.14	**-0.84**	Putative uncharacterized protein	MATERNAL PROTEIN EXUPERANTIA (PTHR12384:SF1)
E9HM79	27	-0.24	-0.43	-0.82	**-0.87**	Putative uncharacterized protein	EUKARYOTIC TRANSLATION INITIATION FACTOR 4G, ISOFORM B-RELATED (PTHR23253:SF9)
E9HM84	13	-0.99	-1.29	-1.92	**-2.50**	Putative uncharacterized protein	MITOCHONDRIAL 2-OXOGLUTARATE/MALATE CARRIER PROTEIN (PTHR24089:SF86)
E9HN52	30	-0.10	-0.59	-0.56	**-1.12**	Putative uncharacterized protein	TRIFUNCTIONAL ENZYME SUBUNIT ALPHA, MITOCHONDRIAL (PTHR23309:SF9)
E9HNC5	16	-0.12	-1.42	-1.64	**-1.16**	Putative uncharacterized protein	ARGININE--TRNA LIGASE, CYTOPLASMIC (PTHR11956:SF1)
E9HNC7	17	-0.53	-1.27	-0.46	**-1.00**	Putative uncharacterized protein	ATP-BINDING CASSETTE SUB-FAMILY E MEMBER 1 (PTHR19248:SF16)
E9HP73	15	0.63	-0.23	-1.47	**-1.52**	Putative uncharacterized protein	
E9HR84	40	-1.02	**-1.89**	**-1.81**	**-2.28**	Putative uncharacterized protein	CALCIUM-TRANSPORTING ATPASE SARCOPLASMIC/ENDOPLASMIC RETICULUM TYPE (PTHR24093:SF163)
E9HRX9	13	0.52	-0.57	-1.32	**-1.79**	Putative uncharacterized protein	SUBFAMILY NOT NAMED (PTHR13711:SF174)
E9HTS2	10	0.35	-0.11	-0.84	**-0.84**	Putative uncharacterized protein	60S RIBOSOMAL PROTEIN L9 (PTHR11655:SF16)
E9HTT3	17	**-4.54**	0.64	-1.37	0.32	Putative uncharacterized protein	CYSTATHIONINE BETA-SYNTHASE (PTHR10314:SF48)
E9HUP6	7	0.10	-0.55	0.05	**-0.91**	Putative uncharacterized protein	CYSTATHIONINE GAMMA-LYASE (PTHR11808:SF15)
E9HX82	4	0.02	0.63	**1.46**	**0.21**	Putative uncharacterized protein	
E9HZI5	19	1.28	-0.32	0.41	**-0.95**	Vitellogenin fused with superoxide dismutase (Fragment)	SUPEROXIDE DISMUTASE [CU-ZN] (PTHR10003:SF32)
E9I5Q2	4	-0.43			**-4.04**	Putative uncharacterized protein (Fragment)	TRANSLOCATION PROTEIN SEC62 (PTHR12443:SF9)

Supplementary table 5. (see original manuscript) Results of NCBI-BLAST homology search against the UniProtKB/Swiss-Prot database (processed with Blast2GO) for the 189 significantly regulated proteins.

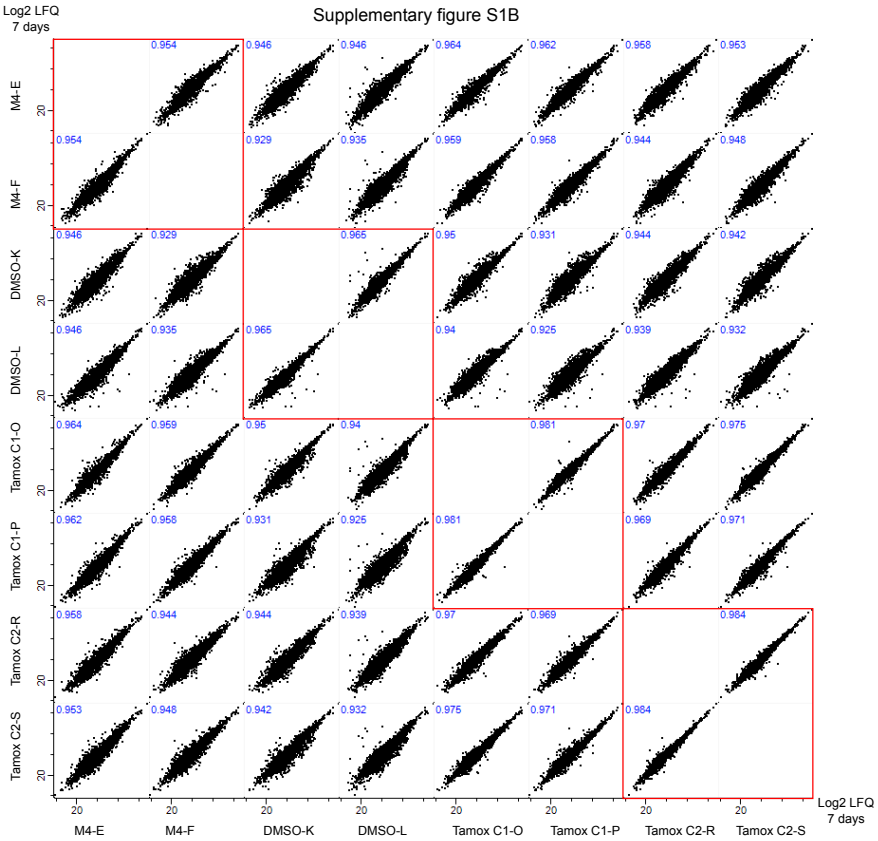
Supplementary table 6. (see original manuscript) List of the proteins showing a significant enrichment of their Gene Ontology (GO) categories in Biological Process (BP) or Molecular Function (MF) classes compared to the whole *D. pulex* genome. Only the significant categories are shown here as GO annotations. These proteins were used for the Figures 3A and 3B, and the supplementary Figures S3 and S4.

Supplementary table 7. (see original manuscript) List of the 57 proteins showing a significant fold change between blank (M4) and solvent control (DMSO) samples with LPE test, after multiple-testing correction and removal of outliers. PANTHER annotations are included and both 2 and 7 days ratios are shown, with significant ones highlighted in grey.

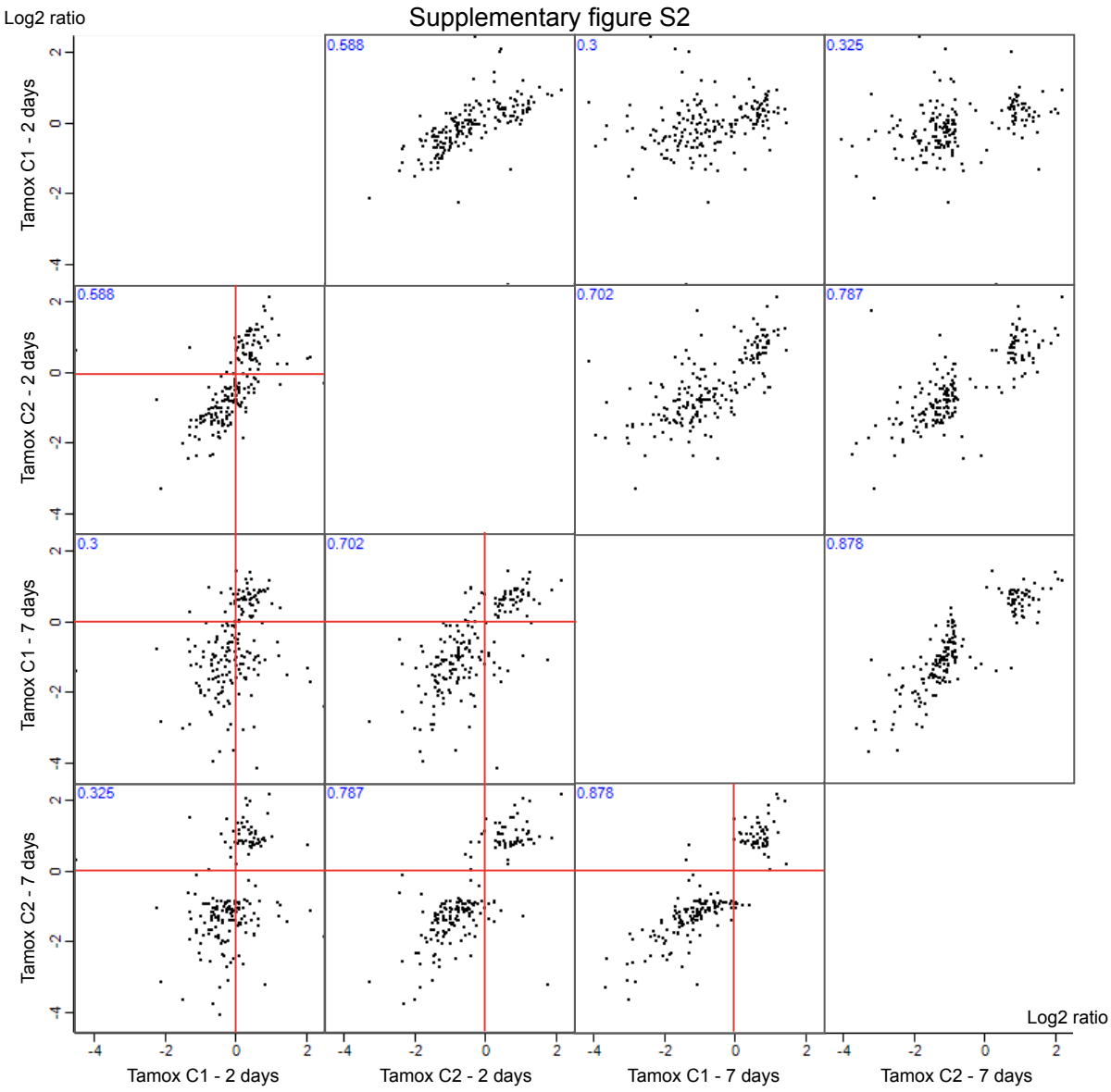
Supplementary figure 1. Correlation of protein intensities (in log2 scale) between all experiments at 2 (A) and 7 (B) days. Pearson correlation factor was superior to 0.9 for all duplicates (red frame), showing a good reproducibility of replicate experiments.



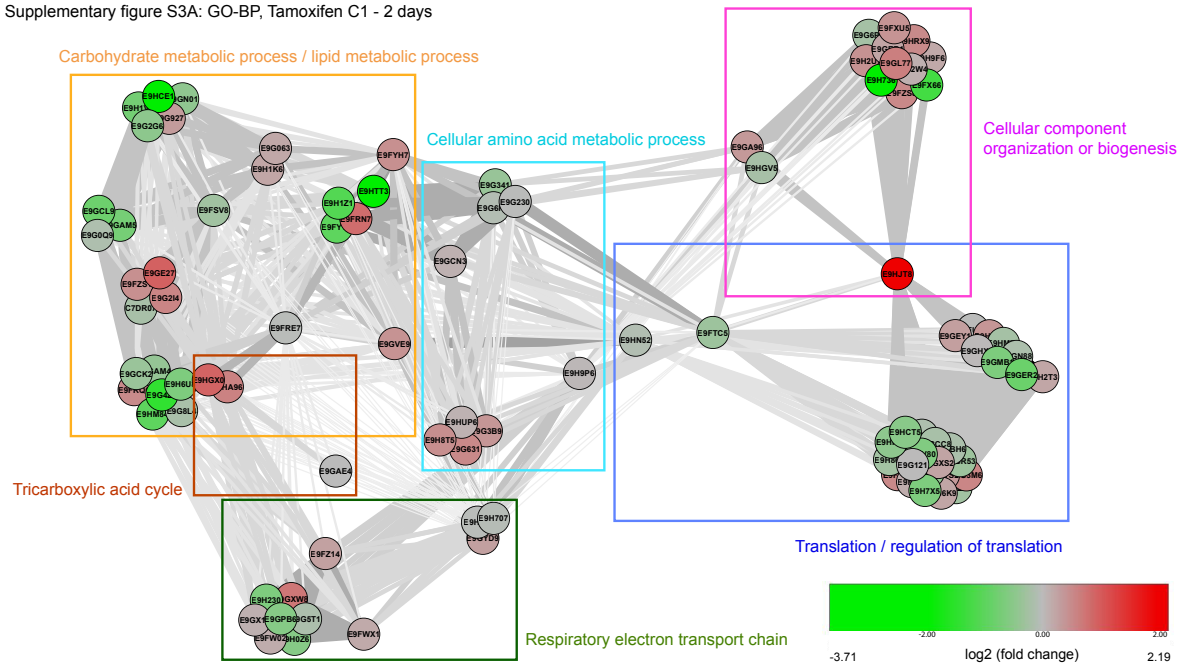
Supplementary figure S1B

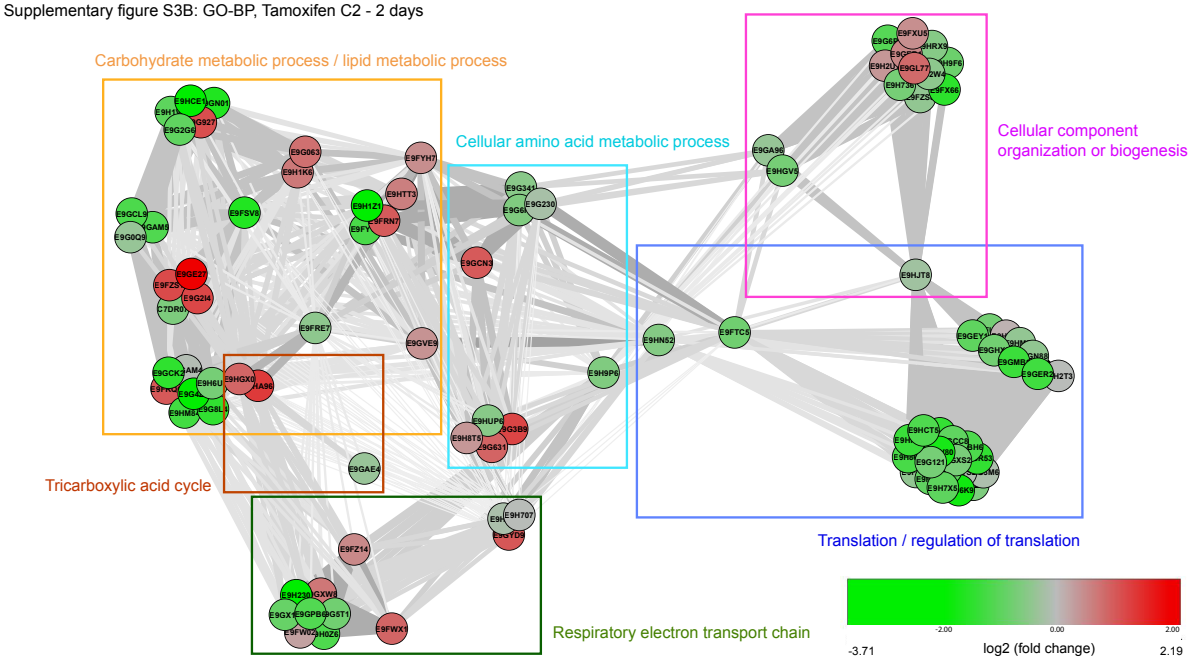


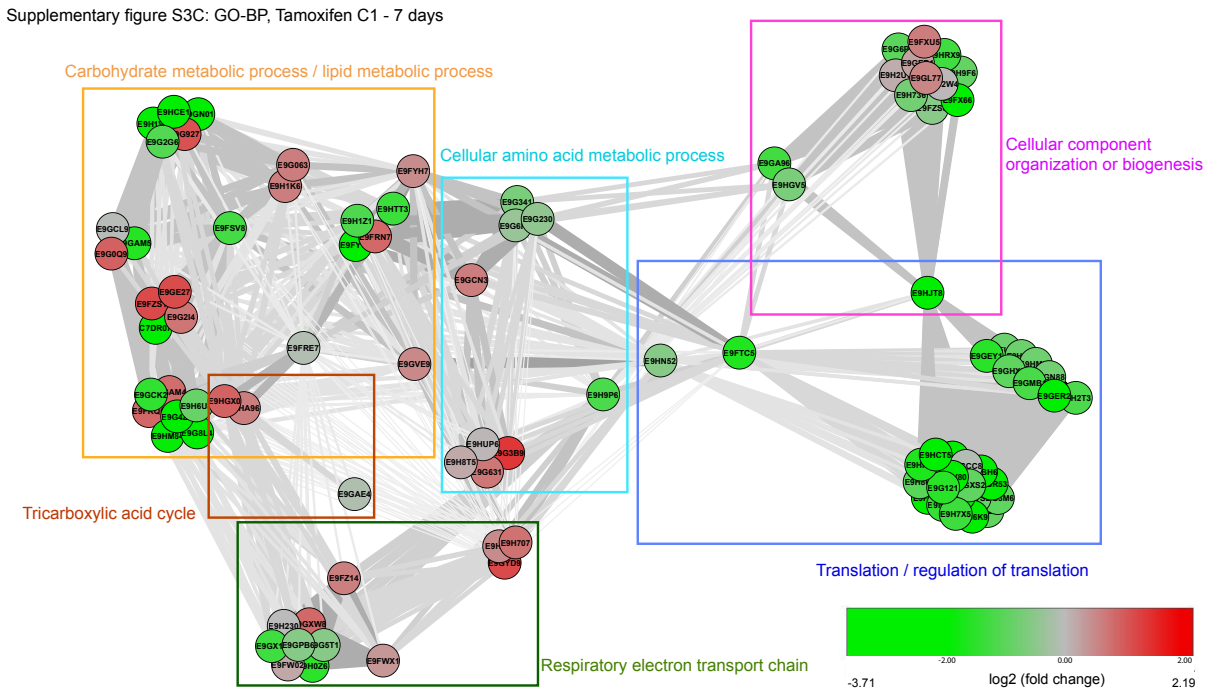
Supplementary figure 2. Graphical representation of relations between proteins and significant Biological Process GO categories with proteins as nodes and shared annotations as edges. Edge width between 2 proteins was proportional to the minimum percentage of shared annotations and node color scaled according to log2 of protein fold change, A: 2 days – C1, B: 2 days – C2, C: 7 days – C1. Results for 7 days – C2 are shown in main (Figure 6.5). Only significant GO categories are displayed, but too general annotations, such as metabolic process, primary metabolic process and protein metabolic process, were not included for a better clarity of the graphs.



Supplementary figure 3. Graphical representation of relations between proteins and significant Molecular Function GO categories with proteins as nodes and shared annotations as edges. Edge width between 2 proteins was proportional to the minimum percentage of shared annotations and node color scaled according to log2 of protein fold change, A: 2 days – C1, B: 2 days – C2, C: 7 days – C1. Results for 7 days – C2 are shown in main Figure 6.6. Only significant GO categories are displayed, but too general annotations, such as catalytic activity, structural molecule activity and binding, were not included for a better clarity of the graphs.







Supplementary files. (see original manuscript) Cytoscape (version 2.8.3, www.cytoscape.org) files (.cys) used to create figures 6.5 and 6.6, and supplementary figures 2 and 3. GO Biological Process: Fig_BP_GO.cys ; GO Molecular Function: Fig_MF_GO.cys.

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Chapter 7

Synthesis and discussion

The studies presented in this thesis focused on the toxicity of anticancer drugs on non-target organisms at the organism and the protein levels of the biological organisation. At the organism level, the primarily objective was to assess the sensitivity of *D. pulex* exposed to anticancer molecules. Imatinib, tamoxifen and two of its potent metabolites, i.e., 4OHTam and endoxifen, were used in acute and long-term experiments. Prospective assays were also performed to observe how daphnids reacted after being withdrawn from stress chemical or after being exposed to a mixture of tamoxifen and 4OHTam. At the protein level, the objective was to determine changes of protein expression in D. pulex after short (2 days) and mid time (7 days) exposures to tamoxifen.

Further experiments are also proposed and briefly described in this last chapter to increase knowledge on tamoxifen mode of action and effects on daphnids. The relevance of using daphnids as model organisms and of using measured or predicted concentrations in ecotoxicology are also addressed in this discussion. Finally, these issues lead us to courses of action that may be undertaken to reduce the input of pharmaceuticals in the aquatic system.

Synthesis and discussion

This thesis was a multidisciplinary project that involved a tripartite collaboration between scientists from ecotoxicology, toxico-pharmacology and proteomics. The project integrated pharmacokinetic and pharmacodynamic aspects along with ecotoxicological and proteomics approaches to address the issue of anticancer drug residues in surface water. The determination of tamoxifen, 4-hydroxy-tamoxifen (4OHTam), endoxifen and imatinib, as key molecules to be assessed among all other anticancer drugs was the result of this tripartite collaboration. Several aspects were considered and discussed about these anticancer pharmaceuticals such as their chemical properties, human metabolism, disease incidence and drug consumption, relevance for non-target species and their potential effects at the population or at the protein levels. Compared with other xenobiotics, pharmaceuticals have the advantage to be one of the best investigated and characterised man-made chemicals. Broad pharmacological knowledge (e.g., physical chemical properties, mode of action, side effects, etc.) on commercialised pharmaceuticals is available in the literature. Together with ecotoxicological knowledge, the pharmacological data help to determine the potentially active and harmful molecules for the environment at low concentrations. Also, this exchange of knowledge may facilitate integrated risk assessment of pharmaceutical compounds to the aquatic fauna and flora. For instance transdisciplinary knowledge could lead to scientifically-based testing strategies for the detection, the identification and the quantification of chemicals during ecotoxicological risk assessment.

The experiments that were performed in this thesis were mainly multigenerational and they were related to certain challenges. Adaptation to change and to unexpected situations was probably the most important ability to be developed prior and during these experiments to meet our predetermined objectives. For instance, we had to face different molecule properties that had lowered daphnids' exposure concentrations, limitations in laboratory handling, unexpected toxic effects on daphnids, etc. Moreover, long-term experiments are rarely undertaken in ecotoxicology and detailed procedures are lacking in the literature. Therefore, the experimental test procedures needed to be designed and they were continuously adapted to ease handling. For instance, three main points were improved over the experiments that were performed with tamoxifen, its metabolites and imatinib. First, the period of exposure was reduced from the standard 21 days to 14 days because we observed that significant toxic effects on the reproduction were already observable after the third clutch when compared with controls. Moreover, multigenerational tests are time demanding and shortening of the period of exposure allowed us to increase the number of generations that were followed. Second, three organisms per beaker, in triplicate (= 3 beakers), were treated at each concentrations instead of one individual per concentration in 10 replicates (0 10 beakers), as recommended in the OECD guideline [1]. These three beakers per concentration instead of ten eased laboratory manipulations and provided nine treated organisms per concentration, which is close to the recommended number of individuals in

standard experiments. Third, the generation that was isolated and exposed to a similar maternal medium (with or without chemicals) was pipetted from a unique beaker among the replicates. In other words, offspring from a same beaker was pipetted to form the next generation to be treated, when ever possible, because individuals born at the same time will reproduce at the same time, i.e., the same day. This synchronised reproduction decreased the variability between the mothers exposed to the same chemical concentration, in terms of the total number of neonates produced. The three above improvements had enabled to provide relevant results about the toxicity of four molecules with anticancer properties.

Ecotoxicological experiments

The studies presented in this thesis showed that anticancer drugs induced several toxic effects on daphnids. Tamoxifen, 4OHTam and endoxifen interacted with reproduction, body-length and/or longevity, and some effects were unusual, such as morphological abnormalities on offspring, evisceration or erratic swim-styles. Tamoxifen and its metabolites were teratogen, since treated mothers produced body-deformed neonates. Tamoxifen impaired physiological processes in *D. pulex* and offspring at environmentally relevant concentrations, and this chemical was the most toxic compound among the anticancer drugs tested, followed by 4OHTam, endoxifen and imatinib. Imatinib interacted only with reproduction and at high concentration.

Daphnids from mothers exposed to tamoxifen or 4OHTam were not able to recover when removed from test solution and transferred to incubation medium exempted from the chemicals. Two general hypotheses were proposed to explain why effects were observed in untreated daphnids. Firstly, damages may have been transferred from treated mothers to offspring. Secondly, damages may have occurred during the early exposure period, when eggs and embryo were still in the maternal brood chamber, or later when neonates were swimming in the maternal test solution. Indeed, animals withdrawn from solutions containing the toxic chemical were neonates that were born in test solutions and then removed from within the first 24-h. During this short exposure period, the toxic chemicals may have interacted with biological processes on the exposed developing organisms. These two hypotheses may be further investigated using experiments where daphnia eggs are removed from the brood chamber of treated mothers [2]. Eggs from the same mother and therefore of the same age could be individually deposited in a multi-well plate. At different times, and thus at different developmental stages of the embryos, each egg could be exposed to tamoxifen or 4OHTam at a concentration that is known to have effects in neonates (Figure 7.1). The critical window (here the most sensitive embryogenesis period) could be established depending on the observed neonatal and adult effects (i.e., malformation or not, size impairment or not, reduced reproduction or not, etc.).

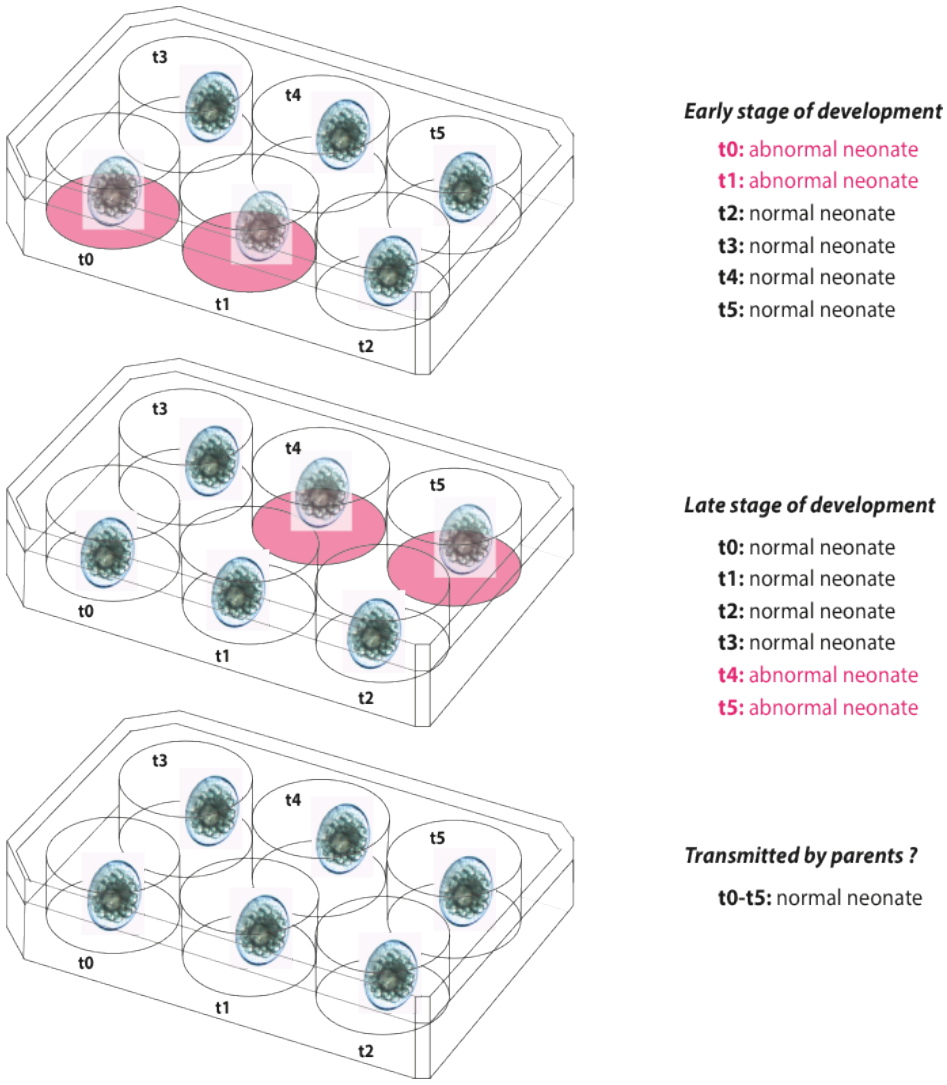

Figure 7.1: Daphnia eggs experiment. Each egg is exposed to toxic chemical-bearing solutions at a different time of the embryogenesis to determine the critical window or the potential transmission of side effects from treated mothers. The studied endpoints in neonates could be morphological abnormalities, size impairment, survival, etc.

The results found in the mixture experiment (tamoxifen and 4OHTam combined) are consistent with the hypothesis postulating that side effects could be induced directly in the developing embryo and might not be transmitted by parents. Indeed, in this study, the effects were similar in adult daphnids that were previously exposed or not to the potentially toxic chemical at embryogenesis. Once adult, the reproductive performance of both groups was decreased. This result showed that tamoxifen and 4OHTam mixture was interacting with developing organisms. Besides, the combination of tamoxifen and 4OHTam induced effects in offspring while no effects were observed when these chemicals were tested individually at the same concentration. Although a synergic potential of these chemicals in daphnids need to be verified in complete mixture experiments (i.e., with complete individual dose response curves), their combined potential cannot be excluded. This hypothesis is in accordance with

authors who discussed the beneficial effect of tamoxifen in patients as the result of an aggregate effect of tamoxifen and its metabolites 4OHTam and endoxifen [3–5]. Also, experiments with endoxifen used in mixture would be interesting to carry out since this metabolite seems to be involved in the combined potential of tamoxifen and 4OHTam in cancer patients [6].

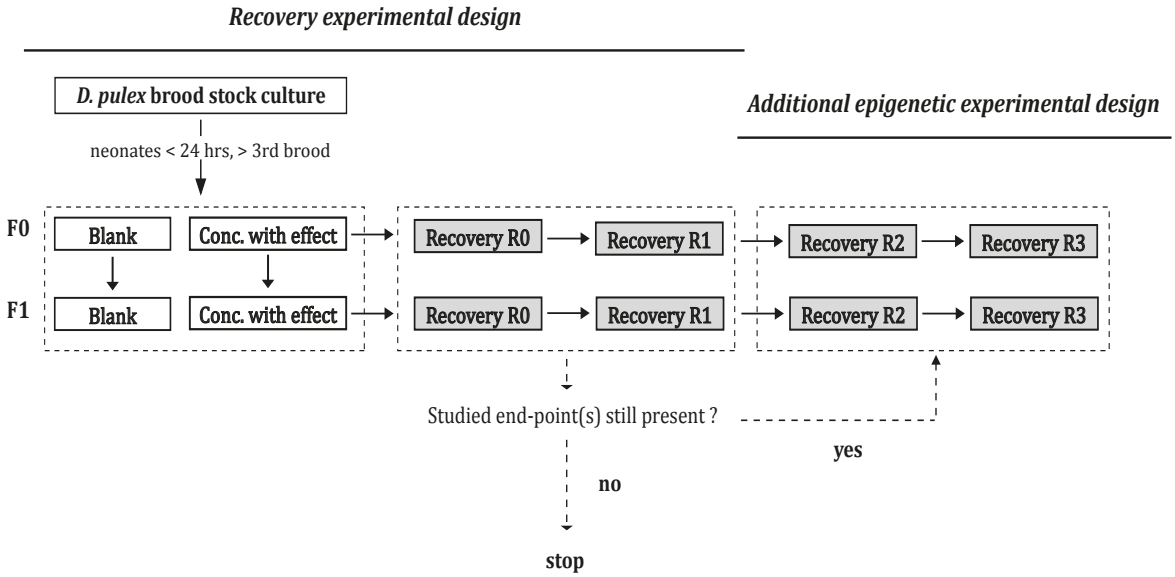

Figure 7.2: *D. pulex* recovery experimental design followed by an additional experiment if epigenetic process is suspected.

Several mechanisms may be involved in and responsible of the observed effects on daphnids and their offspring exposed to tamoxifen and its metabolites, such as endocrine system interactions, DNA modifications but also epigenetic regulations that caused changes in phenotype or gene expression without changes in DNA sequence [7–9]. At the endocrine level, chemicals may have blocked or induced mechanisms that had disturbed the normal development of physiological functions of daphnids, reducing their size or reproduction for instance. Also, epigenetic regulations were discovered in cells exposed to tamoxifen [10–12] and recently, Vandegehuchte et al. [13] showed that epigenetic dysregulations (e.g., DNA methylation) occurred in daphnids that were exposed to various compounds. It is possible that epigenetic inheritance was one reason explaining the adverse effects that were observed in the epigenetic experiment, i.e., in offspring withdrawn from the solution with chemical. To confirm this hypothesis, we recommend to perform recovery experiments with more than two generations, as suggested by Harris et al. [9]. Indeed, if the adverse effects persist into nonexposed generations beyond the third, a true transgenerational effect can be confirmed. Figure 7.2

summarizes the conceptual framework of recovery experiments with a possible extension if epigenetic mechanism is suspected. This experimental design could be integrated in any multigenerational ecotoxicity experiment or it could be run individually, as a distinct experiment. Briefly, we suggest following up neonates that were withdrawn from the two lowest effective concentrations because at higher concentrations the effects may be the result of severe internal lesions rather than subtle epigenetic mechanism. Neonates from the first and the second generations (F0 and F1) could be used because mothers of the F0 were not exposed to chemical during the embryonic stage, while mothers of the F1 were. This parental exposure differences may also induce differences in the outcomes, which would be interesting to compare. Indeed, it is known that epigenetic dysregulation with health effects on several generations can occur because of prenatal exposure to chemical [7]. Therefore, neonates of the F1 may be more sensitive and less fit than neonates of F0, and therefore less able to recover. If a choice has to be taken between the generations to be tested, e.g., due to work feasibilities, the second generation would be more appropriate than the first to avoid missing latent effects resulting from epigenetic process.

However, a direct action of the chemical in the endocrine system may be another reason of the adverse effects that were observed in treated and recovering daphnids. In invertebrates, hormones control development, reproduction and other physiological aspects such as growth [14]. Tamoxifen and 4OHTam may have interfered with hormone production, release or bioavailability, such as other chemicals. These chemicals may also have interacted with signal transductions or with hormone binding to receptors by either blocking (antagonizing) or activating (agonizing) them. In vertebrates, tamoxifen and 4OHTam interact with the estrogen receptors (ERs) but also with the estrogen-related receptor (ERRs, [15]). Because daphnids lack ER, we hypothesized that these molecules could act on the dappu-ERR [16]. Unfortunately, neither synthetic nor endogen ligands of ERR are known in *D. pulex*, and no experiment can be undertaken at this stage to assess potential interactions of chemicals with this receptor in daphnids. However, interactions with dappu-ERR should be kept in mind if experiments that consider other mechanisms of action do not show relevant results, e.g., experiment with ecdysone receptors. Indeed, disruption at the ecdysteroids receptor level is often considered to be responsible of adverse effects when daphnids are exposed to endocrine disruption compounds [17–20]. Ecdysteroids are polyhdroxylated ketosteroids that are present in crustaceans and other arthropods [17,21] and that are involved in important processes such as molting regulation, embryo development, covering organism formation (cuticle), etc [19,22,23]. Ecdysone has structural similarity with vertebrate estrogen [24] and tamoxifen and its metabolites bind with estrogen receptors. Therefore, ecdysteroids may be involved in the adverse effects that were observed in *D. pulex* exposed to these chemicals. Studies reported that ecdysteroid levels such as ecdysone synthesis in daphnids can be measured by radioimmunoassay after its extraction and its radiolabeling [17,25]. Such experiment could be performed to assess ecdysteroid levels in embryos exposed to tamoxifen or its metabolites

and to observe whether the morphological abnormalities that were found later in offspring resulted from ecdysone dysregulation. At organogenesis for instance, when daphnia glands become functional, embryos start to synthesize ecdysteroids that are used for egg maturation [19,25]. A decrease in these signalling molecules may impair healthy development of the embryo and induce morphological abnormalities, such as found after daphnia exposures to tamoxifen and its metabolites.

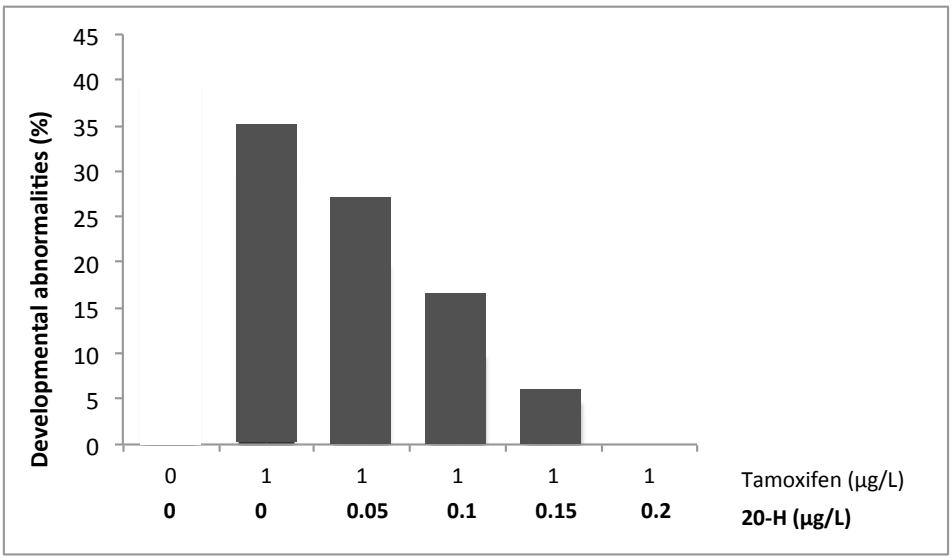

Figure 7.3: Illustration of potential mitigation of tamoxifen-induced developmental abnormalities by 20-hydroxyecdysone (20-H) during *D. pulex* exposure. The exposure period is 21 days and individual offspring is evaluated microscopically to observe any side effects (fictive data)

A second study may also be undertaken to determine whether the effects of tamoxifen or its metabolites on daphnids are due to an ecdysteroid activity. If these chemicals interact with the ecdysone receptor in daphnids, adverse effects on daphnids would indeed be observed. This second experiment could consist of using 20-hydroxyecdysone (20-H), an ecdysone receptor ligand that was successfully used as co-exposure molecule during ecotoxicological experiments with endocrine disruptors [17–20]. For instance, a concentration that is known to induce effects (e.g., morphological abnormalities) is chosen for the test and increasing concentration of 20-H is co-administered (Figure 7.3). Treated and untreated neonates (< 24-h, > 3 broods) would be reared to maturity (approximately six days in our laboratory conditions), and then the number of individual offspring production would be counted daily. These newborn individuals would be evaluated microscopically to identify any morphological abnormalities for instance. The experiments can stop after 21 days of exposure because sufficient number of neonates would have been produced and assessed. If the effects of tamoxifen are mitigated or totally absent by co-exposure to 20-hydroxyecdysone, this would mean that tamoxifen interferes with ecdysteroid control of development.

The above two experiments would help to understand whether the endocrine system is involved in the adverse responses that were observed in daphnids exposed to tamoxifen or its metabolites. Proteomics experiments can also be undertaken to better understand the interaction between toxic chemicals and the biology of treated animals. Indeed, proteomics may highlight changes at sub-organism level long before they are observed on whole organisms or populations.

Ecotoxicoproteomics *vs* multigenerational experiments

In the ecotoxicoproteomic experiment, we tried to relate phenotype effects on *D. pulex* with potential alterations at the protein level. Daphnids were exposed 2 and 7 days to tamoxifen at the predicted test concentrations of 0.1 (C1) and 2.4 (C2) µg/L. At these concentrations already, protein dysregulations were found. After multiple proteomics analyses, a total of 3940 proteins were identified and quantified in *D. pulex* using the UniProt database (www.uniprot.org). Among them, 189 proteins were significantly regulated and possibly related to a general stress induced by tamoxifen exposure. For instance, the expression of the vitellogenin fused with superoxide dismutase domain (E9GVW7) was overexpressed in daphnids exposed to tamoxifen. Some authors hypothesised that stress situations, such as exposure to chemicals, are associated with oxidative stress, which results in increased levels of anti-oxidative enzymes [26], such as copper/zinc superoxide dismutases. These enzymes prevent organisms from cell and tissue damages by catalysing the dismutation of superoxide free radicals. Therefore, the increase of E9GVW7 expression may be a physiological response of cells upon tamoxifen stress [27].

Proteins that play a role in reproduction were down-regulated in this study, such as some of the vitellogenin-2 family. Vitellogenins are major precursors of the egg-yolk proteins, vitellins, which are sources of nutrients during embryonic development [28,29]. The decrease in expression of reproductive proteins may be a strategy of stressed daphnids to keep energy for their primary functions. Because chemical exposure is an environmental stress and because stress responses are energy demanding, living organisms may have developed protective strategies to preserve their species from extinction. For instance, Rowe et al. [27] hypothesised that upon stress, organisms shift their energy for reproduction and/or energy for storage to survival. Jansen et al. [26] used this hypothesis to explain decreased levels of vitellogenin precursor in *D. magna* exposed to natural and anthropogenic stressors. Hence, to maintain their basic metabolic rate, which is considered as a catabolic pathway [27], treated daphnids may have temporary reduced other cost-energy functions. Since basic metabolism cannot be suppressed without compromising survival, it needs to be satisfied before energy is used for reproduction. This hypothesis is in line with the results that were found in the two-generational ecotoxicity experiment performed with tamoxifen in which the reproduction of the first daphnia generation dropped drastically when exposed to a concentration of the same order of

magnitude as C2. Therefore, the protein modifications identified in our studies confirmed and complemented the effects that were observed in treated animals during ecotoxicological experiments with tamoxifen. These modified proteins were identified in daphnids exposed to a concentration that corresponded approximately to one order of magnitude of the environmental concentration. Although specific biomarkers for early detection of tamoxifen harmful effects on *D. pulex* were not discovered in this study, proteins of the vitellogenin-2 family (E9H8K5) and the ryanodine receptor (E9FTU9) were determined as promising potential biomarkers because their expression was already modified after 2 days of treatment. Further experiments that focus on these proteins are recommended to verify their relevance as early warning proteins. In the same vein, experiments with tamoxifen at lower concentrations and in other aquatic organisms are suggested to improve knowledge on tamoxifen potential mechanism of action in non-human species. Also, the ecotoxicoproteomics analyses that were performed in this thesis provided the largest dataset from *D. pulex* that have ever been published up to now. These results are available for the scientific community and they may help further investigations on *D. pulex* exposed to tamoxifen or to other anticancer or endocrine disrupting compounds.

Compared to proteomics that may increase understanding of potential mechanism of action of a chemical, ecotoxicological tests based on long-term exposures and on several generations increase knowledge on potential effects at the population level. Indeed, multigenerational experiments are close to realist situations/exposures that are useful in future hazard assessments of chemicals in the aquatic environment [24]. This is in accordance with Kim et al. [30] who recently underlined that the number of generation is an important factor for chemical toxicity assessments. For instance, the first treated generation are organisms that were not previously exposed to the toxic chemical may be less sensitive towards such compounds [30–32]. Also, the sensitivity of the treated organisms may increase over generations, as stated in the two-generational experiment that was performed with tamoxifen metabolites.

Based on these indications, we propose to use at least two generations of daphnids to consider and to compare the chemical toxicity in aquatic experiments. If effects are magnified in the second generation, two additional generations may be followed to ascertain the long-term toxicity of chemicals (Figure 7.4). For instance, the results obtained during the two-generational study that was performed with 4OHTam did not provide new relevant effects when compared with the four-generational experiment. By contrast, the fourth generations of treated daphnids was adversely affected by tamoxifen at lower concentration than the two first generations. A case by case assessment is suggested to decide whether additional generations should be followed or not.

In this thesis, this follow up was done in a separate experiment, in which tamoxifen and 4OHTam was assessed during four generations because previous results suggested magnified effects over generations. It would also have been possible to follow next generations in the same experiment, as

time magnified effects were suspected, instead of restarting a whole four-generational experiment. But a new experiment allows the results that were obtained during the first assay to be confirmed. This is particularly relevant when molecules with unknown effects on aquatic organisms are evaluated, as it was indeed the case with tamoxifen metabolites.

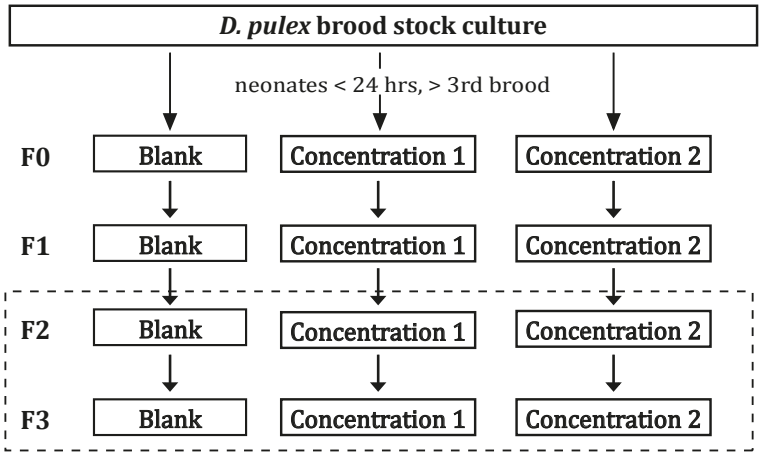

Figure 7.4: Multigenerational experimental design with *D. pulex*. The two first generations (F0 and F1) are required, but the next two generations (F2 and F3) are strongly recommended if magnified effects are suspected in the F1.

Prodrugs, metabolites and specific anticancer drugs

In general, metabolites are poorly studied, although some of them have higher potency to induce effects on living organisms than their parent drug. Prodrugs are chemicals for which the metabolites are known to be active. After administration, these metabolites may be body-excreted in wastewaters. Therefore, prodrugs are interesting molecules to focus on in ecotoxicology. For instance, clopidgrel is an antiplatelet agent administered during several months to prevent vascular diseases, such as heart attacks or strokes, in people at risk [33,34]. This pharmaceutical needs *in vivo* enzymatic activation (cytochrome P450) to be converted in a potent metabolite, named as clopidogrel active metabolite [34,35]. A small percentage of clopidogrel dose is ultimately converted to its active metabolite (i.e., 10%), but because the number of people with cardiovascular risk is high, particularly in industrial countries, the consumption may be high and so its release in wastewaters. This prodrug is indeed the most antiplatelet therapy prescribed in the world [36–38]. Another example is the prodrugs that are used in oncology. These prodrugs are attractive molecules because they provide an alternative therapy with low cytotoxic effects, when compared with cytotoxic anticancer drugs that are systemic antiproliferative agents that target dividing cells [39,40]. For instance, miproxifene phosphate, capecitabine, cyclophosphamide, are anticancer prodrugs that could potentially release active

metabolites through human excreta [40–42]. Besides, it would be interesting to assess the effects of other anticancer compounds with endocrine disruptive activities, such as fluvestrant. Fulvestrant is an oestrogen receptor antagonist with no agonist effects that is used in the treatment of hormone receptor-positive metastatic breast cancer [43]. Approximately 80 and 90 % of this chemical is excreted in faeces, in the intravenous and intramuscular trials, respectively [44]). Fulvestrant and its 17-ketone metabolite seem to be the major excretory compound. 17-Ketone metabolite has a similar anti-estrogenic activity as its parent drug, while all other metabolites do not have any. Besides, other TKIs than imatinib may be assessed and monitored in the aquatic environment because a second generation of "tinib" has reached the market after imatinib commercialisation, such as gefitinib, sunitinib, nilotinib, dasatinib, sorafenib and lapatinib. These new generations of molecules are used against various cancers but with a broad prevalence for chronic myelogenous leukemia and gastrointestinal stromal tumours. Because these molecules demonstrated a definite efficacy on survival of advanced cancer patients - however less impressive than for imatinib in its specific indications - a progressive increase in their use and in their release in the environment can be expected.

Nominal *vs* measured concentrations

In this thesis, the need to measure the exposure level of lipophilic molecules during ecotoxicological experiments was highlighted because notable differences between nominal (i.e., theoretical) and measured concentrations were found with tamoxifen and its metabolites at all test concentrations. We hypothesised that tamoxifen, 4OHTam and endoxifen adsorbed on surfaces of the glass flasks that were used during ecotoxicological assays. This phenomenon might have induced intra- and inter-concentrations variability as well as systematic reduction in the expected test exposure. Therefore, it was necessary to find a method that was able to reflect the exposure level of the test solutions and that was cost and time sustainable. Subsamples of the nominal solutions were therefore analysed and the corresponding measured concentrations were plotted against the nominal levels. This polynomial regression was used to establish the so-called predicted concentrations. Our method predicted well the low exposure levels when these predicted concentrations were compared with those measured. Although it is interesting to know the concentration in acute exposure experiments, this thesis primarily focused on long-term experiments that are environmentally relevant. Because predicted concentrations were used instead of nominal, comparisons with environmental concentrations were possible. In the experiment that was performed with imatinib, the nominal concentrations corresponded to those measured and environmental comparison would be possible if this chemical is monitored in natural waters. Furthermore, the method that was proposed to predict tamoxifen and its metabolites also avoided enormous number of analyses. Based on standard guidelines [1] indeed, a strict follow up of the concentrations would have required sampling at solution renewal and after 48h

at least, which was not feasible during long-term experiments. In the two-generational experiment (21 days of exposure) that was performed with tamoxifen metabolites for instance, such a follow up would have required a total of approximately 900 samples to be collected and analysed (i.e., 8 concentrations, at t0 and t48, in triplicate, during 2 generations). Using our predictive method, a total of only 520 samples (318 and 202 for 4OHTam and endoxifen, respectively) were analysed for all experiments that were carried out in this thesis with these metabolites, which was cost and time sustainable.

Predicted concentrations instead of nominal concentrations were therefore used in this thesis. The chemical lost and the variability in measured concentrations raise questions about the importance of assessing the test solutions in ecotoxicological studies. Indeed, in the literature review that was undertaken in a master project involved in this thesis [45], it was shown that results based on nominal concentrations are still published nowadays. In articles that were published in the journal Environmental Toxicology and Chemistry in 2012, we found that 45% of the test chemicals were not measured. This means that the conclusions on the efficiency or the toxicity of these molecules were based on concentrations that may not reflect real exposure levels. Also, a risk may be assessed and decisions may be taken based on these results. Indeed, several national and international organisations involved in environmental protection [46] use ecotoxicological results to make decisions. If the latters are based on nominal concentrations, underestimation of the chemical toxicity and of the risk of certain chemicals to the aquatic flora and fauna is possible [47].

From single aquatic species to ecosystem

The choice of the test species is also crucial when hazardous assessment of a chemical is undertaken. *D. pulex* and *D. magna* have been described in the scientific literature since 1960 [46] and they have been used in acute and chronic tests for years. In major cases, there is no difference in the overall sensitivity of the *D. pulex* and the *D. magna* exposed to several chemicals [48]. Daphnids are also good animal models in ecotoxicology because of their parthenogenetic life cycle. This mode of reproduction eases recovery, epigenetic and proteomics experiments primarily because confounding genetic differences are avoided [9]. Indeed, it is easy to observe phenotype modifications that are induced by epigenetic changes in gene expression when there are no genetic differences between individuals of any one strain. Finally, *D. pulex* may be more and more used in ecotoxicology, not only because *D. pulex* can be used a sensitive model to toxicants [49], but also because its sequenced genome offers the possibility to better understand chemical toxicities and chemical mode of actions [50,51].

Nevertheless, experiments with other organisms are required to assess chemical harmfulness because the sensitivity between species to a same molecule can be totally different [52]. In our study, two-generations of daphnids exposed to imatinib did not show adverse effect at environmental

concentrations but other species may have been sensitive at lower exposure levels. It is possible that daphnids were highly tolerant to this molecule or it is possible that imatinib's mode of action was too specific and did not target normal daphnia cells. Indeed, imatinib is specific to certain cancer cells only. Nevertheless, further ecotoxicological experiments are recommended to confirm the low toxicity of imatinib to aquatic species, but two other species may be sufficient (e.g., on algae and fish). Indeed, as it is not possible to test all chemicals in each species, choices have to be made. In this sense, multidisciplinary collaboration between biologists, pharmaco-toxicologists and ecotoxicologists may help to find the best species and the most relevant drug residues, with the goal to protect major aquatic species. Indeed, biodiversity is a key factor for humans and environmental health and well-being. It ensures the supply of ecosystem services and facilitates ecosystem stability, which the basis of sustainable development [53,54]. Water quality assessment is thus an important part of the risk assessment process to minimize the occurrence, the fate and the effects of hazardous substances in aquatic ecosystems.

Increased knowledge may lead scientists and non-scientists to recognise some pharmaceuticals such as anticancer drugs as serious threat to freshwater ecosystems. This awareness may be reinforced by their potential magnified release due to increasing urban and medicinal therapy developments. Indeed, new synthetic molecules, including pharmaceuticals are discovered at an exceptional speed. Backhaus et al. [53] reported as an example that more than 700 new chemicals were inscribed, in a 24-h period, into the Chemical Abstracts Service database of the American Chemical Society website (www.cas.org), which means a discovery rate of more than 30 new chemicals per hour. Such as wild organisms, humans are directly and indirectly exposed to thousands of molecules through breathing, skin, mucosal lining, food, drinking water, etc. Living organisms exposure to environmental chemicals and metals is ubiquitous. For instance, results of body fluid analyses showed that humans are exposed to several xenobiotics [55–59] and that women may be exposed during their pregnancy to 43 different chemicals at least, among 163 analysed chemicals from 12 different chemical classes (e.g., polybrominated diphenyl ethers, PBDEs; perfluorinated compounds, PFCs; organochlorine pesticides, phthalates, etc [60]). Therefore, the problematic of xenobiotics, including pharmaceuticals, in the environment is of growing concern for the general public and the scientific community. Scientists and non-scientist are increasingly focused on the potential impact of chemicals on ecosystems but also on drinking water. For instance, the Institut National de la Consommation [61] published a short communication about the presence of tamoxifen traces in drinking water, which underlines that this family of molecules can also reach water intended for human consumption. In general, pharmaceuticals in drinking water present a low risk for human health [62], but to the best of our knowledge, the risk assessment of molecule that is a pharmaceutical, an endocrine disruptor and a carcinogen agent, like tamoxifen, has never been addressed by experts so far. Therefore, we wonder how these molecules would be assessed and whether their double potential risks (i.e., endocrine

disruptor and carcinogen agent) would be considered. Such as in ecotoxicology however, little or nothing is known regarding long-term effects on human exposed to pharmaceuticals through drinking water.

Three levels of action to reduce pharmaceutical release in waters

The risk of pharmaceuticals to the aquatic environment and to humans cannot be excluded because: 1) pharmaceuticals can reach natural and drinking waters, 2) pharmaceuticals are considered as pseudo-persistent, 3) living organisms including humans may be exposed to these active residues throughout their entire lifetimes, for generations [30]. For these reasons, efforts should be undertaken to reduce pharmaceutical exposures. The best effort would obviously be to strictly avoid the release of pharmaceutical molecules into aquatic systems. This action is not realistic at this stage and other actions at different levels may help to reduce the occurrence of these compounds in waters, and thus the stress they induce in living organisms. Here are presented three levels of action that could reduce the total input of pharmaceuticals in wastewaters and natural waters.

Pharmaceutical industries may be considered as one level where actions can be undertaken. Indeed, these companies, which are considered as one of the three main sources of pharmaceutical pollution [63], have financial and technological resources to improve industry wastewater management, drug conception and sales. For instance, reconsideration of the doses may be an interesting action to reduce pharmaceutical residue inputs into the environment. Indeed, the standard dose is not necessarily the lowest dose that could be prescribed to patients, as observed by Decensi et al. [64,65]. Further studies on the minimum therapeutic dose may therefore lead to dose reduction. Development of pharmaceuticals with more specific modes of action would also help to reduce the amount of residues in waters [66]. In addition, increment of industrial wastewater treatment processes, particularly after tanks cleaning, may also be beneficial for the water cycle.

A second level of action to reduce pharmaceutical release in waters may be health facilities and medicine/nursing training. Physicians and nurses familiar with the toxicology of the environment, aware of the water cycle and conscious of the potential exposure to pharmaceutical residues to the general population are professionals that could control the release of substances to the environment. For example, a responsible and optimal use of pharmaceuticals may ensue because concerned physicians may pay more attention to drug prescription and doses. Prescriptions of the right dose or only if the real need exists, like with antibiotics, may decrease the amount of drug consumed and therefore the amount of residues body-excreted into wastewaters. Also, responsible medical staff should avoid direct spillage of pharmaceuticals and personal care products. Among several examples, direct spillage of intravenous bags in wastewaters is one practise widely done in medical units. Indeed, intravenous infusions that are not or partially used are often poured into sink to lighten waste bags

(personal communication, intensive care nurses). Knowledge on the potential impact of pharmaceutical releases in the environment may limit these behaviours and therefore decrease the total amount of drug residues in wastewaters. Indeed, hospital wastewater effluents are already highly charged with various active residues that were excreted by patients or released after immersion into a bath of medical equipment such as stethoscopes, urinal, etc. A pre-treatment of hospital effluents may relieve urban station treatment plants that are not always able to treat all wastewaters. For instance, source separation of excreta is an interesting option to decrease pharmaceuticals released in hospital wastewater. In Germany, a 20 weeks experiment was run to avoid the release of x-ray contrast media in water cycle [67]. Patient excreta were separated using different concepts and two are briefly described here as examples. First, specific toilets for patients were "built" in the unit where the considered pharmaceutical was given. These toilets were separated from the toilets of other patients. Second, the excreta were collected using containers. Patients took the containers also to their home and returned them to the hospital after a certain period of time. In both cases (i.e., special toilets and containers), the excreta were burned and the pharmaceutical was not released into wastewaters. Wastewater effluents were regularly sampled and the analysed concentrations of iodine from x-ray contrast drug were drastically reduced during the period of the study. These two concepts could be applied in health facilities to avoid the release of molecules of high concern, such as tamoxifen, in the water cycle.

Efforts can be undertaken at the urban sewage treatment plant level. This level of action seems to be the favourite, although pharmaceuticals are already in the aquatic system. Most studies primarily focus on sewage treatment plants as the level at which water quality improvements can be done [68,69]. Insufficient or inappropriate sewage treatment processes are indeed one factor, among others, that explain the occurrence of pharmaceuticals in natural water. This level of action is thus important, although end-of-pipe. Ideally, future processes should be 100% efficient, avoiding thus production of unknown metabolites, which seems poorly realistic nowadays. Nevertheless, new technological treatment processes are now able to remove some pharmaceuticals from wastewaters and with low metabolite production [70]. However, a combination of efforts is probably the best way to reduce pharmaceutical occurrence in natural and drinking waters. A better awareness on pharmaceutical pollution may therefore encourage globally pre-commercialization assessment of drugs in terms of eco-conception, biodegradation and risk management, with local actions such as relevant recommendations on drug prescriptions, and relevant improvements on hospital and urban wastewater treatment management. Thereby, this thesis fitted well with the current role of environmental management to protect ecosystems and their inhabitants because our results provided additional scientific knowledge about anticancer drugs, metabolites and multigenerational experiments.

To summarise, anticancer drugs and their metabolites should be considered as priority substances for risk assessment because:

- their metabolites could play a role as important as parent compounds in aquatic species
- parent compound and metabolites can induce effects when considered in mixture
- daphnids are particularly sensitive towards anticancer drugs with endocrine disrupting properties because some daphnia receptors have some sequence homology with human receptors
- these lipophilic molecules are difficult to assess and therefore the experimental framework has to be well designed

We hope that the results of this thesis will contribute to further work regarding integrated risk assessment of anticancer drugs, which is still poorly developed at present.

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Annexe 1

Pharmaceutical residues may interfere with the endocrine system of non-target organisms at the:

- pre-receptor level (ligand availability) by affecting the synthesis, the metabolism and the excretion of hormones, i.e.., altering the concentration of endogen hormones.
- receptor level (structure of ligand binding site) by either [7]:
    - mimicking the action of a naturally-produced hormone (i.e. as agonists), such as estrogen or testosterone, and thereby set off similar effects. E.g., diethylstilbestrol or methoxyclor are estrogen receptors agonists
    - or blocking the hormone receptors (i.e. as antagonists), which prevents the effect of endogen hormones. E.g., diethylstilbestrol or linuron are androgen receptors antagonists.
- post-receptor level by interfering with co-regulators, extracellular signal, amplified signal, regulation in time by messenger inactivation, convergence or divergence of the signal, compartmentalisation of the signal and cross-talk

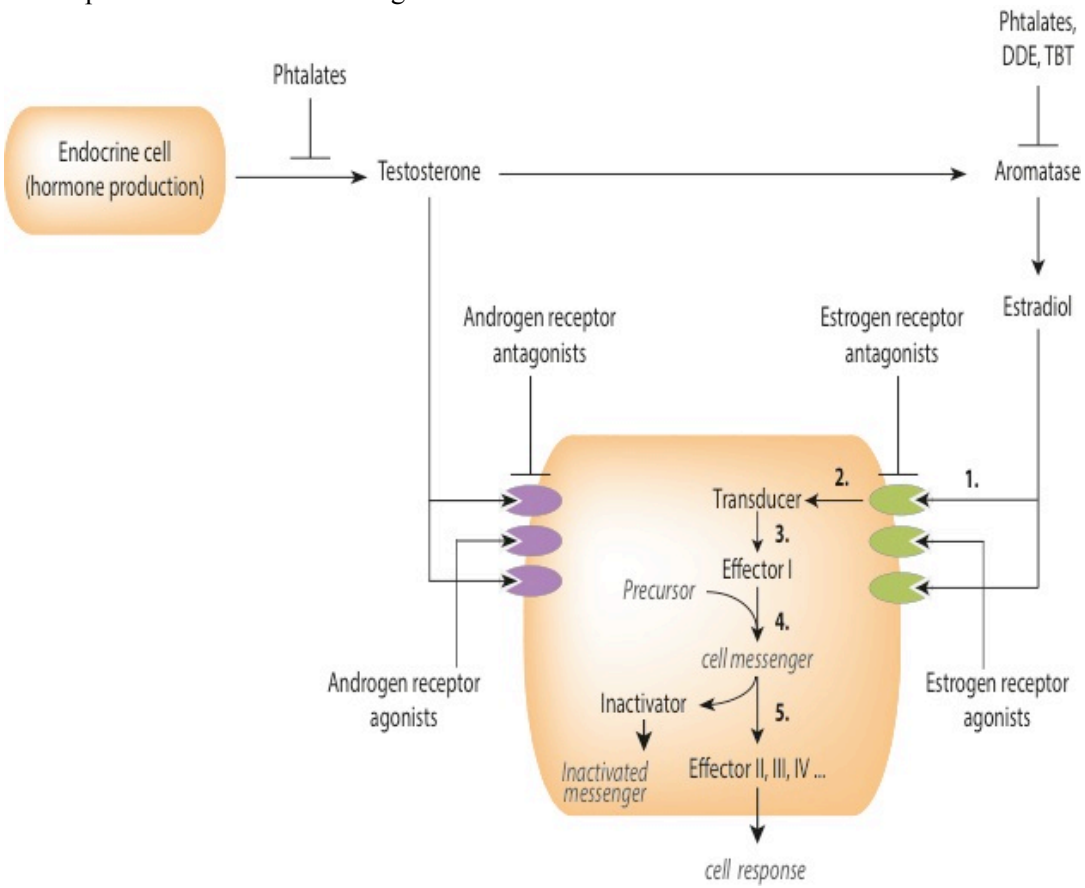

Figure 1: Endocrine disruptive pathways. Endocrine disruptors can interact at: the pre-receptor level (ligand availability), the receptor level (structure of ligand binding site), and the post-receptor level (co-regulators). 1. Extracellular signal, 2. Amplified signal, whose response depends on the proteins that are expressed within the cell, 3. Regulation by messenger inactivation, 4. Convergence/divergence of the signal, 5. Compartmentalisation of the signal and cross-talk. Example of androgen receptors agonists: methyl-testosterone, androgen receptors antagonists: diethylstilboestrol, linuron, estrogen receptors antagonists: tamoxifen. Phthalates are inhibitors of the testosterone production. Phthalates, tributyltin (TBT), and 1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene (DDE) are inhibitors of the bioconversion enzyme aromatase.

Annexe 2

Water hardness and survival

Before conducting any test on organisms, particularly multigenerational tests, the physiological behavior of *D. pulex* needed to be known in conditions exempted from stressors. Therefore, survival and reproduction were assessed under our laboratory conditions.

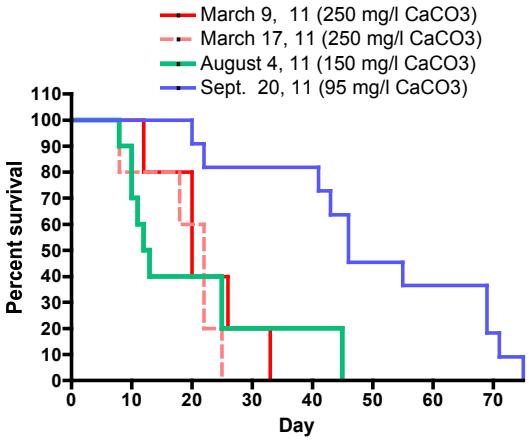

Figure 2: *D. pulex* survival in different reconstituted water hardness media (95, 150 and 250 mg/l $CaCO_3$)

In 2011, *D. pulex* survival in Elendt M4 [4] was observed from birth to death at three different water hardness: 95, 150 and 250 mg/L $CaCO_3$ (Figure 2). In March, survival experiment was performed at hard-reconstituted medium. Sixteen individuals were reared in Elendt medium with 250 mg/L $CaCO_3$. Only one individual survived until the 33rdday and mean survival was about 21 days. This longevity did not correspond to *D. pulex* survival, which was reported as 50 days by Smith [8].
In August, when the water hardness was reduced to 150 mg/l, the mean decreased to 12.5 days but two animals lived until the 45th day. In September finally, *D. pulex* were cultured in a reconstituted low water hardness corresponding to 95 mg/L $CaCO_3$. The mean survival increased to 46 days and one daphnia lived until the 75th days. These results corresponded to Smith data [8] and confirmed that *D. pulex* is species that prefers to live in low water hardness.
The reproductive capacity of the individuals that were reared in a medium at 95 mg/l $CaCO_3$ was also higher than at moderate or hard water hardness. The number of young released at each laying is summarized in Table 1. A total water hardness of 95 mg/l $CaCO_3$ was therefore chosen for the experiments that were performed in this thesis.

Table 1: mean neonates produced by a same healthy batch of mothers exposed to 95, 150 and 250 mg/L $CaCO_3$ (n = 4, 4 and 7, respectively)

mg/L CaCO3	Number of laying																Total neonates
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	
250	5.8	6.3	9.3	14	8.8	9.3	13	9.7	5	10							91
150	4.3	8	9.5	6	14	10	11	16	12	17	8.5	2	8	1	1		128
95	8	14	13	10	15	16	18	18	7	11	18	20	19	9.5	18	12	227

Annexe 3

Daphnia pulex medium

Daphnia medium contains additive trace nutrients to prevent deficiency symptoms [3]. Four synthetic media M4, M5, M6 (which is one-tenth that of the medium M4) and M7 are accepted as standardized media, but M4 and M7 are recognized as suitable for long-term ecotoxicological tests. Elendt [4] assessed the response of *Daphnia magna* to a toxicant in M4 and M6, and noticed that the production of live young was slightly higher in M4 than in M6 meaning that the tolerance of daphnids in M6 was lower than in M4. Also, EDTA is known to influence on the availability of metals. Its use in medium M4 seems to reduce the toxicity of metals by complexation process [3]. The OECD [5] does not recommend the M4 and the M7, which also contains EDTA, for testing compounds containing metals. In this thesis, the chemicals that were tested did not contain metal, and the M4 was chosen as unique medium for experiments and mass-culture. In this medium, the water harndess was adapted for *D. pulex* (Figure 3). Indeed, *D. pulex* prefers moderate or low water hardness, i.e., 40 to 48 mg/l CaCO3 [6] or 80 to 100 mg/l CaCO3 [7], respectively.

Moderate water hardness

Milieu M4		Stock solution		Daphnia medium	
N° solution	Substances	Quantity [mg]	Volume [ml]	Volume [ml] for 5 L	Final concentration [mg/l]
1	**NaHCO3** (Hydrogénocarbonate de Sodium)	6480.0	100 H2O	5.0	64.8
2	**KCl** (Chlorure de Potassium)	580.0	100 H2O	3.5	4.1
3	**MgSO4·7H2O** (Sulfate de Magnésium heptahydraté)	8000.0	100 H2O	4.50	72.0
4	**CaCl2·2H2O** (Chlorure de Calcium dihydraté)	8000.0	100 H2O	4.85	77.6
5	**Na2EDTA·2H2O** (EDTA disodique)	500.0	100 H2O	2.5	2.5
6	**FeSO4·7H2O** (Sulfate de fer heptahydraté)	199.1	100 H2O	2.5	0.996
7	**H3BO3** (Acide borique)	2860.0	100 H2O	0.5	2.860
8	**MnCl2·4H2O** (Chlorure de manganèse tetrahydraté)	72.1	94 H2O + 5ml of the a solution + 1ml of the b solution	2.5	0.361
	LiCl (Chlorure de Lithium)	61.2			0.306
	RbCl (Chlorure de Rubidium)	14.2			0.071
	SrCl2·6H2O (Chlorure de Strontium exahydraté)	30.4			0.152
	NaBr (Bromure de Sodium)	a			0.016
	Na2MoO4·2H2O (Mobildate de Sodium dihydraté)	12.6			0.063
	CuCl2·2H2O (Chlorure de Cuivre dihydraté)	a			0.017
	ZnCl2 (Chlorure de Zinc)	a			0.013
	CoCl2·6H2O (Chlorure de Cobalte)	a			0.010
	KI (Iodure de Potassium)	b			0.003
	Na2SeO3 (Selenate de Sodium)	b			0.002
9	**Na2SiO3** (Metasilicate de Sodium anhydrate)	85.9	95 H2O + 5ml of the c solution	2.5	1.000
	NaNO3 (Nitrate de Sodium)	c			0.028
	KH2PO4 (Dihydrogénophosphate de Potassium)	c			0.014
	K2HPO4 (Hydrogénophosphate di-Potassium)	c			0.018
10	**Thiamin-hydrochlorid** (Thiamin-(HCl) Vitamin B1)	120.0	97 H2O + 3ml of the d solution	0.315	0.075
	Cyanocobalamine (Vitamin B12)	d			0.001
	(+)-Biotin (Vitamine H)	d			0.001
11	**NH4VO3** (Metavanadate d'Ammonium)	e	98 H2O + 2ml of the e solution	0.315	0.001

Milieu M4		Stock solution	
Solution	Substance	Quantity [mg]	Volume [ml]
a	**NaBr** (Bromure de Sodium)	32.0	50.0
	CuCl2·2H2O (Chlorure de Cuivre dihydraté)	33.0	
	ZnCl2 (Chlorure de Zinc)	26.0	
	CoCl2·6H2O (Chlorure de Cobalte)	25.0	
b	**KI** (Iodure de Potassium)	32.5	50.0
	Na2SeO3 (Selenate de Sodium)	22.0	
c	**NaNO3** (Nitrate de Sodium)	55.0	59.0
	KH2PO4 (Dihydrogénophosphate de Potassium)	29.0	
	K2HPO4 (Hydrogénophosphate di-Potassium)	37.0	
d	**Cyanocobalamine** (Vitamin B12)	26.7	50.0
	(+)-Biotin (Vitamine H)	20.0	
e	**NH4VO3** (Metavanadate d'Ammonium)	22.5	50.0
f	**NaOH**	2.0	500.0

Must be renewed every month

Must be renewed every 3 months

Must be renewed every 6 months

Figure 3: *D. pulex* medium with a water hardness of 90 mg/L $CaCO_3$

Annexe 4

Pseudokirchneriella subcapitata culture

The Soluval Santiago laboratory in Couvet, Switzerland, kindly provided *Pseudokirchneriella subcapitata*. This algae was cultured in the OCDE medium [1] in the following conditions: light intensity of 4000 lux (~60-80 µE/m2/s), cool white light (non stop), pH 8, temperature of 22 ± 1°C, manual stirring one or twice a day. Every week, five hundred thousand cells of *P. subcapitata* per ml were added to a fresh medium. Algae growth was exponential up to the 10th day (**Figure 4**). After 7 or 8 days, i.e. when absorbance of light at a wavelength of 690 nanometers was range between 0.140 (min) and 0.170 (max), five hundred thousand cells of *P. subcapitata* was re-injected into a new medium. The rest of the algae was centrifuged at 3000 rpm for 20 minutes. The seaweed concentrate was stored in the dark at 4°C up to 1 weeks, in autoclaved glass bottles. After 7-8 re-injections, a new *P. subcapitata* culture was started from algae stored in Agar.
Daphnids were fed daily with 0.2 mg C/daphnia of *P. subcapitata* algae. In our culture, the *P. subcapitata* contained about 28% carbon. The amount of cells per daphnid was set at a minimum of 2.34E+07 cell/daphnia/day (0.15 mgC / Daphnia / day).

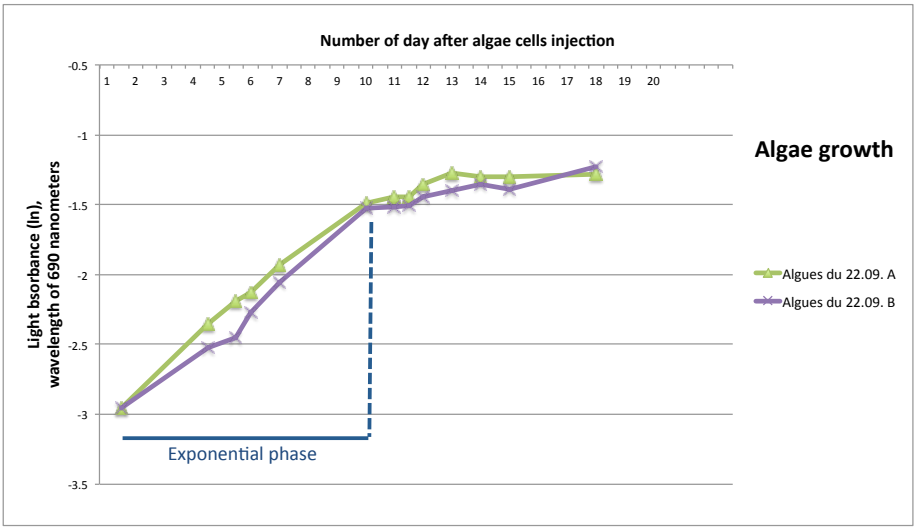

Figure 4: *Pseudokirchneriella subcapitata* growth (x = day number after inoculation; y = ln(absorbance))