WASTEWATERS: OCCURRENCE OF PHARMACEUTICAL SUBSTANCES AND GENOTOXICITY

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Abstract

The major pathways of human pharmaceuticals into the environment are from their use by individuals either at home or under medical supervision in hospitals, and to a lesser extent by the disposal of unwanted or out of date drugs.

The administered pharmaceuticals will be excreted as the parent compound, metabolite or conjugate and will be transported to sewage treatment works. In sewage treatment, the compound may be degraded or partially degraded, adsorbed to sludge if lipophilic, be deconjugated or pass through sewage treatment unchanged. Once in the environment the substance will be subject to further degradation processes.

The first part of this research is dedicated to the occurrence and fate of five substances widely consumed in Switzerland: Clofibric acid (metabolite), Ibuprofen, Ketoprofen, Mefenamic acid and Diclofenac (Chapter 2). These acidic drugs were analyzed in three sewage treatment plants (STPs) over four to seven consecutive days. Ibuprofen, Ketoprofen, Mefenamic acid and Diclofenac are non-steroidal anti-inflammatory drugs (NSAIDs). Clofibric acid is an active metabolite of Clofibrate, Etofibrate, Etofyllinclofibrate which are drugs used as blood lipid regulators. The anti-inflammatory drug Ibuprofen and Mefenamic acid are the most sold substances of this study with 17 tons per year and per substance in Switzerland.

Recoveries after filtration, extraction, derivatisation and clean-up generally exceeded 70%. Limits of detection (5-15 ng/l) and quantification (15-50 ng/l) were in a range which allows the detection and the quantification of these compounds in wastewaters.

The results of samples analysis point out that the five substances were persistent in wastewater effluents after municipal wastewater treatment. At the most, half of Mefenamic acid was eliminated. Ibuprofen was well removed (80%) by one sewage treatment plant. The removal of Ibuprofen is depending on the residence time of wastewater in the STPs. A long raining period induce a important decrease of removal of Ibuprofen and Ketoprofen. Removal

rates showed a great variability according to sewage treatment plants and types of treatments (e.g. biological, physico-chemical). The concentrations of Ibuprofen, Mefenamic acid and Diclofenac were relatively high in the effluents (150-2000 ng/l), showing a potential contamination of surface water.

An environmental risk assessment is presented. Mefenamic acid seems to present the most important risk, followed by Ibuprofen, Clofibric acid, Diclofenac and Ketoprofen. But the risk ratio for surface water calculated with a dilution factor was above one only for Mefenamic acid. Since that toxicity of a single drug might be enhanced by the occurrence of other pharmaceuticals with similar activity, the overall risk of these drugs could be significant. To our knowledge, chronic ecotoxicity data are available only for Diclofenac and Clofibric acid. These kind of data are needed for the other chemicals to confirm our results.

The second part of this thesis is dedicated to anticancer drugs (Chapter 3).

Since the occurrence of anticancer drugs in the environment are few studied and that these substances are extremely toxic (teratogen, mutagen, etc.), it was interesting to evaluate the contamination of wastewaters by two of the most used anticancer drugs.

Two methods were set up to analyse Tamoxifen and 5-Fluorouracil in wastewaters. A Liquid-liquid extraction (LLE) followed by a purification on OASIS® MCX cartridge and gas chromatography and mass spectrometry detection (GC-MS) were used for the analysis of Tamoxifen. 5-Fluorouracil was extracted with an ENV+ (Isolute) cartridge (solid-phase extraction), derivatised with pentafluorobenzyl bromide (PFBBr) and detected by GC-MS. Both methods showed good recoveries (>70%), reproducibility (RSD<10%) and limits of detection (LOD$\leqslant$15 ng/l).

Wastewaters from a residential area, an hospital, and two sewage treatment plants (STPs) were analysed with the analytical methods developed in this study. Tamoxifen was detected in wastewaters of the hospital, residential area and influent of STPs, but not in treated wastewaters. All wastewaters showed no contamination with 5-Fluorouracil.

The risk evaluation was not possible for these drugs, since no ecotoxicity data (even acute data) is available.

The third part of this research is dedicated to toxicity and mutagenicity of wastewaters (Chapter 4).

As pharmaceutical compounds, including anticancer drugs that are geno-

toxic, are discharged in wastewaters, the mutagenic potential of wastewaters from various origins (hospital, two different sewage treatment plants (STPs) and a residential area) was evaluated using the Ames test.

The samples were not concentrated prior the analysis to determine the overall effects of these waters. The survival and the reversion frequencies of strains TA98, TA100, TA102 and TA1538 following treatment with the different wastewaters were determined. Survival was obtained by two methods. The first method was by comparing the number of reversions induced by a known mutagen in the presence and absence of wastewater. The second was by determining the colony forming ability of dilutions of treated and non-treated cultures.

The samples from the hospital were on the whole more toxic than samples from the STPs and residential area. The different strains showed varying sensitivities to the toxic effects of the wastewater, with TA98 exhibiting the highest sensitivity (<5% survival). The results from the reversion assays indicated that TA102 was the most sensitive, followed by TA1538 and TA100. More hospital wastewater than influents of sewage treatment plants were mutagenic, indicating a higher mutagenic activity in the wastewater of the hospital. These wastewaters have not to be released in the environment without an adequate treatment. Comparison of the mutagenicity of the influents and effluents of the STPs showed that less effluent samples were mutagenic. This result indicates that biological treatments were relatively efficient in decreasing the mutagenicity of wastewaters.

Due to their beneficial health effects and economic importance, the actions taken to reduce inputs of drugs into the environment are much debated. The use of pharmaceutical compounds is expected to grow with the increasing age of the population. A solution for pollution control is to add sewage treatments in hospital and to avoid that municipal wastewaters are released without any treatment. Another solution is to focus on reduction at source, by developing a clear labeling on medicinal products, guidelines for the disposal and awareness campaign. These recommendations would have the potential benefit of improved consumer health (by minimizing the intake of active substances), as well as reduced health care spending.

Version abrégée

La contamination de l'environnement par les substances pharmaceutiques est liée à leur utilisation (à la maison ou dans les hôpitaux) ainsi qu'à l'élimination des produits non utilisés ou dépassés de date.

Les stations d'épurations (STEPs) sont des sources importantes d'apport de médicaments, car une partie de la dose du médicament administré est excrétée via l'urine ou les matières fécales sous forme inchangée, sous forme conjuguée ou de métabolites. Dans les STEPs, les substances peuvent être partiellement ou entièrement dégradées, absorbées aux particules en suspension formant les boues d'épuration, déconjuguées ou alors elles peuvent passer sans modification au travers des différents traitements. Une dégradation de ces substances peut aussi survenir lorsqu'elles se trouvent dans l'environnement aquatique.

Dans la première partie de cette recherche, la présence et le devenir de cinq médicaments très utilisés (Acide Clofibrique, Ibuprofène, Kétoprofène, Acide Méfénamique et Diclofénac) ont été analysés dans trois STEPs durant quatre à sept jours consécutifs (Chapitre 2). L'Ibuprofène, le Kétoprofène, l'Acide Méfénamique et le Diclofénac sont des anti-inflammatoires (NSAIDs). L'Ibuprofène et l'Acide Méfénamique sont les médicaments les plus vendus de cette étude: 17 tonnes par an et par substance en Suisse. L'Acide Clofibrique est un métabolite du clofibrate, de l'étofibrate et du clofibrate d'étofylline. Ces substances hypolipémiantes sont utilisées pour abaisser les concentrations plasmatiques élevées de cholestérol et de triglycérides.

La méthode analytique développée pour analyser ces cinq médicaments permet de récupérer généralement plus de 70% de ces composés. Les limites de détection (5-15 ng/l) permettent la détection de ces substances dans les échantillons d'eaux usées.

Les résultats de l'analyse des échantillons montrent que ces cinq substances étaient persistantes et se retrouvaient dans les effluents des STEPs. La moitié de l'Acide Méfénamique était éliminée au travers des STEPs étudiées. L'Ibuprofène était bien éliminé (80%) dans une STEP. L'élimination de l'ibu-

profène était dépendante du temps de résidence des eaux usées dans les STEPs. Une importante période de pluie a induit une diminution de l'élimination de l'Ibuprofène et du Kétoprofène. Les taux d'élimination étaient très variables en fonction des STEPs et du type de traitement (p.ex. biologique, physico-chimique). Les concentrations d'Ibuprofène et d'Acide Méfénamique mesurées dans les effluents étaient relativement élevées (150-2000 ng/l), pouvant induire une contamination non négligeable des eaux de surface.

Une évaluation du risque pour l'environnement est présentée dans ce travail. L'Acide Méfénamique semble présenter le risque le plus important suivi par l'Ibuprofène, l'Acide Clofibrique, le Diclofénac et le Kétoprofène. Mais le risque pour les eaux de surfaces n'est probable que pour l'Acide Méfénamique. La toxicité d'une substance peut être augmentée par la présence d'autres médicaments avec des modes d'actions similaires, ainsi le risque général de ces substances pharmaceutiques dans l'environnement pourrait être significatif. Des données d'écotoxicités chroniques ne sont disponibles que pour le Diclofénac et l'Acide Clofibrique. Ce type de données est absolument nécessaire pour les autres substances afin de confirmer nos résultats.

La deuxième partie de cette thèse est consacrée aux substances utilisées pour le traitement des cancers (Chapitre 3).

Étant donnée que la présence dans l'environnement des médicaments contre le cancer est très peu étudiée et que ces substances sont extrêmement toxiques (tératogène, mutagène, etc.), il était intéressant d'évaluer la contamination des eaux usées par quelques'uns de ces médicaments.

Deux méthodes d'analyse chimique ont été développées pour analyser la présence du Tamoxifène et de 5-Fluorouracil dans les eaux usées. Une extraction liquide-liquide (LLE) suivie par une purification sur une cartouche OASIS® MCX et une analyse par chromatographie en phase gazeuse avec une détection par spectrométrie de masse (GC-MS) ont été utilisées pour l'analyse du Tamoxifène. 5-Fluorouracil a été extrait grâce à une cartouche ENV+ (extraction en phase solide), derivatisé avec du bromide de pentafluorobenzyl (PFBBr) et détecté par GC-MS. Ces méthodes permettent de récupérer plus de 70% de ces substances dans les eaux usées et les limites de détection (LOD$\leqslant$15 ng/l) sont bonnes.

Des eaux usées d'une zone résidentielle, d'un hôpital et de deux STEPs ont été analysées avec les méthodes développées. Le Tamoxifène a été détecté dans les échantillons de l'hôpital, de la zone résidentiel et des influents des STEPs, mais pas dans les eaux traitées (eaux de sortie de STEPs). Aucun des échantillons d'eaux usées n'était contaminé par le médicament 5-Fluorouracil.

L'évaluation du risque pour l'environnement n'a pas été possible pour ces médicaments, car aucune donnée sur leur écotoxicité n'était disponible.

La troisième partie de ce travail est consacrée à la toxicité et à la mutagénicité des eaux usées (Chapitre 4).

Étant donné que certains médicaments mutagènes, comme ceux utilisés contre les cancers, sont excrétés dans les eaux usées, il était intéressant d'évaluer le pouvoir mutagène (avec le test de Ames) des eaux usées de différentes origines (hôpital, deux STEPs, et une zone résidentielle).

Les échantillons n'ont pas été concentrés avant l'analyse afin de déterminer l'effet global de ces eaux. Les souches TA98, TA100, TA102 et TA1538 ont été utilisées pour déterminer les taux de survie et les fréquences de réversion induites par les échantillons. La survie a été mesurée grâce à deux méthodes. La première méthode consistait à comparer le nombre de révertants induits par une substance mutagène connue en présence ou en l'absence des échantillons. La deuxième méthode déterminait la capacité à former des colonies à partir des cultures diluées, traitées ou non-traitées par les échantillons.

Les échantillons d'hôpitaux étaient plus toxiques que ceux des STEPs et de la zone résidentielle. La sensibilité à la toxicité variaient en fonction des différentes souches, TA98 était la souche la plus sensible (<5% de survie). Les résultats du test de mutagénicité montrent que TA102 était la souche la plus sensible, suivie par TA1538 et TA100. Les eaux usées de l'hôpital étaient plus souvent mutagènes que les eaux usées des STEPs. Il est par conséquent très important que les eaux usées d'hôpitaux ne soient jamais déversées dans l'environnement sans un traitement adéquat. La comparaison de la mutagénicité des eaux usées et des eaux traitées des STEPs montre qu'un nombre plus faible d'eaux traitées étaient mutagènes. Ces résultats indiquent que les traitements biologiques des STEPs étaient relativement efficaces pour diminuer la mutagénicité des eaux usées.

A cause de l'effet bénéfique présumé et de l'importance économique, les actions élaborées pour réduire les rejets de médicaments dans l'environnement sont souvent contestées. De plus, l'utilisation des substances pharmaceutiques va très probablement augmenter avec l'accroissement de l'age de la population. Une solution pour limiter la pollution par ces substances serait d'ajouter des traitements d'eaux usées à la sortie des hôpitaux et d'éviter tout débordements dans les STEPs communales. D'autres solutions seraient de diminuer la pollution à la source, en développant un label clair sur les médicaments, des directives pour l'élimination des médicaments non-utilisés et des campagnes de sensibilisation de la population à l'(éco)-toxicité de ces substances. Ces recommandations pourraient améliorer la santé de la population en diminuant l'ingestion de substances actives, et diminuer les coûts de la santé liés à cette sur-consommation.

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Glossary of Terms

Acute exposure Contact with a substance that occurs once or for only a short time (compare with chronic exposure).

Additive effect A biologic response to exposure to multiple substances that equals the sum of responses of all the individual substances added together (compare with antagonistic effect and synergistic effect).

Aneuploid Having a chromosome number that is not a multiple of the haploid number for the species.

Antineoplastic Drugs that control or kill neoplastic cells; used in chemotherapy to kill cancer cells.

Cancer Group of diseases that occur when cells in the body become abnormal and grow or multiply out of control.

Carcinogen A substance, factor or situation that causes or induces cancer.

Chronic exposure Contact with a substance that occurs over a long time (more than 1 year for humans).

DNA Deoxyribonucleic acid. DNA constitutes the molecules inside cells that carry genetic information and pass it from one generation to the next.

Cytostatic Inhibiting or suppressing cellular growth and multiplication.

Ecotoxicology The science that deals with the possible impact of chemicals on the environment.

Effluent Wastewater that flows out of a treatment plant, sewer, or industrial outfall. Generally refers to wastes discharged into surface waters.

Endocrine disruptor A natural or man-made chemical that can interfere with endocrine glands and their hormones or where the hormones act - the target tissues.

Frameshift mutation Type of mutation consisting of the insertion or deletion of one or more nucleotides in the nucleic acid structure of a gene, when the number of base pairs inserted or deleted is not a multiple of three. If the addition or deletion occurs in multiples of three, the unaffected nucleotides in the genome remain in the proper order ("frame") to be correctly translated into protein; in such cases of insertions or deletions not causing a frame shift, a functional though altered protein may be produced by the organism. Frameshift mutations cause more profound changes in the composition of the protein resulting from translation of the mutated gene.

Gene The functional and physical unit of heredity passed from parent to offspring. Genes are pieces of DNA, and most genes contain the information for making a specific protein.

Genotoxic (genotoxicity) Toxic (damaging) to DNA. Substances that are genotoxic may bind directly to DNA or act indirectly leading to DNA damage by affecting enzymes involved in DNA replication, thereby causing mutations which may or may not lead to cancer or birth defects (inheritable damage). Genotoxic substances are not necessarily carcinogenic.

Germ cell a spermatozoon or an ovum.

Haploid Having the same number of sets of chromosomes as a germ cell or half as many as a somatic cell.

Influent Water, wastewater, or other liquid flowing into a reservoir, basin, or treatment plant.

In vitro In an artificial environment outside a living organism or body. For example, some toxicity testing is done on cell cultures or slices of tissue grown in the laboratory, rather than on a living animal.

Mutagen (mutagenic, mutagenicity) A substance or physical agent that causes mutations, i.e. permanently alters the DNA of a cell.

Mutation (gene mutation, genetic mutation, chromosomal mutation) Any permanent change in the DNA of a cell. Mutations may be caused by mistakes during cell division, or they may be caused by exposure to DNA-damaging agents in the environment. Mutations can be harmful, beneficial, or have no effect. If they occur in cells that make eggs or sperm, they can be inherited; if mutations occur in other types of cells,

they are not inherited. Certain mutations may lead to cancer or other diseases.

Nucleotide One of the structural components, or building blocks, of DNA and RNA. A nucleotide consists of a base (one of four chemicals: adenine, thymine, guanine, and cytosine) plus a molecule of sugar and one of phosphoric acid.

Oncogene A gene that is capable of causing the transformation of normal cells into cancer cells.

Prodrug An inactive precursor of a drug, converted into its active form in the body by normal metabolic processes.

Somatic cells All body cells, except the reproductive cells.

Strain A group of organisms of the same species, having distinctive characteristics but not usually considered a separate breed or variety.

Synergy (synergistic or synergic effect) A biologic response to multiple substances where one substance worsens the effect of another substance. The combined effect of the substances acting together is greater than the sum of the effects of the substances acting by themselves.

Teratogen A substance that causes defects in development between conception and birth. A teratogen is a substance that causes a structural or functional birth defect.

Tumor (tumour, solid neoplasm) An abnormal mass of tissue that results from excessive cell division that is uncontrolled and progressive. Tumors perform no useful body function. Tumors can be either benign (not cancer) or malignant (cancer).

Abbreviations

ACN	Acetonitrile
CA	Clofibric acid
CH	Switzerland
CHUV	Centre Hospitalier Universitaire Vaudois / University hospital of Lausanne
d	day
DE	Deutchland
DK	Denmark
DNA	DeoxyriboNucleic Acid
EC_{50}	median Effect Concentration or 50% effective concentration
ECOSAR	Ecological Structure Activity Relationships
effl	effluent (treated wastewater)
5-FU	5-Fluorouracil
GC	Gas Chromatography
h	hour
HC_5	Hazardous Concentration for 5% of the species
IHA	Institut für Haushaltsanalysen
inf	influent (wastewater)
inh	inhabitants
LC	Liquid chromatography
LD_{50}	lethal dose 50% kill
LLE	Liquid-liquid Extraction
LOD	limit of Detection
LOEC	Low Observed Effect Concentration
LOQ	Limit of Quantification
MEC	Measured Environmental Concentration
MeOH	Methanol
MR	Mutagenicity Ratio

MS	Mass spectrometry
ND	Not Detected
NOEC	No Observed Effect Concentration
NSAID	Non-Steroidal Anti-Inflammatory Drug
OFEFP	Office Fédéral de l'Environnement, des Forêts et du Paysage / Swiss Agency for the Environment, Forests and Landscape (SAEFL)
PEC	Predicted Environmental Concentrations
PNEC	Predicted No Effect Concentration
RA	Residential area
RSD	Relative Standard Deviation
SD	Standard Deviation
SIM	Selected ion monitoring
SPE	Solid Phase Extraction
STP	Sewage Treatment Plant
SW	Surface Water
TAM	Tamoxifen
TOL	Toluene
triet.	trietylamine
UK	United Kingdom
WW	Wastewater
WWTP	Wastewater Treatment Plant
y	year

Chapter 1

General introduction

Nowadays tons of pharmacologically active substances are consumed yearly in human medicine for diagnosis, treatment, or prevention. In animal and fish farming there is greater reliance on drugs for preventing illness, as growth promoters, or as parasiticides. Consequently, pharmaceuticals are continuously being released into the environment, mainly as a result of excreta, disposal of unused or expired products, and manufacturing processes.

The contamination of the environment by drugs is partly a function of the quantity administered, the excretion efficiency of the parent compound and metabolites, propensity of the drug to adsorb to solids, and the biodegradation in sewage treatments (or in landfill).

Since drugs are used to "take care" of humans, drugs were rarely viewed as potential environmental pollutants, even tough they can be toxic. Nevertheless, recent studies have shown a wide contamination of water environments by pharmaceutically active substances [for reviews see Halling-Sorensen *et al.*, 1998; Daughton & Ternes, 1999]. Thus, the occurrence of drugs in the environment is now a subject of concern.

1.1 Usage and Consumption

Several thousands of active ingredients are used for drugs, which are represented in even more products. For example, 6 900 human pharmaceuticals and 820 veterinary products were authorized to be sold in Switzerland in 2002 [Swissmedic, 2003]. More than half of them were only delivered with prescription. In Belgium and Luxembourg, more than 11 000 drugs but only 2 200 active substances were registered for human used, and 2 700 brand name were available on the market[1]. In Germany, about 50 000 drugs were

[1] www.aventispharma.be

registered for human use, 2 700 of which accounted for 90% of the total consumption and which, in turn, contained about 900 different active substance [Kümmerer, 2001b].

Large quantities of pharmaceutical substances are prescribed in human medical care, and they are sold in high amounts without prescription or in internet-available products. In Switzerland, the Institut für Haushaltsanalysen (IHA) - IMS HEALTH produces precise statistic in term of quantities of pharmaceutical and active compounds sold. Some of these data are presented in Tables 2.1 and 3.1 and in Appendices A.17 and A.17. For most of the countries, the estimation of drug consumptions were less precise. A synthesis of the literature data is presented in Table 1.1.

The highest consumed drugs were the analgesics, e.g. paracetamol and acetylsalicylic acid (Aspirin®). In UK analysis, it was estimated that paracetamol was used between 1130 to 2000 tonnes per annum in 2000 and 1995, respectively, followed by aspirin with 770 tonnes in 1995 and 79 tonnes in 2000 [Webb, 2001a; Sebastine & Wakeman, 2003]. Ibuprofen was used in UK at 163 tonnes and in Switzerland at 18 tonnes. The consumption of acid mefenamic was the same level in Switzerland and in UK.

The antibiotics, as amoxycillin and penicillin V, are also highly prescribed. According to Halling-Sorensen *et al.* [1998], antibiotics were the most consumed group in human therapy in UK (see Table 1.2). In addition, a total of 110 tonnes of antibiotics in UK were used as growth promoters in livestock production, as feed additives in fish farms or as coccidiostatica in poultry production. Therefore the risk that the antibiotics may end up in the environment is not negligible.

Synthetic steroids are frequently prescribed as oral contraceptives but because of their high pharmacological potency the total amounts annually sold are relatively low. Indeed, the annual prescriptions of 17α-ethinylestradiol in Germany amount only to approximately 50 kg [Ternes *et al.*, 1999b].

There is significant differences in the type of drugs which are prescribed in differing countries (see Table 1.1). For example, in the UK amoxycillin is the highest prescribed antibiotic whereas in Denmark this drug is rarely used with penicillin V being the most prescribed antibiotic [Ayscough *et al.*, 2000].

While already used in vast quantities, the consumption of drugs is expected to increase for the following reasons: expanding population, increasing age of the population, increased per capita consumption, expiration of patents.

TABLE 1.1: Quantity of substance sold or used [t/year] in different countries

Substances	CAS NR	Therapeutic class	CH[b]	UK[c]	UK[d]	DK[e]	DE[f]	AU[g]
			2001-2002	1995	2000	1997	1995 or 1997	1998
Paracetamol	103-90-2	analgesic		2000	1130	248		295
Aspirin	50-78-2	NSAID[a]		770	79	212	>500	20
Metformin	657-24-9	anti-diabetic		106	206			90
Ibuprofen	15687-27-1	NSAID[a]	18		162	34	105-180	14
Cimetidine	51481-61-9	ulcer treatment		72	36			4
Amoxycillin	26787-78-0	antibiotic			71		25-128	46
Erythromycin	114-07-8	antibiotic		68	27			11
Penicillin V	87-08-1	antibiotic			22		140	9
Sulfamethoxazol	723-46-6	antibiotic					17-76	7
Ketoprofen	22071-15-4	NSAID[a]	0.25				0.7	4
Mefenamic acid	61-68-7	NSAID[a]	17		15			
Diclofenac	15307-86-5	NSAID[a]	3.9		26		75	4
Clofibric acid	882-09-7	Antilipemic	0.14				16	

[a] Non-Steroidal Anti-Inflammatory Drug
[b] CH: Switzerland: value from the Institut für Haushaltsanalysen (IHA) - IMS
[c] Webb [2001b]
[d] Sebastine & Wakeman [2003]
[e] DK: Denmark (5.2 millions inhabitant) [Stuer-Lauridsen *et al.*, 2000]
[f] DE: Deutschland [Ternes, 1998; Stan & Heberer, 1997; Hirsch *et al.*, 1999; Ternes, 2001a]
[g] AU: Australia [Khan & Ongerth, 2004]

TABLE 1.2: Consumption in UK of medical substances per therapeutic groups [Halling-Sorensen *et al.*, 1998]

Substances	Applied weight [t/year]
Antibiotics	38
Analgesics (NSAID-type)	28
Diuretics	3.8
Anti-asthmatics	1.7
Psychleptics	7.4

1.2 Source and fate

1.2.1 Source

The main routes for human pharmaceuticals to reach the environment are expected to be through the use by patients in hospitals, medical centres or at home, and disposal of unwanted or out-of-date drugs by users (see Figure 1.1). Another way that drugs enter the environment are as waste effluents of the manufacturing processes and from accidental spills during manufacturing or distribution.

Following its use, a medical substance will be excreted in urine or faeces as a mixture of unchanged substance, metabolites or conjugated products. Metabolism is partly depending on the type of pharmaceutical compound and on the individual patient. The conjugates (i.e. glucuronide and sulphate formation) are more soluble forms. There is some evidence that conjugates entering the sewage treatment plants may be hydrolysed and reactivated to the parent drug or metabolite [Heberer, 2002a], via enzymatic, acidic or alkaline hydrolysis (see Appendix C.2 on page 208).

The substances then enter the sewage system and pass through sewage treatment before release via sludge, or effluent discharge to surface waters. Sewage treatment plants (STPs) therefore serve as an important pathway of pharmaceutical contaminations.

Veterinary products vary considerably from human pharmaceuticals in their pathways to the environment. For example, fish farm chemicals pass directly to water and are not subject to STP processes; antibiotics are added to cattle feed as growth enhancers which are then excreted and dispersed on fields in manure [Hirsch *et al.*, 1999]. The fate of veterinary products is discussed in more detail in several papers [Halling-Sorensen *et al.*, 1998; Hirsch *et al.*, 1999].

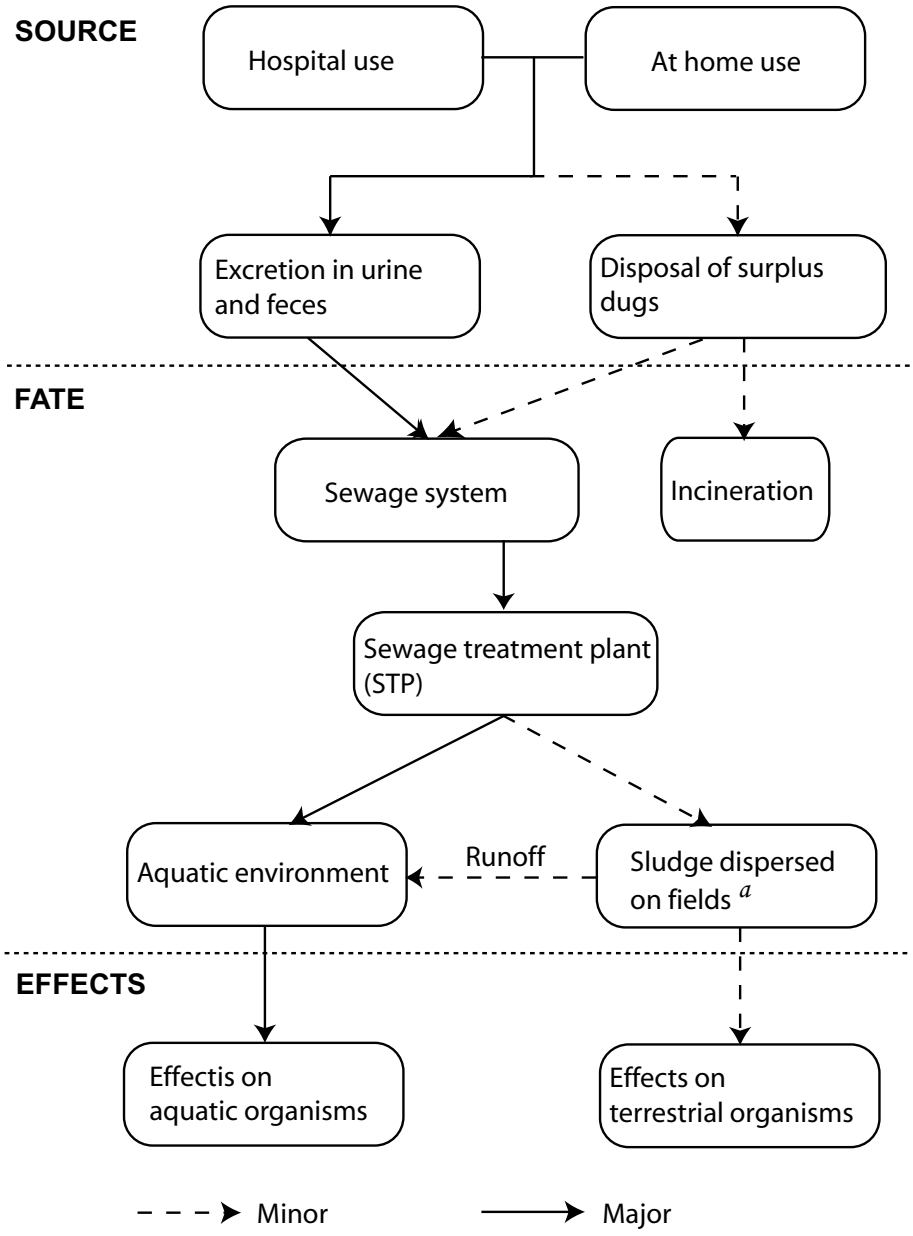

FIGURE 1.1: Possible pathways of human pharmaceuticals in the environment

[a] In Switzerland, the agriculture use of sewage sludge is no more allowed (Ordinance on substances)

1.2.2 Fate in sewage treatment plants (STPs)

The aim of a sewage treatment plants (STPs) is to remove suspended and degradable material from the wastewater. STPs that function on a biological principle are the most widespread. The various treatments are (see Figure 1.2):

Screens: remove large and medium sized clogging constituents.

Sand trap: remove sand and other heavy sediments.

Fat separator: remove floating substances as fats or oils.

Primary sedimentation tank: is used for settling of a significant portion of the organic solids. This settled material is called sludge and follow several treatments (e.g. thickening, digestion).

Biological and chemical treatment: use a large population of microorganisms or chemical (e.g. $FeCl_3$) to convert the remaining organic material into other forms which can easily settled in the secondary sedimentation tank.

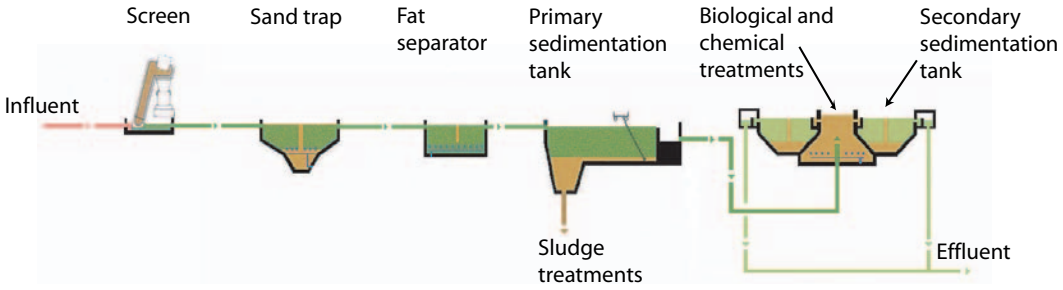

FIGURE 1.2: Description of a sewage treatment plant

In a sewage treatment plant, a pharmaceutical compound and its metabolites can follow one of three patterns in behaviour (see Figure 1.1):

- Hydrophilic (often formed by metabolism, e.g. clofibric acid) and persistent substances, remain in the aqueous phase and may pass though the STP and therefore reach the aquatic environment.

- Degradable substance, transformed into smaller molecular entities or into carbon dioxide and water. During the treatments of the STP, chemical and biological degradation could occur extensively. Aerobic

degradation by microorganisms could lead to biotransformation of the drug substance and its metabolites, with a conversion into breakdown products and some mineralisation could occur (conversion into CO_2 + H_2O). Hydrolysis and photolysis, two main chemical degradation processes, could lead to chemical transformation of the drug. Some pharmaceutical substances are susceptible to these chemical and/or biological degradation processes while others are not.

- Part of the lipophilic and not readily degradable substances will be settled in the tanks of the STPs and retained in the sludge. Hydrophobic compounds are concentrated (by several orders of magnitude) in the sludge compared with the sewage from which the sludge was derived. When the sludge is used as fertiliser and dispersed on fields, pollutants or drugs, or their biologically active metabolites, may threaten the ground water (depending on their mobility in the soil system) and hence affect terrestrial and aquatic organisms. For this reason, the agriculture use of sewage sludge is no more allowed in Switzerland (ordinance on substances).

1.2.3 Disposal

Large quantities of drugs are never consumed and many of these are eventually disposed down the toilets or via domestic refuse [Daughton & Ternes, 1999]. If a patient should have any medicines left over, the correct procedure is to return them to the pharmacy, which is then responsible for disposal. In the Canton of Vaud, 20 tonnes of unused and out-of-date pharmaceuticals are collected annually by pharmacies (pers. comm. of E. Maillefer, CRIDEC SA, Eclepens). Nevertheless, according to Kuspis & Krenzelok [1996], the majority of people in the United States will either flush unused drugs down drain or dispose of them in domestic refuse which will be incinerated.

The disposal of waste pharmaceuticals is subject to control in the cases of manufacturers, wholesalers and retailers of such products, and hospitals.

In the case of pharmacies and hospitals, disposal is done through the use of special containers to be collected by licensed waste disposal contractors. Depending on the nature of the waste, it may then undergo incineration, sent to STPs or be taken to designated landfill sites. In Switzerland, these containers are incinerated.

1.2.4 Manufacture

Wastage from manufacturing units (to landfill, incinerators or sewage treatment plants (STPs)) is minimal due to the careful handling and packaging of expensive pharmaceutical products. For example, it has been estimated that a manufacturer or packer of a pharmaceutical will only incur 1-5% wastage of their product [Richardson & Bowron, 1985]. Similarly, the distribution of pharmaceutical products is likely to lead to releases to the environment only in exceptional circumstances (e.g. an accident).

1.3 Occurrence and fate in the environment

Pharmaceutical substances were detected in hospital effluents, sewage effluents, surface waters and even in ground and drinking waters. The occurrence of pharmaceuticals has been investigated in several countries: Brazil [Stumpf *et al.*, 1999], Canada [Ternes *et al.*, 1999a], England [Ashton *et al.*, 2004], France [Andreozzi *et al.*, 2003], Germany [Heberer *et al.*, 2002; Ternes, 1998; Ternes *et al.*, 2001], Greece [Koutsouba *et al.*, 2003], Italy [Calamari *et al.*, 2003], Spain [Farré *et al.*, 2001], Switzerland [Soulet *et al.*, 2002; Golet *et al.*, 2002; Buser *et al.*, 1998a], Sweden [Andreozzi *et al.*, 2003] and US [Kolpin *et al.*, 2002].

The occurrence of pharmaceuticals in different environmental compartments, especially water, were reviewed by Halling-Sorensen *et al.* [1998]; Daughton & Ternes [1999]; Kümmerer [2001a]; Jones *et al.* [2001]; Heberer [2002a], among others.

Where pharmaceuticals have been detected in sewage effluents or surface waters, the levels are in trace amounts at the ng/l or, at most, low μg/l level.

Only a few studies looked for the presence of pharmaceuticals in groundwater [Heberer *et al.*, 1997; Sacher *et al.*, 2001; Heberer, 2002b]. Most often these have been associated with contamination via older landfills over vulnerable aquifers and therefore may represent isolated and rather specific circumstances. No quantitative data were located on concentrations of pharmaceuticals in sewage sludge.

The groups of pharmaceuticals detected are broad e.g. contraceptive hormones, lipid regulators, pain killers, antibiotics, anticancer drugs, antiepileptic drugs and those regulating blood pressure. The occurrence of drug metabolites has not been studied in detail apart from a few specific compounds (e.g. clofibric acid, fenofibric acid and salicylic acid) [Buser *et al.*, 1998b; Ahrer *et al.*, 2001; Koutsouba *et al.*, 2003; Farré *et al.*, 2001] and, therefore, current knowledge of metabolites contamination is limited.

1.3.1 Analgesics and anti-inflammatory drugs

As a pro-drug, Aspirin® (acetylsalicylic acid) is easily degraded into its more active form salicylic acid. This metabolites is detected in sewage influent, effluent and river samples at concentration up to 54 μg/l [Farré *et al.*, 2001; Ternes, 1998; Heberer, 2002b]. Ternes [1998] observed that Aspirin® was efficiently removed by the municipal STPs. According to Henschel *et al.* [1997], salicylic acid is biodegradable.

To a lesser extent, the pain killer paracetamol (acetaminophen) is also easily biodegraded [Henschel *et al.*, 1997; Richardson & Bowron, 1985]. Ternes [1998] and Kolpin *et al.* [2002] detected paracetamol in less than 24% of all samples at a maximum concentration up to 10 μg/l.

Ibuprofen, diclofenac and ketoprofen has been detected in STP influents and effluents and in surface water samples [Soulet *et al.*, 2002; Buser *et al.*, 1999; Sacher *et al.*, 2001; Öllers *et al.*, 2001; Stumpf *et al.*, 1999]. Ibuprofen showed high removal rates with biological treatment measured by several authors [Ternes, 1998; Buser *et al.*, 1999; Stumpf *et al.*, 1999]. For diclofenac, Heberer *et al.* [2002] showed a removal rate of 17% in different STPs in Berlin. On the other hand, in the STPs studied by Stumpf *et al.* [1999] and Ternes [1998], up to 75% of diclofenac was removed. In addition, diclofenac was sensitive to photodegradation [Buser *et al.*, 1998a; Andreozzi *et al.*, 2003] and ozone [Zwiener & Frimmel, 2000; Huber *et al.*, 2003; Ternes *et al.*, 2002]. Stumpf *et al.* [1999] measured a removal rates of 48% for ketoprofen with an activated sludge treatment.

Several other analgesics such as, for instance, codeine and naproxen, have also been detected in sewage and surface water samples [Ternes, 1998; Kolpin *et al.*, 2002; Öllers *et al.*, 2001; Rodriguez *et al.*, 2003a; Tixier *et al.*, 2003]. These drugs were non-biodegradable [Richardson & Bowron, 1985].

Phenazone, diclofenac or ibuprofen, have also been detected in some ground water or drinking water samples [Heberer *et al.*, 1997; Sacher *et al.*, 2001; Heberer *et al.*, 2001; Ternes, 2001a].

The Chapter 2 is focused on mefenamic acid, ibuprofen, diclofenac and ketoprofen.

1.3.2 Antibiotics

Hirsch *et al.* [1999] combined overviews on antibiotics in water with results from their own investigations and Thiele-Bruhn [2003] reviewed the contamination of soils.

Kolpin *et al.* [2002] detected a high number of antibiotics in U.S. surface water samples. Golet *et al.* [2001] found ciprofloxacin and norfloxacin in

wastewater sample at a maximum concentrations up to 400 ng/l. Ciprofloxacin has been detected at high concentrations (3 to 87 μg/l) in hospital effluents [Hartmann *et al.*, 1998]. Golet *et al.* [2002] detected ciprofloxacin and norfloxacin in surface water, but less consumed antibiotics as fleroxacin or lomefloxacin were not detected.

The occurrence of sulfamethoxazole, dehydroerythromycin and sulfamethazine in groundwater samples in Germany was reported by Sacher *et al.* [2001] and Hirsch *et al.* [1999].

Most of the tested antibiotics (ciprofloxacin, ofloxacin, metronidazole, erythromycin, tetracycline and sulphamethoxazole) were biodegradable, with the exception of penicillin G [Richardson & Bowron, 1985; Kümmerer *et al.*, 2000; Al-Ahmad *et al.*, 1999].

1.3.3 Antiepileptic drugs

The antiepileptic drugs carbamazepine and primidone have frequently been detected in wastewater and surface water samples [Öllers *et al.*, 2001; Ahrer *et al.*, 2001; Heberer, 2002b; Ternes, 1998]. Several studies showed that carbamazepine was not significantly removed during sewage treatment [Heberer, 2002b; Ternes, 1998] and can be detected in groundwater and drinking waters [Sacher *et al.*, 2001; Ternes, 2001a].

1.3.4 Beta-blockers

Several beta-blockers (metoprolol, propanolol and bisprolol) have been found in sewage effluents and in surface waters [Hirsch *et al.*, 1996; Ternes, 1998]. Sotalol was detected in three groundwater samples by Sacher *et al.* [2001].

1.3.5 Blood lipid regulators

The first detections of clofibric acid, the active metabolite of the blood lipid regulators clofibrate, etofyllin clofibrate and etofibrate, in wastewaters from STPs have already been reported in the 1970s [Hignite & Azarnoff, 1977]. Since then, this substance was one of the most frequently reported pharmaceuticals in monitoring studies. It has been detected in waste, surface, ground and drinking water samples [Stan *et al.*, 1994; Buser *et al.*, 1998b; Heberer, 2002b; Stumpf *et al.*, 1996]. Several studies showed no or little removal rate by different STPs [Heberer *et al.*, 2002; Ternes, 1998; Stumpf *et al.*, 1999]. Winkler *et al.* [2001] and Richardson & Bowron [1985] found

no evidence for biotic degradation of clofibric acid and clofibrate. The occurrence and behaviour of clofibric acid are discussed in detail in Chapter 2.

Bezafibrate and gemfibrozil have been detected in wastewater and in surface water samples [Stumpf *et al.*, 1999; Ahrer *et al.*, 2001; Farré *et al.*, 2001]. Ternes [2001a] and Heberer [2002b] detected gemfibrozil in groundwater samples.

1.3.6 Anticancer drugs

Ifosfamide and cyclophosphamide have been found in sewage samples from hospitals and STPs [Ternes, 1998; Steger-Hartmann *et al.*, 1997, 1996; Kümmerer *et al.*, 1997]. 5-Fluorouracil has been detected at high concentration (900 ppm) in wastewater from a 5-Fluorouracil plant [Anheden *et al.*, 1996]. Until now, cytostatics have not been detected in surface water [Ternes, 1998]. Ifosfamide, cyclophosphamide and methotrexate exhibited poor biodegradability [Steger-Hartmann *et al.*, 1997; Kümmerer *et al.*, 1997; Henschel *et al.*, 1997]. Chapter 3 is focused on anticancer drugs.

1.3.7 Oral contraceptives

17α-ethinylestradiol and mestranol were detected at trace-level concentration (<1-3 ng/l) in sewage effluents, surface waters and ground waters in various countries [Ternes *et al.*, 1999a; Hohenblum *et al.*, 2004; Adler *et al.*, 2001]. Baronti *et al.* [2000] measured a removal rate of 85% for 17α-ethinylestradiol with an activated sludge treatment.

1.4 Effect to the environment

The evidence supports the case that pharmaceutical compounds, refractory to degradation and transformation, do indeed have the potential to reach the environment. What is not known, however, is whether these chemicals and their transformation products can elicit physiologic effects on biota at low concentrations at which they are observed to occur.

1.4.1 Ecotoxicity

Although pharmaceutical chemicals receive considerable pharmacological and clinical testing, information on the ecotoxicity of these biologically active substances is generally limited. Acute toxicity values are in the mg/l range

for most of the pharmaceuticals detected in the environment [for reviews see: Halling-Sorensen *et al.*, 1998; Webb, 2001a]. But, reported levels in surface water are at least three orders of magnitude below the mg/l levels which cause acute toxicity. It is more difficult to assess whether there is any environmental significance with regard to long-term effects as chronic toxicity data are lacking [for review see Webb, 2001a].

1.4.2 Antibiotic resistance

Antibiotics have a different effect than common xenobiotics, because bacteria are the target organism of antibiotics. The increased use of antibiotics during the last five decades has caused a genetic selection of more harmful bacteria [Jorgensen & Halling-Sorensen, 2000]. Moreover, it seems that development of antibiotics resistance is favoured by pollution or concentrations of antibiotics in waters or sediments [Kümmerer & Henninger, 2003; Leff *et al.*, 1993; Attrassi *et al.*, 1993]. Nevertheless, according to Kümmerer [2004], there is insufficient information available to reach a final conclusion on the impact of antibiotics on bacterial populations in the environment.

1.4.3 Endocrine disruption

Endocrine disrupters, i.e., chemicals which can disturb the normal function of hormones, cause environmental damages even if they are found in very low concentration. As several pharmaceuticals (17α-ethinyloestradiol or tamoxifen) show hormonal activities, it might not be excluded that this type of effect is associated with the use of drugs [Jorgensen & Halling-Sorensen, 2000].

1.4.4 Genotoxicity

In recent years there have been an increasing interest in the genotoxicological effect connected to the release of genotoxins in the environment. Genotoxicity is a measure of the ability of a substances to damage the DNA and chromosomes of cells. Several pharmaceutical substances are genotoxic, for instance cytostatic substances [for review see Sorsa *et al.*, 1985] and antibiotics [Ehlhardt *et al.*, 1988; Giuliani *et al.*, 1996]. The Section 1.6 (page 14) is focused on a description of genotoxicity tests.

1.5 Choice of pertinent pharmaceuticals

Given the large number of pharmaceuticals in use, it is impractical to monitor samples for all the active substances involved. The use of information on metabolism/excretion, environmental fate and toxicity in addition to pharmaceutical use data would help us to make our choice on pertinent pharmaceuticals.

1.5.1 Acidic drugs: NSAIDs and clofibric acid

This work has been first focused on several pharmaceutical substances widely consumed in Switzerland and not readily biodegradable: Ibuprofen, Mefenamic acid, Diclofenac and Ketoprofen (see Table 1.1). They are non-steroidal anti-inflammatory drugs (NSAIDs) and possess analgesic and antipyretic activities. They are used for relief of the signs and symptoms of rheumatoid arthritis and osteoarthritis and are indicated for relief of mild to moderate pain. They are also indicated for treatment of primary dysmenorrhea.

This work has also been focused on the first and highly persistent substance detected in the environment: Clofibric acid. Clofibric acid is an active metabolite of Clofibrate, Etofibrate, Etofyllinclofibrate, which are drugs used as blood lipid regulators. These substances are used to decrease the plasmatic concentration of cholesterol and triglycerides.

Description sheets of these pharmaceutical substances were presented in the Appendices A.1 to A.5 (pages 120 to 132).

1.5.2 Anticancer drugs

According to Daughton & Ternes [1999] and Heberer [2002a], antineoplastics is a class of drugs of potential concern for environmental effects, not only for their acute toxicity but for their ability to effect subtle genetic changes. Indeed such compound often exhibit carcinogenic, mutagenic or embryotoxic properties.

Drugs used to treat cancer inhibit the mechanisms of cell proliferation. There is approximatively 50 active substances which are classified in several groups depending of their modes of action [Pratt *et al.*, 1994; Atkins & Gershell, 2002]. For instance:

Alkylating agents (e.g. cyclophosphamide, busulfan, carboplatin) add alkyl groups to DNA bases, causing cross-linking of DNA strands, abnormal base pairing, or DNA strand breaks, thus preventing the cell from dividing.

Anticancer antibiotics (e.g. daunorubicin) intercalate between the DNA base pairs, preventing DNA replication.

Steroid hormones (e.g. prednisolone, tamoxifen), via receptor binding, interfere with DNA synthesis and alter intracellular metabolism.

Antimetabolites (e.g. methotrexate, 5-fluorouracil) is an analog of a normal metabolite. By being mistaken for a metabolite during the synthesis of DNA, they inhibit nucleotide biosynthesis, preventing cell division or DNA synthesis.

The anticancer drugs interfere with the function of cancer cells, but also with healthy cells, inducing an important toxicity.

To select several anticancer drugs, description sheets of the ten most used cytostatic drugs were prepared and are available in the Appendices A.6 to A.15 (pages 135 to 170). The usage of cytostatic drugs in Switzerland and in the university hospital of Lausanne (CHUV) are presented in the Appendices A.17 and A.18 (pages 175 and 177).

Hydroxycarbamide (Appendix A.6) could be a substance with a high potential of contamination. Nevertheless, this compound is not stable [Havard *et al.*, 1992] and loss of 40% after 72h in water was observed [Iyamu *et al.*, 1998]. It is degraded to pyridine by pyrolyse [Elyazigi & Alrawithi, 1992].

5-Fluorouracil (Appendix A.8) is an interesting substance, since it was one of the most used anticancer drug. In addition Capecitabine (the most used) is metabolised to 5-Fluorouracil in the tumor. Adjei [1999] presented a review of Capecitabine and 5-Fluorouracil metabolism. According to Kümmerer & Al-Ahmad [1997], 5-Fluorouracil was not biodegradable. Capecitabine and 5-fluorouracil are recommended for the treatment of skin, breast and colo-rectal cancers.

Tamoxifen (Appendix A.7) is the second anticancer drug which is chosen, seeing its high consumption and its classification as human carcinogen. Tamoxifen is recommended for the treatment of breast cancers.

Cyclophosphamide (Appendix A.10) and Ifosfamide (Appendix A.9) were already studied by several scientists in Switzerland (pers. communication of H.-R. Buser, Wädenswil) and in Germany [Steger-Hartmann *et al.*, 1996]. It is the reason why these substances were not chosen in this work.

1.6 Genotoxicity

Genetic toxicity assays are used to identify germ and somatic cell mutagens and potential carcinogens. These assays can complement chemical

analysis because genotoxic chemicals are usually present as complex mixtures [Theodorakis *et al.*, 2000]. Measuring the concentrations of all genotoxic chemicals is impossible, and the additive or synergistic effects of complex mixtures are largely unknown. In addition, there may be unknown or unsuspected genotoxic chemicals present.

Human health effects commonly associated with exposures to genotoxic compounds include cancer, birth defects and heart disease [Houk, 1992]. DNA damage has been associated with perturbations in fecundity, longevity and growth of affected organisms [Shugart & Theodorakis, 1994; Barja, 2002; Steinert *et al.*, 1998].

Genotoxicity assays detect diverse kinds of genetic alterations that are relevant for human health:

Mutagenesis refers to gene (or point) mutations, which are changes in the DNA sequence within a gene.

The assays developed to measure mutagenicity are: Ames test, *Escherichia coli* reversion assay, mouse lymphoma tk assay, HPRT assay.

Chromosomal aberration refers to changes in chromosome structure, usually resulting in a gain, loss, or rearrangement of chromosome pieces (clastogenesis) or in a gain or loss of intact chromosomes (aneuploidy).

Chromosomal aberration test or micronucleus test in human or animal cells are examples of tests.

DNA Damages refers for instance to DNA fragmentation (Comet assay) and to activation of cellular system as stress or repair (*UmuC* test, SOS chromotest).

1.6.1 The *Salmonella* Mutagenicity Test

Developed by B.N. Ames and coworker in the early 1970s, the *Salmonella* mutagenicity assay (Ames test) has been used worldwide to evaluate the mutagenicity of pure chemicals and complex environmental mixtures. Of the numerous genetic bioassays available, the *Salmonella* assay is selected most often to evaluate the genotoxicity of industrial effluents, wastes and discharges.

The assay uses *Salmonella typhimurium* strains carrying a defective (mutant) gene (His^-) that prevents them from synthesising the essential amino acid histidine from the ingredients in standard bacterial culture medium. Therefore, these strains (His^-) can only survive and grow on medium that contains excess histidine. However, in the presence of a mutagenic chemical,

the defective histidine gene may be mutated back to the functional state (His^+), allowing the bacterium to grow on standard medium that does not contain supplemental histidine:

$$\underbrace{His^-}_{\text{no growth without histidine}} \xrightarrow{mutations} \underbrace{His^+}_{\text{growth without histidine}}$$

These mutations, which lead to a regaining of normal activity or function, are called "back" or "reverse" mutations and the process is referred to as "reversion". The mutant colonies, which can make histidine, are called "revertants".

To increase the susceptibility of the *Salmonella* tester strains to mutagens, various modifications to the wild-type strains have been made (see Table 1.3). Modifications include the removal of the bacteria's DNA excision repair system ($\Delta uvrB$) resulting in a decrease in the ability of the bacteria to correct DNA damage and the partial loss of the lipopolysaccharide structure of the bacteria cell wall (rfa), permitting large mutagens (such polycyclic aromatic hydrocarbons) to penetrate the cell. In addition, an extra-chromosomal piece of DNA called a plasmid (pKM101), which codes for error-prone DNA repair enzymes, has been inserted into some of the tester strains. Plasmid-containing strains incorrectly repair certain types of DNA damage and are more likely to express a mutation. Some of the *Salmonella* tester strains contain frameshift mutations (the addition or deletion of nucleotides). Other strains contain base pair substitution mutations. Frequently used strains are TA97, TA98, TA100, TA102, TA104, TA1535, TA1537, and TA1538. During the past 15 years, two strains (TA98 and TA100) have emerged as the most sensitive for analysing the mutagenicity of both pure chemical compounds and complex environmental mixtures.

Table 1.3: Commonly used *Salmonella* tester strains and their characteristics

Stain	Target allele	rfa	$\Delta uvrB$	Plasmid	Mutations
TA98	$hisD3052$	+	+	pKM101	Frameshifts
TA100	$hisG46$	+	+	pKM101	Base-pair substitutions
TA102	$hisG428$	+	-	pKM101, pAQ1	Base-pair substitutions, oxidative and alkylating mutagens
TA1538	$hisD3052$	+	+	none	Frameshifts

Thus each strain is genetically different, so using several strains in a test increases the opportunity of detecting a mutagenic sample.

Many chemicals are not mutagenic (or carcinogenic) in their native forms, but they are converted into mutagenic substances by metabolism in the liver. The bacteria do not have many of the metabolic enzymes present in mammals. Mammalian metabolism can be imitated in vitro by adding rodent liver homogenate (called S9). This permits to determine if a chemical must be metabolised to express mutagenic activity.

To perform the *Salmonella* assay, the tester strains are exposed to incremental doses of the test substance or to the environmental sample. The solution are poured onto a selective agar medium plate without histidine that allows only the revertants (His^+) to grow and form colonies. Spontaneous mutations (not induced by chemical treatment) will appear as colonies on the control petri dishes, where the sample is replaced by purified water. If the sample was mutagenic, the number of colonies of revertants arising on those plates will be significantly greater than the control plate.

2-fold rule

It is frequently recommended that, for an agent or sample to be judged as positive, it should result in at least a doubling of the spontaneous mutation rate. This 2-fold rule has the advantage to be simple. However, there are several limitations. Indeed, the results of several authors show that use of the 2-fold rule can be too radical for a low spontaneous reversion frequency (e.g. TA1538) [Cariello & Piegorsch, 1996], but also too conservative for a large spontaneous reversion frequency (TA100) [Cariello & Piegorsch, 1996; Takanashi & Urano, 1998; Hamada *et al.*, 1994; Mahon *et al.*, 1989; Helma *et al.*, 1996]. Thus the use of the 2-fold rule can increase the number of "false positive" and of "false negative" results with the strains with low or large spontaneous mutations, respectively [Chu *et al.*, 1981]. Instead of using the 2-fold rule and to avoid these false results, all our results are tested with statistical analysis.

Predicting carcinogenicity

Nearly all of the agents determined to be human carcinogens are mutagenic in the *Salmonella* assay, except hormones, metal and fibers [Shelby & Zeiger, 1990]. In comparison to four assays for genetic toxicity (*Salmonella typhimurium*, mutagenesis in mouse lymphoma cells, chromosome aberrations and sister chromatid exchanges in Chinese hamster ovary cells), *Salmonella* Ames test performed best, achieving a 66% concordance, an 89% positive

predictivity, and a 55% negative predictivity with regard to rodent carcinogenicity [Haseman *et al.*, 1990].

The rapidity and the low cost of the test make it an important tool for screening substances or samples for potential carcinogenicity.

1.7 Objective and outline

The occurrence of drugs in the environment are now a subject of concern. This thesis aimed at investigating the potential of contamination of wastewaters by several of the most frequently used and several of the most toxic pharmaceuticals and at estimating the environmental risk associated with these contaminations. The first general aim was to develop or to improve analytical methods to measure the concentration of these substances in wastewaters. These methods were applied on influents and effluents of two or three sewage treatment plants (STPs). The second aim was to adapt a genotoxicity assay for environmental samples as wastewaters, and to evaluate the contamination by genotoxins in wastewaters of hospital and of municipal STPs.

Chapter 2 presents the article "Occurrence of several acidic drugs in sewage treatment plants in Switzerland and risk assessment". This chapter discusses the wastewater contamination by four anti-inflammatory drugs (Mefenamic acid, Ibuprofen, Ketoprofen and Diclofenac) and one metabolite of blood lipid regulators (Clofibric acid). The efficiency of different STPs to remove these substances was investigated. In addition, the environmental risk that these substances may pose for the aquatic environment was assessed.

Chapter 3 presents the article "Trace determination of Tamoxifen and 5-Fluorouracil in hospital and urban wastewaters". This article describes the method developments for the analyse of Tamoxifen and 5-Fluorouracil in wastewaters. These methods were applied to evaluate the contamination of hospital and municipal wastewaters, and the removal efficiency of STPs for those two compounds.

Chapter 4 presents the article "Mutagenicity of hospital and sewage treatment plants wastewaters". This chapter discusses the mutagenicity and the bacterial toxicity of hospital and municipal wastewaters. The sensitivity of different strains of *Salmonella typhimurium* was compared. Two tests of toxicity evaluation using *Salmonella* were developed and compared.

Chapter 2

Occurrence of several acidic drugs in sewage treatment plants in Switzerland and risk assessment

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Motivations

The present chapter discusses the wastewater contamination by highly used drugs and by one of the first drug found in the environment. Since the consumption of these substances are important, the probability to detect these compound is high. The efficiency of different STPs to remove these substances during a relative long period of time was few studied. In addition, the aquatic risk in Switzerland that these substances may pose was not yet investigated.

The protocol of the developed method, the chromatographical condition and the regression lines are available in the Appendix B.1 (page 181). As these substances are also excreted in a conjugated form, an additional step of hydrolysis was tested (see Appendix C.2, page 208).

One sample of Geneva Lac was also analysed, the results are shown in Appendix C.3 (page 210).

Abstract

The occurrence and fate of five acidic drugs (Mefenamic acid, Ibuprofen, Ketoprofen, Diclofenac and Clofibric acid) were analyzed in three sewage treatment plants (STP) over 4-7 consecutive days. The results point out that the five substances were persistent in wastewater effluents after municipal wastewater treatment. At the most, half of Mefenamic acid was eliminated. Ibuprofen was well removed (80%) by one sewage treatment plant. The removal of Ibuprofen is depending on the residence time of wastewater in the STPs. A long raining period induce a important decrease of removal of Ibuprofen and Ketoprofen. Removal rates showed a great variability according to sewage treatment plants and types of treatments (e.g. biological, physico-chemical). The concentrations of Ibuprofen, Mefenamic acid and Diclofenac were relatively high in the effluents (150-2000 ng/l), showing a potential contamination of surface water. An environmental risk assessment is presented. Mefenamic acid seems to present a risk for the aquatic environment, with a ratio PEC/PNEC higher than one.

Keywords pharmaceutical, non-steroidal anti-inflammatory drugs, lipid regulation agent metabolite, wastewater, sewage treatment plant

2.1 Introduction

The occurrence of drugs in the environment is a subject of concern from several years. In comparison with the quantity of authorized drugs to be sold in Switzerland (7753 in 2002, Swissmedic [2003]), relatively few substances were analysed in the Swiss aquatic environment or in wastewaters: several antibiotics [Hartmann *et al.*, 1998; Golet *et al.*, 2002; Giger *et al.*, 2003] and anti-inflammatory drugs [Soulet *et al.*, 2002; Tixier *et al.*, 2003; Buser *et al.*, 1998a, 1999], one antiepileptic agent [Tixier *et al.*, 2003], and one metabolite of some blood lipid regulators [Soulet *et al.*, 2002; Buser *et al.*, 1998b]. The contamination is due to the consumption (up to several ten tons) and the excretion via urine and feces in wastewaters. Indeed, many of ingested pharmaceutical is excreted in the same form or in a conjugated or slightly transformed form. The unused part of medicine could be also a source of sewage contaminations. Indeed, according to a survey conducted in the USA [Kuspis & Krenzelok, 1996], 35% of people flushed medications down the toilet or sink. However the portion of drugs marketed which are not used are not known [Jorgensen & Halling-Sorensen, 2000; Halling-Sorensen *et al.*, 1998].

This study focused on five substances widely consumed in Switzerland:

Clofibric acid (metabolite), Ibuprofen, Ketoprofen, Mefenamic acid and Diclofenac (for structures see Figure 2.1). The anti-inflammatory drug Ibuprofen and Mefenamic acid are the most sold substances of this study with 17 tons per year and per substance in Switzerland (pers. comm. of Institut für Haushaltsanalysen (IHA) - IMS HEALTH, Switzerland; see Table 2.1 and Appendix A.16).

TABLE 2.1: Quantity of substance sold in Switzerland from July 2001 to June 2002 and excretion in the urine and in the bile of glucuronide and unchanged drugs

Substances		Amount sold [kg/year][a]	Excretion [% of the dose][b]
Clofibric acid	Etofibrate	67	
	Clofibrate	42	95-99%
	Etofylline clofibrate	34	8.5% (8h after administration)
Ibuprofen		17 982	15% (1% unconjugated)
Ketoprofen		254	80%
Mefenamic acid		17 275	11% (<5% unconj.)
Diclofenac		3 883	10-15% (little unconj.)

[a] From Institut für Haushaltsanalysen (IHA) - IMS in Switzerland
[b] From rxlist (http://www.rxlist.com/)

Clofibric acid is an active metabolite of Clofibrate, Etofibrate, Etofyllinclofibrate which are drugs used as blood lipid regulators. These substances are used to decrease the plasmatic concentration of cholesterol and triglycerides. Clofibric acid has already been detected in sewage treatment plant (STP) influents and effluents, in German river waters, in Swiss lakes and in groundwater wells [Soulet *et al.*, 2002; Stumpf *et al.*, 1996; Ternes, 1998; Buser *et al.*, 1998b; Heberer *et al.*, 1997].

Ibuprofen, Ketoprofen, Mefenamic acid and Diclofenac are non-steroidal anti-inflammatory drugs (NSAIDs). They possess analgesic and antipyretic activities. These drugs have been detected in STP influents and effluents and in surface water samples [Soulet *et al.*, 2002; Buser *et al.*, 1999; Sacher *et al.*, 2001; Öllers *et al.*, 2001; Stumpf *et al.*, 1999], except for Mefenamic acid which concentrations and behaviour in STPs has never been reported.

Description sheets of these pharmaceutical substances were available in Appendices A.1 to A.5 (pages 120 to 132).

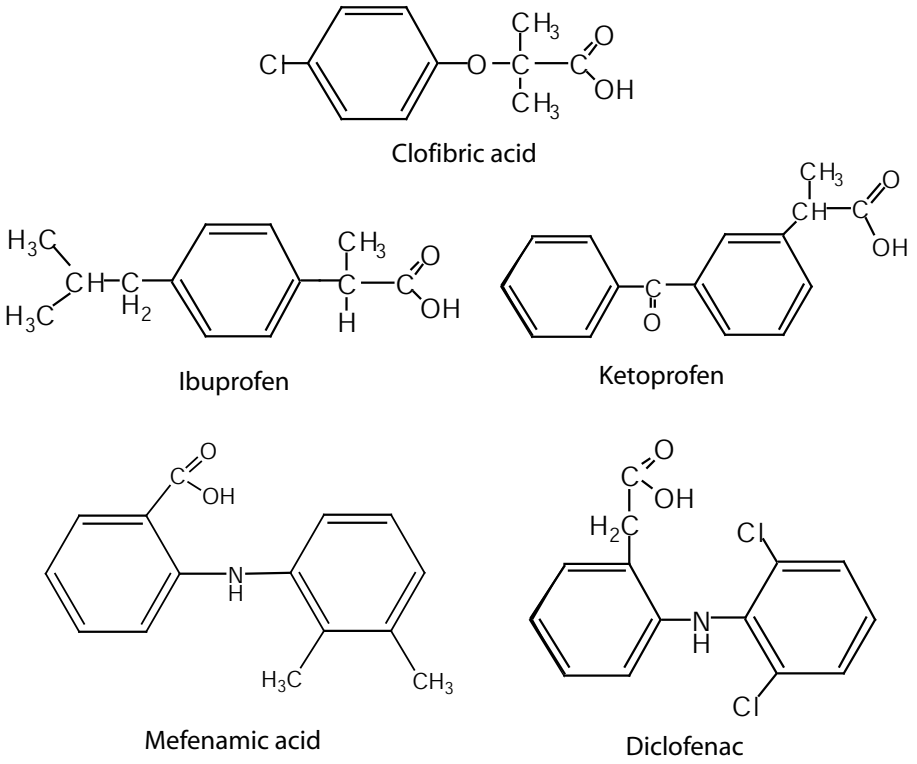

FIGURE 2.1: Structures of studied pharmaceuticals

Variations of the behaviour in various STPs and in different seasons are little known. Therefore, the first aim of our study was to analyze the occurrence of these drugs during a representative amount of days in three STPs. The second aim was to investigate the removal in summer and winter for one STP. The third aim was to measure the removal during different steps of the sewage treatment process. And the fourth aim was to assess the environmental risk that these substances may pose for the aquatic environment in Switzerland.

2.2 Experimental section

2.2.1 Standards and reagents

Ibuprofen, Ketoprofen, Diclofenac sodium salt, Clofibric acid, Mefenamic acid and Pentafluorobenzyl bromide (PFB-Br) were all purchased from Sigma-Aldrich (Steinheim, Germany). Supelclean ENVI-18 (6 ml, 1 g) solid phase

extraction (SPE) cartridges were purchased from Supelco (Bellefonte, USA). SiOH cartridges (3 ml) containing 500 mg of unmodified silica were obtained from Macherey-Nagel Chromabond (Düren, Germany). All solvents were super-purity quality from Romil (Cambridge, England) or analytical-grade from Merck (Dietikon, Switzerland). Stock solutions of all compounds were prepared in methanol.

2.2.2 Analytical procedure

2.2.2.1 Filtration and Solid phase extraction (SPE)

With a Millipore Hazardous Waste Filtration System, 250 ml portions of each sample were filtered (0.45 μm). The pH was adjusted to 2 with HCl. Subsequent extraction of solid matter retained by the 0.45 μm filter with diethyl ether did not show any presence of analytes of interest [Soulet *et al.*, 2002]. Extraction was performed by percolation through a ENVI-18 reverse phase packed tube at a flow rate of approximately 3 ml/min by applying a low vacuum. The solid phase was previously conditioned by flushing with 3 ml acetone, followed by 3 ml methanol and 3 ml of water adjusted to pH <2. At the end of percolation, erlenmeyer flasks were washed with 3 x 15 ml of acidified water, which are also passed through the cartridge. After drying the solid phase for one hour under vacuum, the analytes were eluted with 6 ml of methanol. The methanol extract was evaporated till dryness under a gentle stream of nitrogen.

2.2.2.2 Derivatisation and Clean-up

Derivatisation was performed as described by Heberer *et al.* [1994], at 90°C for one hour using 400 μl of pentafluorobenzyl bromide (2% in toluene) and 4 μl of triethylamine. The derivatised extract was passed through a SiOH cartridge conditioned with toluene. The analytes were eluted with 15 ml of toluene. The eluate volume was reduced under a gentle stream of nitrogen between 100 and 1500 μl, to be inside the range of concentration tested in the calibration curve. If higher/smaller concentration were found, the samples were diluted/concentrated and analyzed a second time.

2.2.3 Gas Chromatography and quantification

GC/MS system (Varian CP 3800 gas chromatograph / Varian 1200L mass spectrometer) was used for quantitative analysis.

The gas chromatograph was equipped with a 60 m x 0.25 mm i.d. x 0.25 μm RTX-5 capillary column connected to a 5 m deactivated fused silica 250 pre-column. Constant column flow mode was chosen.

GC injection parameters: 1 μl on-column; injection port: 250°C; 80°C for 1 min; 30°C/min to 150°C; 3.5°C/min to 280°C; 280°C isothermal 30 min.

MS parameters: Transfer line temperature: 250°C; EI mode, electron energy: 70 eV. For quantification in SIM mode, three characteristic ions were selected for each compound [Soulet *et al.*, 2002] and scanned using corresponding time windows. External standards were used for quantification. Calibration curves were obtained with four to seven standard concentrations (linear regression: $R^2 > 0.99$). Concentration of samples did not differ more than 20% from the concentration of the standard used for quantification. The results were corrected for recovery. The identity of substances in samples was confirmed by checking the relative abundances of the characteristic ions.

2.2.4 Reproducibility, determination of recoveries and detection limits

To determine the initial concentration and to quantify the reproducibility of the whole method, an unspiked sample was analyzed four times. The relative standard deviations are shown in Table 2.2.

To determine the recoveries, samples of wastewater were spiked with the pharmaceutical substances at four concentrations: 50%, 100%, 150% and 200% of the initial concentration or about 5, 10, 15 and 20 times the limit of detection for compounds not found in the wastewater tested (Clofibric acid). Samples were taken through the analytical procedure. The experimental quantities expressed as a function of the theoretical quantities enabled to determine a regression line (see Figure B.5 of the Appendix B.1; page 187). The recovery rate was then derived from the slope. Deviation standards of slopes were calculated with the method of least squares and are also shown in Table 2.2. Recoveries after filtration, SPE, derivatisation and clean-up generally exceeded 70%. Seeing that relative standard deviations on the reproducibility and standard deviation on recoveries varied from 2% to 16%, the precision is sufficient. These results point out that the analytical procedures were suitable for the analysis of these five substances.

Limits of detection (signal/noise ratio of 3) and limits of quantification (s/n ratio of 10) of the entire analytical procedure were calculated with a spiked sample and were corrected for recovery. Limits of detection and quantification (Table 2.2) were in a range which allows the detection and the quantification of these compounds in wastewaters.

TABLE 2.2: Relative standard deviations (RSD) of the method reproducibility (n = 4), recoveries and their standard deviations (SD), limits of detection (LOD) and limits of quantification (LOQ) per liter of wastewater for five pharmaceutical compounds

Substances	Reproducibility (RSD)	Recovery ± SD	LOD [ng/l]	LOQ [ng/l]
Clofibric acid		75% ± 4%	15	50
Ibuprofen	3%	74% ± 2%	8	30
Ketoprofen	16%	76% ± 5%	8	30
Mefenamic acid	11%	91% ± 5%	5	15
Diclofenac	3%	68% ± 3%	6	20

The samples of the STP of Mittleres Emmental were frozen before analysis. To test the stability of the substances during congelation, four freezed samples were analyzed and compared with four non-freezed samples. The freezed samples showed a lower quantity of substances (maximum decrease of 12%). This small decrease was no statistically significative for the five substances (level of significance was evaluated with the non-parametric Mann-Whitney U test: p above 0.05), so it was not taken into account.

2.2.5 Sampling

Eighty-six samples of wastewater were collected: in April 2002 at the STP of Mittleres Emmental in Hasle (Berne, central Switzerland), in January 2003 at the STP of Lausanne and in February and June 2003 and January 2004 at the STP of Morges (Western Switzerland, on Lake Geneva). The characteristics of these STPs are shown in Table 2.3. Seeing that sewer systems were combined for the three STPs, the effluent flow rates depend on the rain. Table 2.3 also shows the wastewater residence time during each of the treatment stages. The STPs of Mittleres Emmental and Morges have a quite similar treatment process (activated sludge and chemical precipitation with $FeCl_3$). In the STP of Lausanne, 70% of wastewater of Lausanne are treated by a chemical ($FeCl_3$) and biological (activated sludge) treatment followed by a secondary clarifier and 30% are treated by a physico-chemical treatment ($FeCl_3$ and sedimentation) followed by a biological filtration on coal.

The samples (24h composite) were collected each day during 4-7 consecutive days, with a flow proportional automatic sampler for the STPs of Mittleres Emmental and Morges and during 5 days with a time-related auto-

Table 2.3: Description of sewage treatment plants

	Mittleres Emmental-Berne	Morges	Lausanne-Vidy
Inhabitants	23 000	29 000	220 000
Effluent flow rate at dry weather (m^3/day)	9 300	8 500	100 200
Hospital	none	180 beds	1200 beds
Effluent discharged into	River (Emme)	Lake Geneva	Lake Geneva
Residence times of water in the following treatments (in hour):			
Screen and sand trap	0.9 - 1.2	X	X
Fat separator		X	
Primary sedimentation tank	2.5 - 3.4	3.9 - 5.2	0.5 - 1.6
Biological (activated sludge) and chemical ($FeCl_3$) treatment	7.0 - 9.7	9.3 - 15.9	1.1 - 1.6 Phy-Ch: 1 - 1.7
Secondary sedimentation tank	7.8 - 10.8	b	1.9 - 2.7 Bio-filter: 0.3 - 0.5

X: unknown residence time
Blank: treatment process is not available in the sewage treatment plant
b: The residence time in biological and chemical treatment included the residence time in the secondary settling tank
Phy-Ch: Physico-chemical treatment ($FeCl_3$ and sedimentation)
Bio-filter: Biological filtration (on coal)

matic sampler for the Lausanne STP (30 ml every 15 minutes). The samples were kept at 4°C during the collection and until the analysis, excepted the samples of Mittleres Emmental which were frozen.

From the STPs of Mittleres Emmental and Morges, we analyse three samples (influent, sample after primary treatment and effluent) per day of the sampling period. Two effluent samples were analysed at the Lausanne STP, one treated by biological treatment and one treated by physico-chemical treatment and bio-filtration.

2.2.6 Risk assessment

The environmental risk assessment aims to evaluate the potential impact of individual substances on the environment by examining both exposures and effects on the ecosystem of such emissions [EC, 2003]. For pharmaceutical products exposures result principally from excretion in wastewaters of active drugs and/or its metabolites by patients, so only the effects on aquatic compartment are taken into account.

The environmental risk was assessed as described by several authors [EMEA, 2001; Stuer-Lauridsen *et al.*, 2000; Ferrari *et al.*, 2003]. Risk quotients were calculated between the predicted environmental concentrations (PECs) or measured environmental concentration (MEC) and the predicted no effect concentration (PNEC), which is the concentration of the substance for which adverse effects are not expected to occur.

PNEC values of the literature are used in our study. For Clofibric acid and Diclofenac, Ferrari *et al.* [2003] estimated the PNEC values from chronic No Observed Effect Concentration (NOEC) values and a statistical extrapolation model providing an estimate of hazardous concentration for 5% of the species (HC_5). For Ibuprofen, Stuer-Lauridsen *et al.* [2000] calculated the PNEC value with the lowest environmental toxicity data and with a safety factor of 1000. For Mefenamic acid, Jones *et al.* [2002] propose a PNEC extrapolated with the Ecological Structure Activity Relationships (ECOSAR) model. For Ketoprofen, the lowest EC_{50} (median Effect Concentration) proposed by Farré & Barceló [2003] and Robin & Soulet [1999] were used to derive a PNEC with a safety factor of 1000 as recommended with short-term toxicity data [EC, 2003].

The estimation of the concentrations in influent (PEC_{inf}), in effluent (PEC_{effl}) and in surface water (PEC_{sw}) were calculated from the following equation

$$PEC = \frac{A \times (100 - R) \times E}{365 \times P \times V \times D \times 10\,000} \tag{2.1}$$

where A is the predicted amount used per year (kg/yr) (Table 2.1), R the

removal rate in percent (due to loss by adsorption to sludge particles, by hydrolysis, by biodegradation during sewage treatment, etc.), E the maximal excretion of the glucuronide and unchanged drug in percent (Table 2.1), P the number of inhabitants of the geographic area considered (in Switzerland: 7 261 000 in 2001), V the volume of wastewater *per capita* and day (0.3 m^3/capita-day), and D the factor for dilution of wastewater by surface water flow. We have chosen four scenarios:

$PEC_{inf\mathbf{a}}$: Worst case scenario. The removal rate (R) was set to zero, the excretion (E) to 100 and the dilution factor (D) to 1.

$PEC_{inf\mathbf{b}}$: Scenario considering the metabolization rate (excretion) and accounts for free and conjugated parent drugs. We calculated a more realistic influent concentration which was compared to the measured influent concentrations.

$PEC_{effl\mathbf{c}}$: Scenario with excretion and STP removal (R), which was determined from the results of the present study.

PEC_{swd}: Scenario proposed by EMEA [2001] for a prediction of an environmental concentration in surface water. The removal rate (R) was set to zero, the excretion (E) to 100 and the dilution factor (D) to 10.

The scenarios b and c did not consider the ecotoxic risk of metabolites. The scenario a and d considered that metabolites are no more ecotoxic than the parent drugs. In presence of toxic metabolites, the risk could be underestimated.

Measured environmental concentrations (MECs) correspond to the maximal concentrations measured in influents or effluents obtained during the present study. The maximal values were chosen to be the most protective in the evaluation of the risk.

2.3 Results and discussion

2.3.1 Clofibric acid

Clofibric acid was not degraded during the treatment process of Lausanne and Morges, whatever the sampling period (Figure 2.2). In the same way, Heberer *et al.* [2002] showed no removal rate in different STPs of Berlin, and Winkler *et al.* [2001] found no evidence for biotic degradation of Clofibric acid. On the other hand, the results of Ternes [1998] and Stumpf *et al.* [1999] pointed out a relatively high removal rate of 15% (biological filtration), 34%

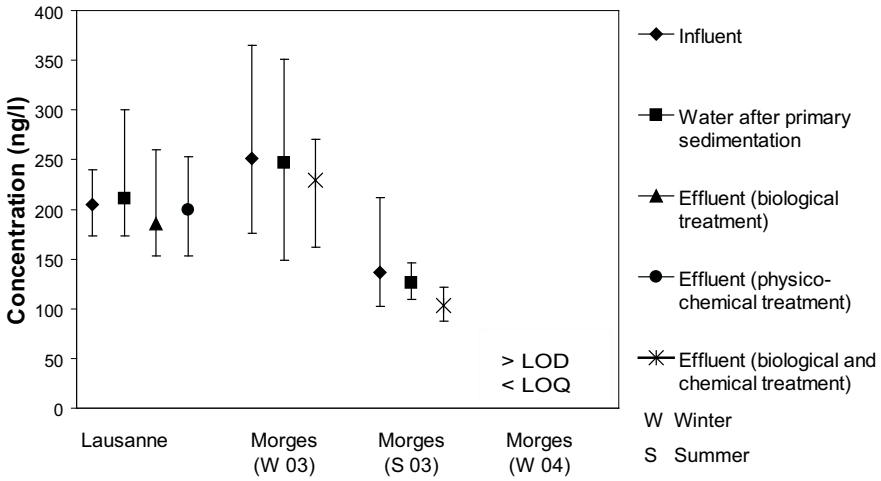

FIGURE 2.2: Ranges of concentrations over 4-7 days (minimal, maximal and average) of Clofibric acid in sewage treatment plants

(activated sludge) and 51% (activated sludge and $FeCl_3$). Thus, Clofibric acid is generally not eliminated from wastewater, but a removal can appear in some sewage treatment plants, which used nevertheless the same treatment processes as in our study.

With the daily flow rate of the effluent during the sampling period, the release in surface water was calculated. The annual release was estimated by making the hypothesis that the sampling period was representative of the whole year. In a large STP as Lausanne, approximately 12 kg per year of Clofibric acid was discharged into Lake Geneva.

This substance was not detected in wastewater of Mittleres Emmental and detected but not quantified in sewage of Morges (Winter 2004). This pharmaceutical was also not detected in STPs of France, Greece and Italy [Andreozzi *et al.*, 2003; Ferrari *et al.*, 2003]. According to these authors, fenofibrate and gemfibrozil are probably alternative parent drugs of Clofibric acid in these countries. For the area of our study, the diminution of the consumption of the Clofibric acid parent drugs is confirmed by the relevant diminution of the load of this pharmaceutical in wastewaters of Morges from 2003 to 2004 (Winter 2003: 0.09 mg/inhabitant/day; Summer: 0.05 mg/inh/d; Winter 2004: below the limit of quantification). The load for a larger STP (Lausanne) was higher (0.16 mg/inh/d). The concentrations of this substance in winter 2003 were in the same order of magnitude than published by several authors [Ternes, 1998; Stumpf *et al.*, 1999; Heberer *et al.*, 2002].

The Predicted Environmental Concentrations (PECs) for Switzerland calculated from Equation 2.1 are close to the mean measured concentrations (PEC_{inf}: 180 ng/l; mean MEC_{inf}: 230 ng/l; see Table 2.4 and Figure 2.2).

The worst case (100% excretion and no STP removal) risk quotient (PEC/PNEC), calculated with the PNEC value of Ferrari *et al.* [2003], is 0.1. Considering that aquatic risks can be suspected when PEC/PNEC ratio is above one, environmental concentrations of this substance do not seem to cause a risk for aquatic ecosystems. According to Ferrari *et al.* [2003], the risk ratio for Germany was close to one (0.92). This value, nine time higher than in Switzerland is due to a no negligible difference in the PEC (3.85 μg/l), calculated on the base of consumption data.

2.3.2 Ibuprofen

Ibuprofen was relatively well removed in the STPs of Mittleres Emmental: from 66 to 93%, mean: 79% (Figure 2.3). The primary sedimentation tank removed already 32% (12-45%). The removal rate of Ibuprofen show an important variation in the STP of Morges depending of the sampling period. This STP was most efficient in summer 2003 (removal from 62 to 79%, mean: 72%) and absolutely no removal was observed during a raining week of winter 2004. The biological treatment of Lausanne decreases slightly the concentration of Ibuprofen (20-43%, mean: 27%), in comparison with concentration after the primary sedimentation. The difference in efficiency between the biological treatment of these three STPs is due to the fact that they do not have the same residence time of water in treatments tanks. Indeed, a linear regression on our data on biological treatment of the three STPs showed that an increased residence time results in a significant increased Ibuprofen degradation (n=27, R^2=0.70, P<0.01; see Appendix C.1). In addition, the residence time is partly depending on the rain, that may have been presumably responsible for the observed decrease in drug elimination rates [Ternes, 1998]. The sampling period of the STP of Lausanne and Morges (winter 2004) was very wet (> 5 mm every day, up to 30 mm), for instance, the flow rate was sometimes three times higher than in a dry period. In our case, the rain induced a dramatic decrease of removal rates, that enhance the contamination potential. On the other hand, the small rainfall events (one or two consecutive days, 3-15 mm), that had occurred during the sampling periods of the STPs of Morges (winter and summer 2003) and Mittleres Emmental, had no relevant effect on the removal rate (non-parametric Wilcoxon test: p>>0.05). The higher removal rates for biological treatment measured by several authors [Ternes, 1998; Buser *et al.*, 1999; Stumpf *et al.*, 1999] are probably due to a relatively dry sampling period and good operating conditions as high

TABLE 2.4: Predicted No Effect Concentration (PNECs), Predicted Environmental Concentrations (PECs) estimated from Equation 2.1, Measured environmental Concentration (MEC) and risk quotients

Substances	PNEC	PEC_{inf} **a** [a]	PEC_{inf} **b** [b]	PEC_{effl} **c** [c]	PEC_{sw} **d** [d]	MEC max
	[ng/l]	[ng/l]	[ng/l]	[ng/l]	[ng/l]	[ng/l]
Clofibric acid	4200[e]	180	-	170	18	360
P(M)EC/PNEC		***0.04***		***0.04***	***0.004***	***0.09***
Ibuprofen	5000[f]	22620	3400	700-3400	2262	4620
P(M)EC/PNEC		***4.5***	***0.7***	***0.1-0.7***	***0.5***	***0.9***
Ketoprofen	15600[g]	320	255	125-255	32	570
P(M)EC/PNEC		***0.02***	***0.02***	***0.01-0.02***	***0.002***	***0.04***
Mefenamic acid	430[h]	21730	2390	1200-2340	2173	4540
P(M)EC/PNEC		***50***	***5.6***	***2.8-5.4***	***5***	***10.6***
Diclofenac	116000[e]	4890	730	730	489	2940
P(M)EC/PNEC		***0.04***	***0.01***	***0.01***	***0.004***	***0.03***

inf: influent; effl: effluent
[a] Without metabolism and STP removal
[b] With metabolism (max)
[c] With metabolism (max) and STP removal (min-max)
[d] Without metabolism and STP removal but with dilution with surface water
[e] According to Ferrari *et al.* [2003]
[f] According to Stuer-Lauridsen *et al.* [2000]
[g] Based on ecotoxicity data of Farré & Barceló [2003] and Robin & Soulet [1999] with a safety factor of 1000
[h] According to Jones *et al.* [2002] (calculated with ECOSAR)

residence times.

Physico-chemical treatment of Lausanne had no effect on Ibuprofen, which is consistent with the laboratory studies of Winkler *et al.* [2001], indicating that abiotic degradation processes were not significant. The physico-chemical treatment of Lausanne is followed by a biological filtration, which had also only little effect (22%) in the study of Stumpf *et al.* [1999]. Indeed, con-

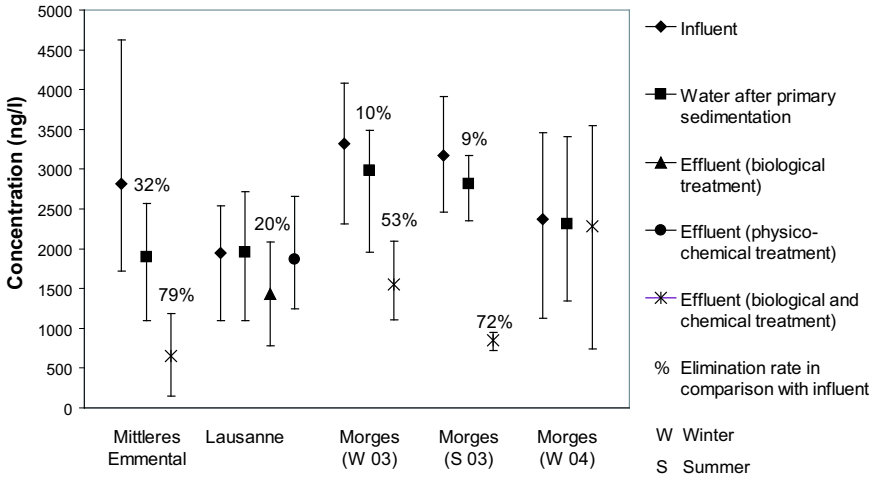

FIGURE 2.3: Ranges of concentrations over 4-7 days (minimal, maximal and average) and elimination rate of Ibuprofen in sewage treatment plants

tact times with microorganisms were certainly to short to be efficient for the removal of such substances.

In our study, Ibuprofen was the most important substance discharged into Lake Geneva by the STP of Lausanne: 140 kg/year (see hypothesis in Clofibric acid section).

Concentrations in influents varied from 1 to 4.6 μg/l, which is in accordance with the values of other Swiss STPs presented by Buser *et al.* [1999], but higher than concentrations in a Brazilian STP [Stumpf *et al.*, 1999]. The variation of concentration from one day to another is very important, especially for the STP of Mittleres Emmental (from 1.7 μg/l for Monday up to 4.6 μg/l for Wednesday). These variations of concentrations are not due to a variation of flow, which was relatively stable during the week (7000-9600 m^3/day). Thereby the load between Monday and Wednesday varied also of a factor three (0.6-1.6 mg/inhabitant/day).

Ibuprofen was measured in lower quantity in the STP of Morges in 1999 by Soulet *et al.* [2002]. In addition, from January 2003 to January 2004, a relevant increase of the average load of Ibuprofen reached the STP of Morges (Winter 2003: 1.2 mg/inhabitant/day; Summer: 1.1 mg/inh/d; Winter 2004: 2.3 mg/inh/d) was observed. The differences in concentrations and loads are likely due to an increase of Ibuprofen consumption in this area. More analysis covering a larger period (with various weather) will be necessary to confirm this state. The load reaching the STP of Lausanne and Mittleres Emmental were the same order of magnitude (1.4 and 1.0 mg/inh/d, respectively).

The Swiss PEC estimated including metabolism ($PEC_{inf\mathbf{b}}$: 3400 ng/l, Table 2.4) was close to the influent concentrations (MEC_{inf} range of various STP means: 2000-3300 ng/l, Figure 2.3). This shows that the values of consumption and excretion rate used in Equation 2.1 are consistent.

PEC/PNEC ratio exceeded one for the worst case scenario (100% excretion and no STP removal), remained close to one for the maximum measured concentration, but was below one for the more realistic scenarios taking into account metabolism or surface water dilution. Risk ratios for Denmark are above one [Stuer-Lauridsen *et al.*, 2000], showing that this substance could pose a potential risk for freshwater ecosystems. Nevertheless, the elimination by sedimentation in the aquatic environment [Tixier *et al.*, 2003], could decrease this risk.

2.3.3 Ketoprofen

Half of Ketoprofen (from 15% to 72%) was removed from the influents of Morges (winter and summer 2003) and Mittleres Emmental (Figure 2.4). The most important part of the elimination took place during the primary sedimentation in the STP of Morges, but after this treatment in the Mittleres Emmental STP. This difference could be due to the fact that the residence time of water in the primary sedimentation tank was higher in the STP of Morges than in Mittleres Emmental (see Table 2.3). The physico-chemical treatment of Lausanne removed 20% (5-36%) of this substance. For a similar treatment, Stumpf *et al.* [1999] found slightly higher elimination rates in a Brazilian STP (48%). During the rainfall events, the biological treatment (Lausanne and Morges winter 2004) had nearly no effect on Ketoprofen. Thus, STP efficiencies to remove Ketoprofen are very sensitive to external perturbations, as pouring rain.

Lausanne sewage treatment plant released annually about 14 kg of Ketoprofen.

The concentrations of Ketoprofen in influents were in the same order of magnitude than in other Swiss STPs [Öllers *et al.*, 2001; Soulet *et al.*, 2002], but lower than in a Brazilian STP [Stumpf *et al.*, 1999]. The used water and the consumption of this substance might be different in the area of the Brazil STP than in Switzerland (Table 2.1). In addition, the consumption of Ketoprofen seems to increase from 2003 to 2004, indeed the reached quantity in the STP of Morges increase continuously (winter 2003: 0.11 mg/inhabitant/day; summer: 0.15 mg/inh/d; winter 2004: 0.26 mg/inh/d). The same quantity of this drug was loaded in the STP of Mittleres Emmental (winter 2002: 0.12 mg/inh/day), with an approximatively equivalent number of connected inhabitants. This value was slightly higher for a larger STP (Lausanne 2003:

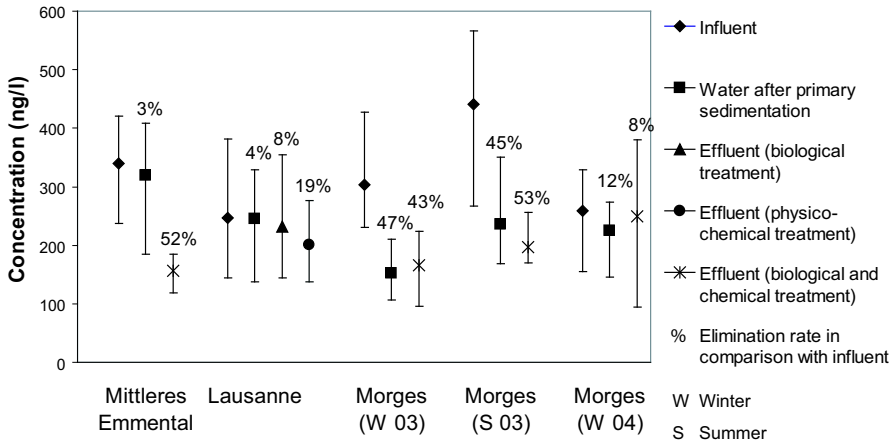

FIGURE 2.4: Ranges of concentrations over 4-7 days (minimal, maximal and average) and elimination rate of Ketoprofen in sewage treatment plants

0.18 mg/inh/d).

The Predicted Environmental Concentrations ($PEC_{inf\mathbf{b}}$: 255 ng/l and $PEC_{effl\mathbf{c}}$: 125-255 ng/l, see Table 2.4) estimated from Equation 2.1 were very close to concentrations measured in the influents (means: 250-440 ng/l) and effluents (means: 160-250 ng/l).

Several ecotoxicty data were found in the literature (ToxAlert 100, *V. ficheri*, EC_{50}=15.6 μg/ml [Farré & Barceló, 2003]; Microtox®, *V. ficheri*, EC_{50}=27 μg/ml; *Daphnia magna* 24h, EC_{50}=101-138 μg/ml; *Daphnia magna* 48h, EC_{50}=52-76 μg/ml [Robin & Soulet, 1999]). The test showing the most toxicity (EC_{50}=15.6 μg/ml) was used with a safety factor of 1000 [EC, 2003]. The risk quotients for Ketoprofen are the lowest of all studied substances, but it has to be confirmed with chronic ecotoxicty data not yet available in the literature.

2.3.4 Mefenamic acid

The Mittleres Emmental STP removed half (28-74%) of Mefenamic acid, while biological and physico-chemical treatments of Lausanne STP showed an elimination rate of 40% (30-50%) and 30% (21-36%), respectively (Figure 2.5). In spite of a high residence time of water in the STP of Morges (winter 2003), no removal was measured, which was already observed in this plant for a limited number of samples [Soulet *et al.*, 2002]. Under bad weather conditions, during the sampling period of Morges 2004, this STP was able to remove 43% of Mefenamic acid (16-69%), slightly less efficient than in

summer (from 38% to 58%; mean: 49%). Thus, the STP of Morges improved significantly its efficiency to remove Mefenamic acid, in spite of rainfall events, which had no harmful effect on the removal of this pharmaceutical. We could not find any data in the literature on the behaviour of this substance in wastewater.

In the region of Lausanne, about 82 kg of Mefenamic acid were discharged per year into Lake Geneva.

The concentrations of Mefenamic acid were as high as those of Ibuprofen. Mefenamic acid is commonly used in Switzerland, (pers. comm. of IHA-IMS in Switzerland, see Table 2.1). The Predicted Environmental Concentrations ($PEC_{inf\mathbf{b}}$: 2390 ng/l and $PEC_{effl\mathbf{c}}$: 1200-2340 ng/l of Table 2.4) are very close to the measured concentrations of influents (means: 1600-2400 ng/l) and effluents (means: 800-2400 ng/l). This shows that the values of consumption and excretion rate used in Equation 2.1 are also consistent. From January 2003 to January 2004, we observed a slight increase of the average load of Mefenamic acid reached the STP of Morges (Winter 2003: 0.9 mg/inh/day; Summer: 1.1 mg/inh/d; Winter 2004: 2.0 mg/inh/d). This increase was relevant for January 2004, due certainly to an important consumption of this drug during such painful bad weather condition. This increase has to be confirmed by the analysis of more samples during a longer sampling period. The load reaching the STP of Lausanne in winter 2003 was the same order of magnitude (1.2 mg/inh/d), but the one reaching the STP of Mittleres Emmental one year before was less important (0.5 mg/inh/d). This difference

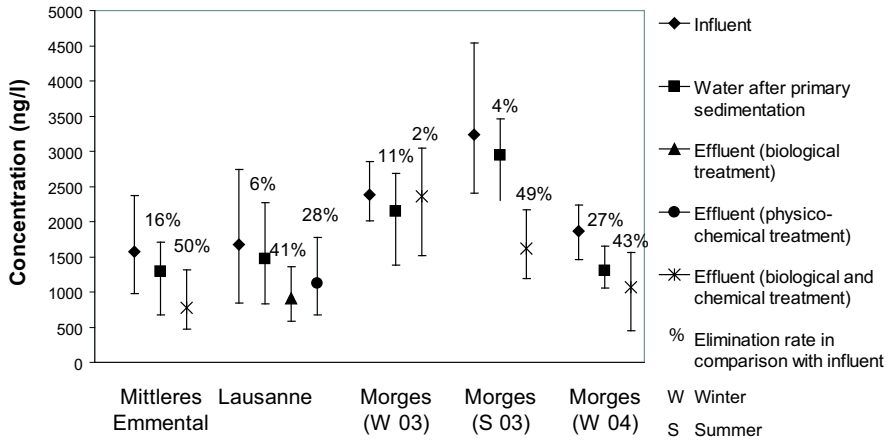

FIGURE 2.5: Ranges of concentrations over 4-7 days (minimal, maximal and average) and elimination rate of Mefenamic acid in sewage treatment plants

could be due to the absence of hospital in the watershed of this STP. Indeed, Mefenamic acid are the most sold substance by hospital (10%, only 3% for Ibuprofen; pers. comm. of IHA - IMS HEALTH, Switzerland; see Appendix A.16 on page 174).

There is no ecotoxicity data published in the literature, but Jones *et al.* [2002] propose a PNEC value calculated with the Ecological Structure Activity Relationships (ECOSAR) model. This program estimates the toxicity of chemicals to aquatic organisms such as fish, invertebrates and algae in the absence of test data, using structures activity relationships (SARs) to predict the aquatic toxicity of chemicals based on the similarity of their molecular structures to other compounds for which the aquatic toxicity is known. Thus, a risk quotients could be estimated (See Table 2.4). PEC/PNEC ratios exceed one for all scenarios. Due to the high consumption of this substance in Switzerland, the PEC is higher than in UK (440 ng/l, Jones *et al.* [2002]), on that account the PEC/PNEC ratio for Switzerland is higher than the one presented by Jones *et al.* [2002] for UK (1.03). PEC/PNEC ratios for Mefenamic acid are ten times higher than for Ibuprofen. On that account, this pharmaceutical seems to be the pharmaceutical of our study presenting the highest risk for the aquatic environment. Since PNEC estimated with ECOSAR are overconservative for a large amount of substances [Salvito *et al.*, 2002; Cash, 1998] including several pharmaceuticals [Sanderson *et al.*, 2003; Cleuvers, 2003], the risk estimated could be overprotective for this drug. In addition, the validation and the statistical robustness of this model are debated [Kaiser *et al.*, 1999]. Thus, these estimations have to be imperatively confirmed in future with ecotoxicity tests.

2.3.5 Diclofenac

None of the studied sewage treatment plants were able to remove Diclofenac from wastewater (Figure 2.6). In addition, the removal of this pharmaceutical was not dependent on the sampling period. Heberer *et al.* [2002] pointed out a slightly higher elimination rate of 17% in different STPs in Berlin. On the other hand, in the STPs studied by Stumpf *et al.* [1999] and Ternes [1998] with biological treatment, up to 75% of Diclofenac was removed. Thereby, the removal of this substance was very variable between different STPs studied by various authors.

The sewage treatment plant of Lausanne discharged 50 kg per year of Diclofenac, which are probably degraded in surface water by photodegradation [Buser *et al.*, 1998a; Andreozzi *et al.*, 2003] or in drinking water treatment plants by ozone [Zwiener & Frimmel, 2000; Huber *et al.*, 2003; Ternes *et al.*, 2002].

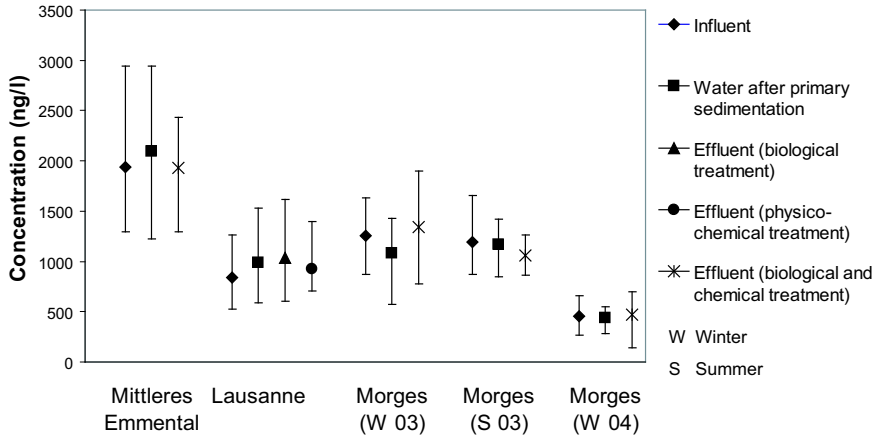

FIGURE 2.6: Ranges of concentrations over 4-7 days (minimal, maximal and average) of Diclofenac in sewage treatment plants

The concentrations of Diclofenac were close to Ibuprofen and Mefenamic acid, especially for the Mittleres Emmental STP, in spite of a lower consumption (Table 2.1). Indeed, the measured concentrations of this STP (mean: 1900 ng/l) were about three times higher than the Predicted Environmental Concentrations ($PEC_{inf\mathbf{b}}$ and $PEC_{effl\mathbf{c}}$: 730 ng/l, of Table 2.4). But the measured concentrations for the STP of Morges during a dry sampling period (mean: 1250 ng/l) are closer to the PECs. The loads of Diclofenac were the same order of magnitude in the STPs of Lausanne and Mittleres Emmental (0.63 and 0.67 mg/inh/d, respectively). From January 2003 to January 2004, we observed no relevant difference of the average load reached the STP of Morges (Winter 2003: 0.45 mg/inhabitant/day; Summer: 0.42 mg/inh/d; Winter 2004: 0.47 mg/inh/d).

The risk quotients for Diclofenac were as low as the one calculated for Clofibric acid (Table 2.4). The risk quotients calculated for France [Ferrari *et al.*, 2003] with the worst case scenario (100% excretion and no STP removal) were identical to our value in Switzerland (0.04). Nevertheless, the administration of this pharmaceutical had adverse effect on terrestrial ecosystem. Indeed, Oaks *et al.* [2004] observed that vulture population decline was directly due to the administration of this drugs on live stock that died and left for scavengers to remove.

2.4 Conclusion

The concentrations of Ibuprofen, Mefenamic acid and Diclofenac were relatively high in the effluents, and thus, the risk for surface water contamination was important. None of these drugs were well removed (>80%), except Ibuprofen in the STP of Mittleres Emmental. For Ibuprofen, the removal is depending on the residence time of wastewater in biological treatment. The removal of Ibuprofen and Ketoprofen were decreased during a raining period of winter, in comparison to dry periods. None of the studied STPs were able to remove Diclofenac and Clofibric acid of wastewater. For the other substances, the elimination rates depended on the type of treatment available in the different sewage treatment plants. The biological and physico-chemical treatments did not have the same impact. This study showed a high variability of concentrations (up to a factor three) and of removal rates during the week. Thus, to obtain a reliable average removal rate, it is important to analyse a representative amount of samples, which was done in this study by analysing wastewater of four to seven consecutive days.

Mefenamic acid seems to present the most important risk, followed by Ibuprofen, Clofibric acid, Diclofenac and Ketoprofen. But the risk ratio for surface water calculated with a dilution factor was above one only for Mefenamic acid. Since that toxicity of a single drug might be enhanced by the occurrence of other pharmaceuticals with similar activity [Cleuvers, 2003], the overall risk of these drugs could be significant. To our knowledge, chronic ecotoxicity data are available only for Diclofenac and Clofibric acid. These kind of data are needed for the other chemicals to confirm our results.

Acknowledgements

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Chapter 3

Trace determination of Tamoxifen and 5-Fluorouracil in hospital and urban wastewaters

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Motivations

Since the occurrence of anticancer drugs in the environment are few studied and that these substances are extremely toxic, the contamination of wastewaters by two of the most used anticancer drugs were evaluated. The removal efficiency of STPs and the contamination of treated wastewaters were also a subject of interest.

The present chapter and the Appendices C.4 to C.5 (pages 211 to 216) describe the method development for the analyse of Tamoxifen and 5-Fluorouracil in wastewaters. The final protocols are presented in Appendices B.2 and B.3 (pages 189 to 193).

Abstract

Tamoxifen and 5-Fluorouracil are widely used in cancer therapy. They are highly toxic (teratogenic, mutagenic, etc.), as are most of the anticancer drugs. Two methods were set up to analyse these drugs in wastewaters to evaluate the potential for environmental contamination by anticancer drugs. Liquid-liquid extraction (LLE) followed by purification on OASIS® MCX cartridge and gas chromatography with mass spectrometry detection (GC-MS) were used for the analysis of Tamoxifen. 5-Fluorouracil was extracted with an ENV+ (Isolute) cartridge (solid-phase extraction), derivatised with pentafluorobenzyl bromide (PFBBr) and detected by GC-MS. Both methods showed good recoveries ($> 70\%$), reproducibility (RSD $< 10\%$) and limits of detection (LOD $\leqslant$ 15 ng/l). Wastewaters from a residential area, an hospital, and sewage treatment plants (STPs) were analysed using the analytical methods developed in this study. Tamoxifen was detected in wastewaters of the hospital, residential area and influent of STPs, but not in treated wastewaters. 5-Fluorouracil in all wastewaters was below the limit of detection of the analytical method.

Keywords: Tamoxifen; 5-Fluorouracil; Cytostatic; Chemotherapy; Cancer; Pharmaceuticals; Wastewaters; Sewage treatment plants

3.1 Introduction

Many drugs are detected in aquatic environments [for review see Kümmerer, 2001b; Halling-Sorensen *et al.*, 1998]. Most of the studies are based on widely used pharmaceutical compounds such as anti-inflammatory drugs [See Chapter 2; Ternes, 2001b], which have a low toxicity. Few cytostatic substances have been studied [Steger-Hartmann *et al.*, 1997; Kümmerer & Al-Ahmad, 1997; Aherne *et al.*, 1985] and no ecotoxicological data on cytostatic have been published. However, antineoplastic drugs are very toxic (mutagenic, carcinogenic or teratogenic) for humans [for review see Sorsa *et al.*, 1985] and it is a group of potential concern for environmental effects [Daughton & Ternes, 1999].

Our study focused on Tamoxifen and 5-Fluorouracil, since they are two of the most used anticancer drugs (see Table 3.1 and Appendices A.17 and A.18). Tamoxifen is a non-steroidal antiestrogen that is used as adjuvant therapy for breast cancer and it is undergoing several clinical trials as a chemo-preventive agent in healthy women at risk of breast cancer. According to Adjei [1999], colorectal cancer is the third leading cause of cancer deaths in the United States and standard therapy is 5-Fluorouracil modulated with

folinic acid. Capecitabine is a prodrug of 5-Fluorouracil approved in the treatment of colorectal and breast cancer. Capecitabine allows more convenient administration (oral), provides a quality-of-life and economic advantage and offers the potential of less gastrointestinal toxicity as compared with intravenous 5-Fluorouracil chemotherapy [Adjei, 1999; Zufia *et al.*, 2004; Diasio, 1999]. These advantages induce a higher consumption of Capecitabine than 5-Fluorouracil (see Table 3.1).

TABLE 3.1: The most sold antineoplastic drugs in Switzerland: quantities of substances sold from July 2001 to June 2002 by hospitals, pharmacies and self-dispensing doctors

Substances	Amount sold [kg/year][a]			
	Total	Hospitals	Pharmacies	Doctors
Capecitabine	455	145	207	103
Hydroxycarbamide	352	44	216	92
Tamoxifen	156	10	108	38
5-Fluorouracil	81	60	11	10
Cyclophosphamide	34	22	8	5
Methotrexate	13	6	4	3
Ifosfamide	12	12	0.1	0.1

[a] From Institut für Haushaltsanalysen (IHA) - IMS in Switzerland

Tamoxifen causes liver cancer in rats [Greaves *et al.*, 1993]. In Tamoxifen-treated women there is an increase in endometrial abnormalities [Kedar *et al.*, 1994] and in the incidence of uterine endometrial tumours [Fisher *et al.*, 1994]. Tamoxifen and its metabolite, 4-hydroxytamoxifen, exhibit both estrogenic and antiestrogenic activities [Shelby *et al.*, 1996; Jordan, 1995]. 5-Fluorouracil is mutagenic, genotoxic and teratogenic [for reviews: Sorsa *et al.*, 1985; Reifferscheid & Hell, 1996].

Description sheets of these pharmaceutical substances were available in Appendices A.7 and A.8 (pages 139 and 143).

There are several methods described in the literature for the analysis of Tamoxifen. From wastewater, Tamoxifen has been extracted with a SPE column but with low recovery (42%) and a high standard deviation (40%) [Thomas & Hilton, 2004]. Tumor tissues were analysed with a C2 (Bond-Elut) solid phase extraction (SPE) cartridge [MacCallum *et al.*, 1997]. Liquid-Liquid extraction (LLE) methods have been used for the extraction of Tamoxifen from plasma and horse serum [Lee *et al.*, 2003; Manns *et al.*, 1998]. High-

performance liquid chromatography (HPLC) - UV [Jalonen, 1988], HPLC with Fluorescence detection [MacCallum *et al.*, 1997; Fried & Wainer, 1994], LC-MS/MS [Thomas & Hilton, 2004], Gas chromatography (GC) and mass spectrometry (MS) [Murphy *et al.*, 1987; Mihailescu *et al.*, 2000] and GC with flame ionisation detection (FID) [Rodriguez *et al.*, 2003b] have been described for measuring levels of Tamoxifen.

Several methods have been reported for the quantitative analysis of 5-Fluorouracil in various matrices. For environmental samples, Kiffmeyer *et al.* [1998] proposed SPE with various sorbents (Amberlyste, C18 and ENV+). But the limit of detection (LOD) was much higher than environmental concentrations of drugs. Occupational environmental samples (air, glove) were analysed with a SPE cartridge (Isolute ENV+). LLE methods were used for the extraction of 5-Fluorouracil from human or rat plasma and urine [Bruin *et al.*, 1983; Zambonin *et al.*, 1996; Guerrieri *et al.*, 1994; Matsushima *et al.*, 1997]. The extraction was followed either by HPLC and UV detection [Loos *et al.*, 1999; Debruijn *et al.*, 1986; Kiffmeyer *et al.*, 1998; Micoli *et al.*, 2001] or by GC-MS where appropriate derivatisation increases the sensitivity [Anderson *et al.*, 1997; Matsushima *et al.*, 1997; Kok *et al.*, 1985].

The first aim of our work was to set up efficient methods for the analysis of Tamoxifen and 5-Fluorouracil in wastewater. The second aim was to analyse the contamination of hospital wastewater, municipal sewage (residential area, sewage treatment plant (STP) influents and effluents) with these drugs, and to evaluate the removal efficiency by STPs for the two compounds.

3.2 Experimental

3.2.1 Standards and reagents

Tamoxifen, 5-Fluorouracil and Pentafluorobenzyl bromide (PFBBr) were purchased from Sigma-Aldrich (Steinheim, Germany). OASIS® HLB and MCX (150 mg, 6 ml, 30 μm) cartridges were purchased from Waters (Rupperswil, Switzerland). The OASIS® HLB sorbent is a poly(divinylbenzene-co-N-vinylpyrrolidone) copolymer. The OASIS® MCX sorbent is OASIS® HLB sorbent with sulfonic groups. Isolute ENV+ (200 mg, 500 mg, 1 g, 6 ml) and C2 cartridges were purchased from Separis (Grellingen, Switzerland). SiOH cartridges (3 ml) containing 500 mg of unmodified silica were obtained from Macherey-Nagel Chromabond (Düren, Germany). Supelclean ENVI-18 (6 ml, 1 g) solid phase extraction (SPE) cartridges were purchased from Supelco (Bellefonte, USA). All solvents were super-purity quality from Romil (Cambridge, England) or analytical-grade from Merck (Dietikon, Switzer-

land). Stock solutions of both compounds were prepared in methanol.

3.2.2 Handling cytostatic drugs

Since cytostatic drugs are (geno)toxic, their handling requires a number of organisational and technical precautions in order to guarantee the best possible protection of research workers. The workers wore special protective clothing (Chemoprotect® gloves and gowns from CODAN, Germany). All stock solutions were prepared under a biological safety cabinet with laminar airflow. An absorbent paper (BenchGuard®) was used to protect the work surfaces. Waste materials were collected in appropriate sealed containers and were disposed of as contaminated material from hospital pharmacies.

3.2.3 Tamoxifen (Extraction, Purification)

3.2.3.1 Method development

Several solid phase extraction (SPE) cartridges were tested with spiked bi-distilled water (n=1) for the extraction of Tamoxifen: MCX and HLB (OASIS®), ENVI-18 (Supelco), C2 (Isolute), ENV + (Isolute). Many different conditions of column conditioning, elution, sample pretreatment, sample volumes and quantities of sorbent in the cartridge were tested (see Table C.3, page 212).

MCX (OASIS®) cartridge was tested with spiked and filtered (0.45 μm) wastewater (n=1). Since Tamoxifen is lipophilic, the addition of methanol (1%-2% of final volume) was tested to desorb this compound of wastewater particles (see Table C.6, page 215).

Liquid-liquid extraction was performed on spiked bi-distilled water (n=1) using both dichloromethane and diethyl ether (see Table C.10, page 220).

3.2.3.2 Final Method

100 g of sodium chloride was mixed with 1 l of raw wastewater in a LLE separating funnel. The extraction was performed three times with 60 ml of dichloromethane. The dichloromethane emulsion was centrifuged at 2500 rpm for 10 minutes. The dichloromethane phase (bottom layer) was passed through a funnel filled with sodium sulfate and collected in a flask. Two ml of methanol was added and the solution was evaporated to 0.2-0.4 ml in a rotary evaporator (850 mbar, 40°C). The addition of methanol is essential to avoid losses of Tamoxifen during rotary evaporation.

The purification was performed on OASIS® MCX (150 mg, 6 ml, 30 μm) cartridge conditioned with 6 ml of methanol and 1 ml MQ water. Acidified

water (10 ml at pH2) was added to the sample extract and was loaded onto the cartridge at a flow rate of 1 drop/sec (5-10 minutes for 10 ml). The flask was cleaned three times with 5 ml of acidified water, which was passed through the cartridge. The cartridge was washed with 4 ml of 0.1N HCl and dried for 2 minutes under vacuum. The cartridge was washed a second time with 4 ml of Methanol and 4 ml of methanol:acetonirile (30:70, v:v). The analyte was eluted (soak for 4 minutes and then dropwise) with 3 ml of methanol:NH_4OH (95:5, v:v) and collected in a SPE tube. The eluate volume was evaporated to dryness under a gentle stream of nitrogen and re-suspended in toluene.

3.2.4 5-Fluorouracil (Extraction, Derivatisation, Purification)

3.2.4.1 Method development

Several SPE cartridges were tested with spiked bi-distilled water for the extraction of 5-Fluorouracil: ENVI-18 (Supelco), C2 (Isolute), MCX and HLB (OASIS®), ENV + (Isolute). Many different conditions of column conditioning, elution, sample pretreatment, sample volume and quantity of sorbent in the cartridge were tested (see Appendix C.4, page 211).

Various conditions were tested to obtain an optimal and repeatable derivatisation. Three catalysts (triethylamine [Chapter 2; Matsushima *et al.*, 1997], K_2CO_3 [Plagellat *et al.*, 2004] and K_2HPO_4 [Anderson *et al.*, 1997]), various final concentrations of pentafluorobenzyl bromide (PFBBr), different temperatures (20-100°C) and durations (0.5-3 h) of the reaction were tested (see Appendix C.5, page 216).

3.2.4.2 Final Method

The pH of the raw wastewater sample (150 ml) was adjusted to 5 with one (or two) drops of HCl (32%) and phosphate buffer (0.01 mol/l KH_2PO_4 adjusted with 0.1 mol/l phosphoric acid to pH3).

The cartridge (ENV +, 6 ml, 1 g) was conditioned with 12 ml of methanol and 12 ml of phosphate buffer (0.01 mol/l KH_2PO_4 adjusted with 0.01 mol/l KOH solution to pH5). The sample was loaded with a flow rate of 3-5 ml/min (30-50 minutes for 150 ml) by applying a low vacuum. After drying the solid phase for two to three hours under vacuum, the analyte was eluted dropwise with 4x3 ml of methanol. The sorbent was soaked for 4 minutes with each 3 ml. The methanol extract was evaporated till dryness under a gentle stream of nitrogen.

The derivatisation was performed by adding 1 ml of acetonitrile and 100 μl K_2CO_3 solution (25% in MQ-water; w/w). This solution was mixed (vortex) for 30 sec, and 100 μl PFBBr solution in acetonitrile (20:80, v:v) was added. The tube were capped and incubated at 80°C for one hour.

1 ml of toluene was added. The solution was evaporated to 200 μl under a stream of nitrogen before adding 1 ml of isooctane. Purification was performed on a SiOH cartridge completed with 0.5 cm of Na_2SO_4 and conditioned with 5 ml of hexane:acetone (80:20, v:v) followed by 5 ml of hexane. After adding the extract, the cartridge was washed with 8 ml of toluene:hexane (15:85, v:v). The cartridge was dried for 1 min under vacuum. Then, the cartridge was washed with 2 ml of hexane:acetone (80:20, v:v). The analyte was eluted dropwise with the next 2 ml of hexane:acetone (80:20, v:v) and collected in a SPE tube. 0.7 ml of toluene was added, and the eluate volume was reduced under a gentle stream of nitrogen to 100 μl.

3.2.5 Gas Chromatography and quantification

A GC/MS system (Varian CP 3800 gas chromatograph / Varian 1200L mass spectrometer) was used for the quantitative analysis.

The gas chromatograph was equipped with a 60 m x 0.25 mm i.d. x 0.25 μm RTX-5 capillary column connected to a 5 m deactivated fused silica precolumn. Constant column flow mode was chosen (1 ml/min).

GC injection parameters: 1 μl with a SPI - Septum-equipped Programmable injector (on-column); injection port: 85°C for 0.2 min; 100°C/min to 250°C.

GC oven temperature programm: 90°C for 4 min; 50°C/min to 180°C for 1 min; 1.5°C/min to 270°C for 5 min; 50°C/min to 300°C; 300°C isothermal 30 min.

MS parameters: Transfer line temperature: 250°C; EI mode, electron energy: 70 eV; NCI mode, gas: methane. Tamoxifen was detected in the EI mode. A mass spectra is shown in Figure 3.1. Derivative 5-Fluorouracil was detected in NCI and EI mode. Figure 3.2 includes mass spectra in both modes. For identification of the substance in SIM mode, three to four characteristic ions were selected for each compound (see Table 3.2). External standards were used for quantification. Calibration curves were obtained with four to seven standard concentrations (linear regression: $R^2 > 0.99$). The identity of substances in samples was confirmed by checking the relative abundances of the characteristic ions.

TABLE 3.2: GC/MS data for the detection of Tamoxifen and 5-Fluorouracil-PFBBr

Substances	Retention time [min]	Characteristic ions [m/z]
Tamoxifen	63.5	58 / 72 / 371
5-Fluorouracil-PFBBr (NCI)	42.9	308-311
5-Fluorouracil-PFBBr (EI)		114 / 181 / 266 / 490

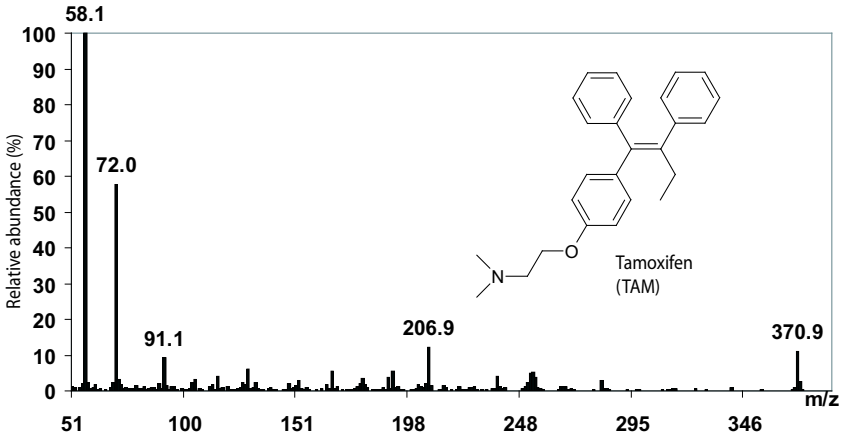

FIGURE 3.1: Structure and mass spectra of Tamoxifen (EI mode)

3.2.6 Reproducibility, determination of recoveries and detection limits

To quantify the reproducibility of the whole method, a spiked sample was analysed four times. The relative standard deviations are shown in Table 3.3.

To determine the recoveries, samples of wastewater were spiked with the pharmaceutical substances at four concentrations: about 5, 10, 15 and 20 times the limit of detection. Samples were taken through the analytical procedure. The experimental quantities expressed as a function of the theoretical quantities enabled to determine a regression line (see Figures B.7 and B.9; pages 192 and 196). The recovery was then derived from the slope. Deviation standards of slopes were calculated with the method of least squares and are also shown in Table 3.3. Recoveries after SPE or LLE, derivatisation and clean-up exceeded 70% for both compounds. Seeing that relative standard

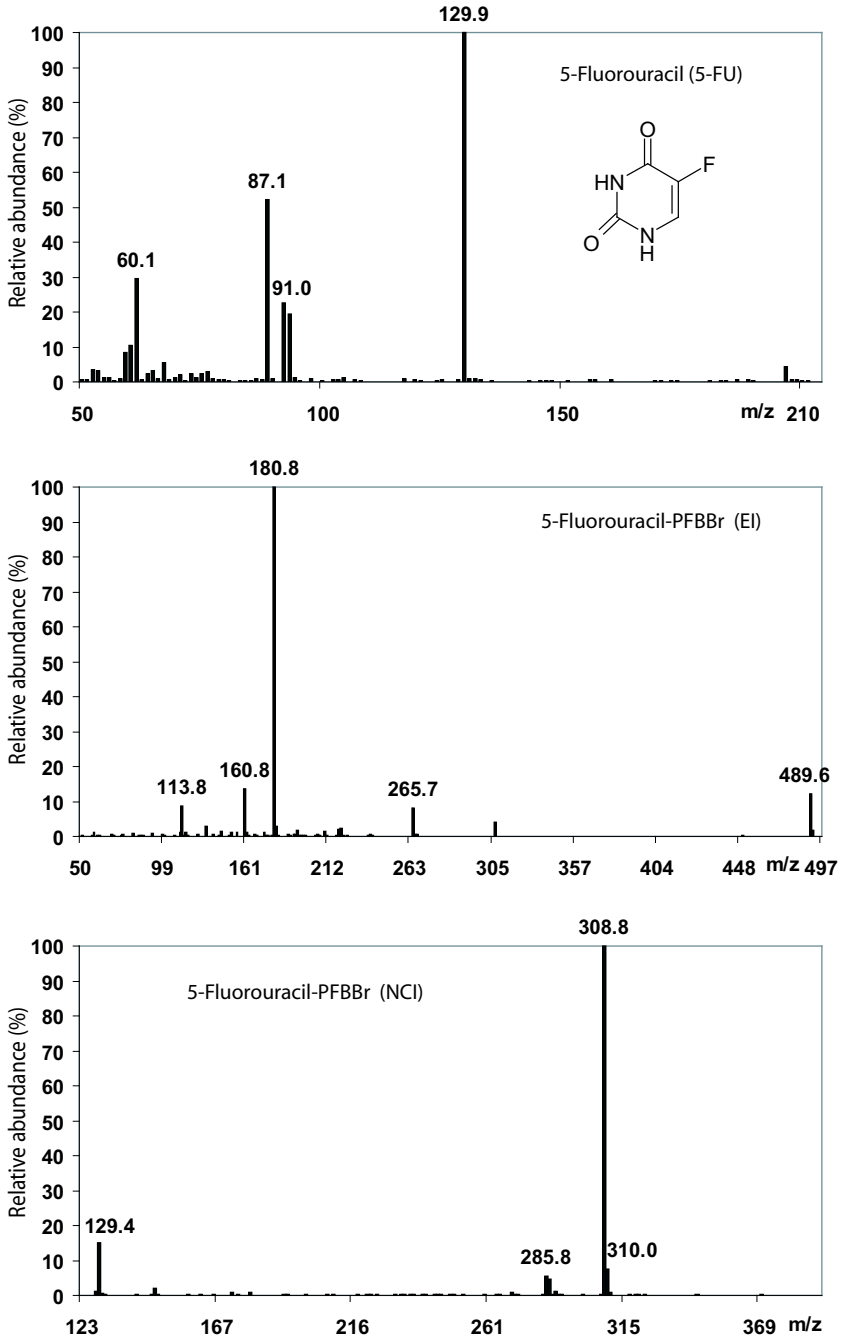

FIGURE 3.2: Structure and mass spectra of 5-Fluorouracil (EI mode) and mass spectra of derivatised 5-Fluorouracil (EI and NCI mode)

deviations on the reproducibility and standard deviations on recoveries varied from 3% to 9%, the precision is sufficient. These results indicate that the analytical procedures are suitable for the analyses of both substances.

Limits of detection (signal/noise ratio of 3) and limits of quantification (s/n ratio of 10) of the entire analytical procedure were calculated from spiked samples, and were corrected for recovery (Table 3.3).

TABLE 3.3: Relative standard deviations (RSD) of the method reproducibility (n = 4), recoveries and their standard deviations (SD), limits of detection (LOD) and limits of quantification (LOQ) per liter of wastewater for Tamoxifen and 5-Fluorouracil

Substances	Reproducibility (RSD)	Recovery ± SD	LOD [ng/l]	LOQ [ng/l]
Tamoxifen	3%	81% ± 4%	1	4
5-Fluorouracil	9%	73% ± 4%	NCI: 15	50
			EI: 30	90

3.2.7 Sampling

Thirty seven samples of wastewater were collected: in June and July 2004 at the University hospital (1200 beds) of Lausanne (CHUV) and at the STP of Lausanne (220 000 equivalent inhabitants), in July 2004 at the STP of Morges (29 000 equivalent inhabitants; Western Switzerland, on Lake Geneva) and in July and August 2004 in a residential area (RA) of Lausanne. Both STPs have a similar treatment process (activated sludge and chemical precipitation with $FeCl_3$ followed by a secondary clarifier). A more precise description of these sewage treatment plants is presented in Chapter 2 (Table 2.3, page 26).

The samples (24h composites) were collected each day during 6-7 consecutive days, with a flow proportional automatic sampler for the STP of Morges and with a time-related automatic sampler for the Lausanne STP (30 ml every 15 minutes) and for the hospital and residential wastewaters (70 ml every 15 min). The samples were analysed immediately.

From the STPs of Morges and Lausanne, two samples (influent and effluent) per day of the sampling period were analysed.

3.2.8 Calculation of predicted environmental concentrations (PECs)

The estimation of environmental concentrations (PEC) in Switzerland were calculated from the following equation, modified from several authors [Chapter 2; EMEA, 2001; Stuer-Lauridsen *et al.*, 2000; Ferrari *et al.*, 2003]

$$PEC = \frac{A \times (100 - R) \times E}{365 \times P \times V \times D \times 10000} \tag{3.1}$$

where A is the predicted amount used per year (kg/yr) (Table 3.1), R the removal rate in percent (due to loss by adsorption to sludge particles, by hydrolysis, by biodegradation during sewage treatment, etc.), E the maximal excretion of unchanged drug in percent (Table 3.5), P the number of inhabitants of the geographic area considered (in Switzerland: 7 261 000 in 2001), V the volume of wastewater *per capita* and day (0.3 m^3/capita-day), and D the factor for dilution of wastewater by surface water flow.

To estimate concentrations in wastewater (influent) and to be able to compare with analytical measurements and limits of detection, two scenarios were chosen:

$PEC_{inf\mathbf{a}}$: Without metabolisation. The excretion (E) was set to 100, the removal rate (R) to zero and the dilution factor (D) to 1.

$PEC_{inf\mathbf{b}}$: Scenario considering the metabolisation rate (Excretion). We calculated a more realistic influent concentration which was compared to the measured influent concentrations.

3.3 Results and discussion

3.3.1 Method development

3.3.1.1 Tamoxifen

Solid phase extraction (SPE) cartridges were tested for the extraction of Tamoxifen. ENV + (Isolute) gave a recovery below 5% (n=1). ENVI-18 (Supelco) provided a slightly higher recovery (<30%, n=1). 80% of extraction was achieved with a C2 cartridge (1 g, Isolute), but only with a mixture of elution solvent (either Methanol:NH_4OH or Methanol:NaCl). MCX cartridge (OASIS®) gave a recovery up to 100% (n=1) in bi-distilled water with methanol:NH_4OH (95:5, v:v) elution. Due to the high lipophilicity of Tamoxifen, losses of 50% were observed with filtered (0.45 μm) wastewater, and the

filtration step was absolutely necessary to avoid clogging of the cartridge. The addition of methanol (1-2%) before filtration did not improve the recovery. Thus SPE cartridges could not be adapted to get acceptable recovery of Tamoxifen. For this reason, liquid-liquid extractions (LLE) were tested. Seventy-five percent of Tamoxifen was extracted from bi-distilled water with diethyl ether (1 x 120 ml and 2 x 60 ml) and 100% with dichloromethane (3 x 60 ml). Before injection into the GC/MS, the LLE extract needed a purification step. Since wash steps could be introduced in the extraction with a MCX cartridge (OASIS®), we decided to combine the LLE and a SPE with several washing steps. More details on the method development are available in the Appendices C.4 to C.6 (pages 211 to 220).

The final conditions were applied to the wastewater samples and the whole methodology was tested for reproducibility and recovery (see Table 3.3). The recovery (81%±4%) and the reproducibility (RSD = 3%) are better than previously published results [Ashton *et al.*, 2004]. A mass spectrum (in EI mode) is presented in Figure 3.1 and is similar to that previously published [Mihailescu *et al.*, 2000].

3.3.1.2 5-Fluorouracil

Most of the tested cartridges gave 0% recovery (n=1) with bi-distilled water: ENVI-18 (Supelco), C2 (Isolute), MCX and HLB (OASIS®). ENV + (Isolute) showed the highest recoveries (2-110%). Losses of 5-Fluorouracil were observed with high sample volumes (see Figure 3.3). The sorption of this drug on the sorbent was weak and even water could elute 5-Fluorouracil. These losses could be reduced by using a cartridge with more sorbent. The best compromise was to use a cartridge of 1 g with only 150 ml of water (see Figure 3.3).

5-Fluorouracil can be detected without derivatisation, but the peak shape is poor. Its mass spectra is provided in Figure 3.2.

Testing of derivatisation revealed that the quantity of PFBBr is a key parameter for a complete and repeatable derivatisation. 400 μl of the solution of PFBBr (2%) was not sufficient. 600 μl of PFBBr (4%) showed a significant amelioration. An increase of temperature was necessary for a complete derivatisation. With the catalyst K_2CO_3 [Plagellat *et al.*, 2004], 60°C could be adequate with a reaction time of three hours. To decrease the duration of this method, one hour of reaction was necessary at 80°C or 100°C. Derivatisation with K_2CO_3 or K_2HPO_4 catalysts worked better than with triethylamine. K_2HPO_4 seemed very efficient, but an impurity was detected very close to 5-Fluorouracil in the GC/MS analysis. Mass spectra in EI and NCI modes are presented in Figure 3.2. The one in NCI mode is similar to one previously

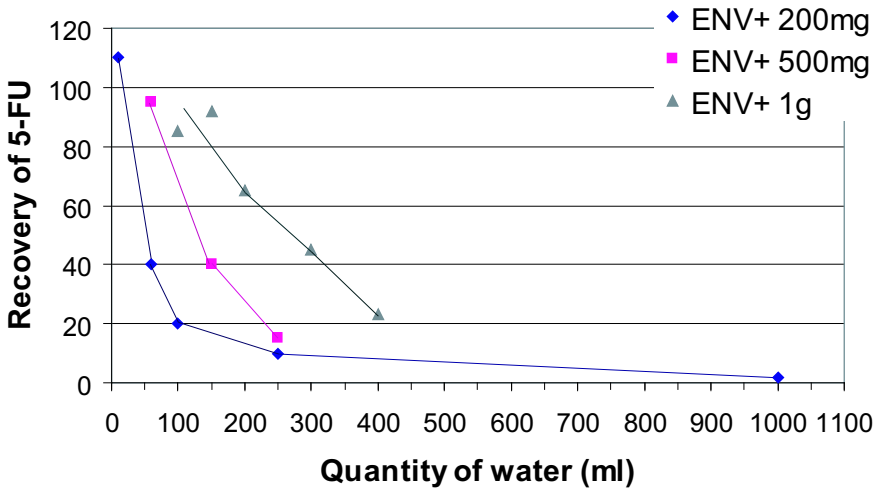

FIGURE 3.3: Recoveries of 5-Fluorouracil using solid phase extraction with ENV+ (200 mg , 500 mg and 1 g)

published [Kok *et al.*, 1985]. The EI spectra demonstrated molecular and fragment ions consistent with the addition of two PFB groups ($CH_2C_6F_5$; m/z=181). The NCI spectra showed one major fragment (M-$C_7H_2F_5$).

Again, a detailed description of the method development results are available in the Appendices C.4 and C.5.

The selected conditions were applied to the wastewater samples and the whole methodology was tested for reproducibility (RSD = 9%) and recovery (73%±4%; Table 3.3). The sensitivity was better in NCI (LOD = 15 ng/l; Table 3.3) than in EI mode (LOD = 30 ng/l), but this difference was lower than expected. The signal was higher in NCI than in EI mode. Nevertheless the noise was also more important in NCI mode that decreased the sensitivity of this mode. The sensitivity of our method is a factor of one hundred times more sensitive than that of a previously published method [Mahnik *et al.*, 2004].

3.3.2 Wastewater contamination

3.3.2.1 Tamoxifen

Tamoxifen was detected in the wastewaters from the hospital, residential area and both STPs (see Table 3.4). The concentrations of this drug were between the limit of quantification and the limit of detection (1 and 4 ng/l).

TABLE 3.4: Percentage of wastewaters of hospital (CHUV) and of two sewage treatment plants where Tamoxifen (TAM) and 5-Fluorouracil (5-FU) were detected

Substances	TAM (>1 ng/l; <4 ng/l)		5-FU
CHUV (7 samples)	70%	(5/7) (sat, sun: ND)	0%
Lausanne influents (7 samples)	100%	(7/7)	0%
Lausanne effluents (7 samples)	0%		0%
Morges influents (6 samples)	100%	(6/6)	0%
Morges effluents (6 samples)	0%		0%
RA Lausanne (4 samples)	100%	(4/4)	0%

ND: Not Detected
RA: residential area

This range of concentration is below the predicted environmental concentrations $PEC_{inf\mathbf{a}}$ and $PEC_{inf\mathbf{b}}$ (see Table 3.5). This difference could be explained in different ways. Firstly, Tamoxifen could be degraded before the analysis. Indeed, Tamoxifen is sensitive to UV light and up to 90% is degraded in five days [Jalonen, 1988]. Our analyses were performed as soon as possible and were protected from light. But some degradation cannot be ruled out. Secondly, Tamoxifen is adsorbed onto particles due to its high lipophilicity (estimated value: Log Kow = 6.3 [Meylan & Howard, 1995]). These particles could settle in sewer systems and would not be analysed. Another explanation could be that the proposed value for excretion of unchanged drug is too high, so the $PEC_{inf\mathbf{b}}$ value is overestimated.

Other authors have tried to detect this drug in wastewaters, but in most samples concentrations were below the limit of detection, with the exception of two samples [Ashton *et al.*, 2004].

Our results indicate that the hospital effluent samples for the Saturday and Sunday were not contaminated by Tamoxifen, resulting probably from a decrease of the hospital activity (work/treatment) during the weekend. Indeed, patients were fewer during the weekend and only a small amount of Tamoxifen was distributed by the hospital. Indeed, most of this substance is sold by pharmacies (see Table 3.1) and patients ingest the drug at home. On that account, we observed no difference in the contamination of hospital wastewaters and municipal sewage.

TABLE 3.5: Excretion in the urine and in the bile of unchanged drugs and Predicted Environmentally Concentrations (PECs) [ng/l] estimated from Equation 3.1, comparison of several cytostatic drugs

Substances	Excretion [% of the dose][c]	$PEC_{inf\mathbf{a}}$ [a] [ng/l]	$PEC_{inf\mathbf{b}}$ [b] [ng/l]
Tamoxifen	20% (F)	196	39
5-Fluorouracil	< 20% (U)	675	< 23
Capecitabine	0.5% of 5-Fluorouracil (U)		
Ifosfamide	12-90% dose-dependent (U)	15	2-14
Methotrexate	50-80% (U)	16	8-13
Hydroxycarbamide	30-60% (U)	443	133-266
Cyclophosphamide	50% (U)	43	22

[a] Without metabolism and STP removal
[b] With metabolism
[c] From rxlist (www.rxlist.com) or Swiss drug compendium (www.kompendium.ch)
F: Fecal excretion
U: Urinal excretion

Due to the high adsorption of Tamoxifen on particles, this drug was removed from wastewater by both STPs (see Table 3.4), avoiding surface water contamination. To our knowledge, no study is available on biodegradability of this compound.

3.3.2.2 5-Fluorouracil

5-Fluorouracil was not detected in any of the wastewater samples (see Table 3.4). Since only a small portion of this pharmaceutical is excreted in the same form, the $PEC_{inf\mathbf{b}}$ was lower than the $PEC_{inf\mathbf{a}}$ (see Table 3.5). The predicted environmental concentrations using excretion ($PEC_{inf\mathbf{b}}$) were in the range of the limit of detection of our method ($PEC_{inf\mathbf{b}}$ = 23 ng/l and LOD = 15 ng/l; see Tables 3.5 and 3.3). Seeing that the calculation of PEC used an approximated value of excretion, and that it did not take into consideration the degradation, the real concentration is below the LOD of the method.

PEC was also estimated for the wastewaters of the hospital (PEC_{CHUV}). These results are presented in Table A.6 of the Appendix A.18 (page 179).

Contradictory results have been published concerning the biodegradabil-

ity of 5-Fluorouracil [Kümmerer & Al-Ahmad, 1997; Kiffmeyer *et al.*, 1998]. According to Kümmerer & Al-Ahmad [1997], it is not biodegradable in the closed bottle test (CBT) nor in the Zahn-Wellens test (ZWT). On the other hand, Kiffmeyer *et al.* [1998] found that 5-Fluorouracil was completely removed from the spiked influent in a laboratory sewage plant within a few days, but the rate seemed dependent on the initial concentration. Nevertheless, this drug can be inactivated by ozonation [Rey *et al.*, 1999].

5-Fluorouracil has been detected in effluents of the oncologic department in Vienna University Hospital [Mahnik *et al.*, 2004]. Due to the absence of dilution with other sources of wastewaters (such as other medical departments), the detected concentrations were high (20-122 μg/l).

3.3.2.3 Potential contamination by other anticancer drugs

PECs for the seven most used chemotherapeutic agents are presented in Table 3.5. These estimations showed that Hydroxycarbamide could be a substance with a high contamination potential. Nevertheless, this risk is decreased by the fact that this compound is labile in water [Elyazigi & Alrawithi, 1992]. Cyclophosphamide $PEC_{inf\mathbf{b}}$ is the same level as 5-Fluorouracil, which is in accordance with reported environmental concentrations (<6 ng/l to 140 ng/l) [Steger-Hartmann *et al.*, 1997]. Ifosfamide has also been detected in few samples of wastewaters (<6 ng/l to 30 ng/l) and in the range of the $PEC_{inf\mathbf{b}}$ [Kümmerer & Al-Ahmad, 1997]. Methotrexate was not detected in river and potable supply samples, but has been reported in one hospital effluent (1 μg/l) [Aherne *et al.*, 1985]. As no STP sample was analysed, a comparison with the calculated $PEC_{inf\mathbf{b}}$ is not possible.

The other antineoplastic drugs were administered in lower quantity in Switzerland, and thus the risk of a detectable contamination in the environment is weak (see PECs in Appendices A.17 and A.18).

3.4 Conclusion

Cytostatic drugs are less used in comparison with other pharmaceutical substances such as anti-inflammatory drugs. Predicted environmental concentrations (PEC) are very low. Powerful methods are necessary to detect these compounds at such low concentrations (ng/l level). The methods developed in our study showed good limits of detection and quantification, recoveries and reproducibility.

Tamoxifen was detected in all wastewaters (hospital, residential area, and STPs), but was not detected in treated wastewaters. Thus, both STPs effi-

ciently removed Tamoxifen. 5-Fluorouracil was not detected in any of the wastewater samples.

Acknowledgments

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Chapter 4

Mutagenicity of hospital and sewage treatment plants wastewaters

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To be submitted

Motivations

As genotoxic pharmaceutical compounds, including cytostatic agents, are discharged in wastewaters, the mutagenic potential of wastewaters from various origins (hospital and municipal) was a domain of interest. The present chapter discusses the mutagenicity and the bacterial toxicity of wastewaters evaluated with Salmonella typhimurium (Ames test). As several strains can be used with the Ames test, their sensitivity to wastewaters was interesting to know. Since wastewater samples could also be toxic in addition to the mutagenicity and that these toxicity could interfere with the results of the mutagenicity, two methods to evaluate the survival of Salmonella were elaborated.

The protocol of the methods and of the solutions are available in the Appendices B.5 and B.4, respectively.

Abstract

As pharmaceutical compounds, including cytostatic agents that are genotoxic, are discharged in wastewater, the mutagenic potential of wastewater from various origins (hospital, two different sewage treatment plants (STP) and a residential area) was evaluated using the Ames test. The samples were not concentrated prior the analysis to determine the overall effects of these waters. The survival and the reversion frequencies of strains TA98, TA100, TA102 and TA1538 following treatment with the different wastewaters were determined. Survival was obtained by two methods. The first method was by comparing the number of reversions induced by a known mutagen in the presence and absence of wastewater. The second was by determining the colony forming ability of dilutions of treated and non-treated cultures. The samples from the hospital were on the whole more toxic than samples from the STPs and residential area. The different strains showed varying sensitivities to the toxic effects of the wastewater, with TA98 exhibiting the highest sensitivity (<5% survival). The results from the reversion assays indicated that TA102 was the most sensitive, followed by TA1538 and TA100. More hospital wastewaters than influents of sewage treatment plants were mutagenic, indicating a higher mutagenic activity in the wastewater of the hospital. Comparison of the mutagenicity of the influents and effluents of the STPs showed that less effluent samples were mutagenic. This result indicates that biological treatments were relatively efficient in decreasing the mutagenicity of wastewaters.

Keywords: Mutagenicity; Genotoxicity; Ames test; *Salmonella typhimurium*; wastewater; sewage treatment plant.

4.1 Introduction

Due to human activity, many pollutants reach the aquatic environment. The contamination of water by xenobiotics is a critical problem regarding the water ecosystem and the human population exposed via drinking water. Indeed, the presence of xenobiotic compounds or of hazardous waste sites may increase the risk of human cancer [Koivusalo *et al.*, 1994; Griffith *et al.*, 1989; Morris *et al.*, 1992]. In addition, sufficient quantities of contaminants, among them some genotoxic compounds [Stahl, 1991; Adams *et al.*, 1992; De Flora *et al.*, 1991; Theodorakis *et al.*, 2000] could result in adverse effects on the ecosystem community. Most chemicals observed to be carcinogenic in humans by clinical or epidemiological studies are genotoxic [Shelby & Zeiger, 1990]. Mutations are implicated in carcinogenesis and the *Salmonella*

mutagenicity assay (Ames test) is highly predictive for rodent carcinogens [Zeiger, 1998; Zeiger *et al.*, 1990; Tennant *et al.*, 1987]. This assay is a short term test used worldwide in more than 2000 laboratories [Edler, 1992]. In addition, the *Salmonella* mutagenicity assay has been shown to be a useful tool in the identification of important pollution sources [Umbuzeiro *et al.*, 2001].

The influence of bacteria survival and growth is seldom used in the quantification of genotoxicity results. However, samples that contain mixtures, such as wastewater or industrial effluents can be toxic [Castillo *et al.*, 2001; Dizer *et al.*, 2002; Giuliani *et al.*, 1996]. This toxicity may mask the mutagenic effect, especially if the sample is a weak mutagen. In addition, some artifacts can occur for instance in the fluctuation assay [Harrington *et al.*, 1983], that could lead to false positive results, suggesting that survival determination should be a routine part of this assay. The normalization of the genotoxic response with toxicity has been proposed for the Ames assay [Kargalioglu *et al.*, 2002] and for other genotoxic assays, such as the umuC test [Baun *et al.*, 1999].

Many studies have reported on the mutagenicity of wastewaters [Rappaport *et al.*, 1979; Helma *et al.*, 1996; Filipic & Toman, 1996b; Jolibois *et al.*, 2003; Jolibois & Guerbet, 2005], surface waters [Pelon *et al.*, 1977; Cerna *et al.*, 1996; Umbuzeiro *et al.*, 2001; Kutlu *et al.*, 2004] and drinking waters [Park *et al.*, 2000]. The absence of mutagenic activity in effluents has been described in several studies [Shishida *et al.*, 2000; Rappaport *et al.*, 1979; Monarca *et al.*, 2000] and several authors had reported a partial removal of mutagens by biological treatment [Hu *et al.*, 2003; Ono *et al.*, 1996]. Most of the studies concentrate the samples through procedures such as resin column (XAD, Separon SE), or liquid/liquid extraction (methylene chloride, dichloromethane), to increase the limit of detection. Nevertheless, the concentration of the samples results in a loss and/or modification of some compounds [Filipic & Toman, 1996a; Filipic, 1995] and in an alteration of the interaction among the substances [Filipic & Toman, 1996a]. Thus, analysis of samples "as is" without any prior treatment is more representative of the real environmental effect.

A variety of sewage sources enter into a municipal wastewater treatment plant. For instance, municipal sewage can contain domestic, commercial, or industrial wastewaters, as surface runoff. Some studies have suggested that the genotoxicity of municipal wastewaters is proportional to the occurrence of industries [Rappaport *et al.*, 1979; Meier *et al.*, 1987]. Nevertheless, seeing that the results (*umu*-test during one week) were positive even on Sunday, the origin of potency seems mainly not to come from industrial wastewater but from human [Ono *et al.*, 1996]. Moreover, other studies indicated that

the mutagenic activity of sludge is independent of the percent of industrialization [Babish *et al.*, 1983] and that over 90% of the genotoxic loading of the Montreal municipal wastewater had a nonindustrial origin [White & Rasmussen, 1998]. Since many chemicals used in hospitals, including cytostatic substances [for review see Sorsa *et al.*, 1985], antibiotics [Ehlhardt *et al.*, 1988; Giuliani *et al.*, 1996], and disinfectants [Giuliani *et al.*, 1996], are genotoxic or carcinogenic, and since urine samples of patients undergoing chemotherapy are also mutagenic [for review see Sorsa & Anderson, 1996], wastewaters of hospitals could be an important source of mutagenic potency.

The first aim of our work was to include different ways to evaluate the bacterial toxicity in the Ames test. The second aim was to compare the sensitivity of different strains of *Salmonella typhimurium* to wastewaters. The third aim was to compare the mutagenicity of hospital wastewaters with municipal sewage treatment plant influents and effluents.

4.2 Materials and methods

4.2.1 Materials

Agar and Benzo(a)pyrene were purchased from Sigma-Aldrich (Steinheim, Germany), LB broth, histidine, biotin, sodium azide, all salts and nutrients from Fluka (Buchs, Switzerland), and 2-nitrofluorene from Merck (Dietikon, Switzerland).

4.2.2 Sampling of hospital and sewage treatment plant (STP) wastewater

Forty-one samples of wastewater were collected: in June and July 2004 at the University hospital (1200 beds) of Lausanne (CHUV) and at the STP of Lausanne (220 000 equivalent inhabitants), in July 2004 at the sewage treatment plant (STP) of Morges (29 000 equivalent inhabitants; Western Switzerland, on Lake Geneva) and in July and August 2004 in a residential area of Lausanne. Both STPs have similar treatment processes (activated sludge and chemical precipitation with $FeCl_3$ followed by a secondary clarifier).

The samples (24h composite) were collected each day during 6-7 consecutive days, with a flow proportional automatic sampler for the STP of Morges and with a time-related automatic sampler for the Lausanne STP (30 ml every 15 minutes) and for the hospital and residential wastewaters (70 ml

every 15 min). The samples were immediately filtered (0.45 μm) and were stored at -20°C.

From the STPs of Morges and Lausanne, we analyse two samples (influent and effluent) per day of the sampling period.

4.2.3 *Salmonella* mutagenicity assay - Ames test

The Ames test was done with preincubation as previously described [Maron & Ames, 1983; Mortelmans & Zeiger, 2000] with the following modifications (see Figure 4.1 and Appendix B.5):

The mixture A was prepared with 1 ml of Mini A 10x (102.4 g Na_2HPO_4-$7H_2O$, 24 g KH_2PO_4, 4 g NaCl, 8 g NH_4Cl and bi-distilled water to a final volume of 800 ml), 20 μl of Growth supplement (200 mg of L-Proline, L-Threonin, L-Arginine, 400 mg of L-Leucine, 10 mg of Thiamine HCl, 100 mg of Biotin in 20 ml of sterile MQ-Water), 10 μl of $CaCl_2$ 0.1M, 10 μl of $MgSO_4$ 1M, 40 μl of glucose (50%), 50 μl of Histidine (20 mg/ml) and 8.8 ml of water sample filtered with 0.22 μm sterile filter.

3 ml (in triplicate) of the mixture A was inoculated with 100 μl of the bacteria culture. The inoculated wastewater were incubated at 37°C for 3 hours with agitation and then centrifuged at 4000 rpm during 5 minutes. The resulting pellet was re-suspended in 3 ml of sterile phosphate buffered saline (PBS) and centrifuged another time to ensure removal of histidine. This pellet was re-suspended in 0.3 ml of PBS (mixture B).

0.1 ml of mixture B was added to 2 ml of top agar (supplemented with histidine and biotin) at 45°C and mixed and poured onto minimal glucose agar plates. The plates were inverted and placed in a 37°C incubator for 48 h.

When the assay was performed with metabolic activation, 0.3 ml of the S9-mix replaced 0.3 ml of wastewater sample in the mixture A. The S9-mix was freshly prepared before each test using lyophilized Aroclor-1254-induced rat liver S9 fraction (Moltox - Trinova Biochem GmbH, Giessen, Germany). S9-mix was prepared with: S9 20%, nicotinamide adenine dinucleotide (NADP) 4mM, glucose-6-phosphae 5mM, sodium phosphate pH 7.4 100 mM, KCl 33 mM and $MgCl_2$ 8 mM.

Spontaneous reversion controls (sterile bi-distilled water) were used as negative controls. Positive controls included 2-Nitrofluorene (5 μg/ml) for TA98 and TA1538 strains without S9, Sodium azide (5 μg/ml) for TA100, Benzo(a)pyrene (5 μg/ml) for TA98 and TA100 with S9.

TA98 and TA1538 strains detect frameshift mutagens, TA100 strain responds to base-pair substitution mutations and TA102 strain detects a large variety of oxidative mutagens, active forms of oxygen and alkylating agents

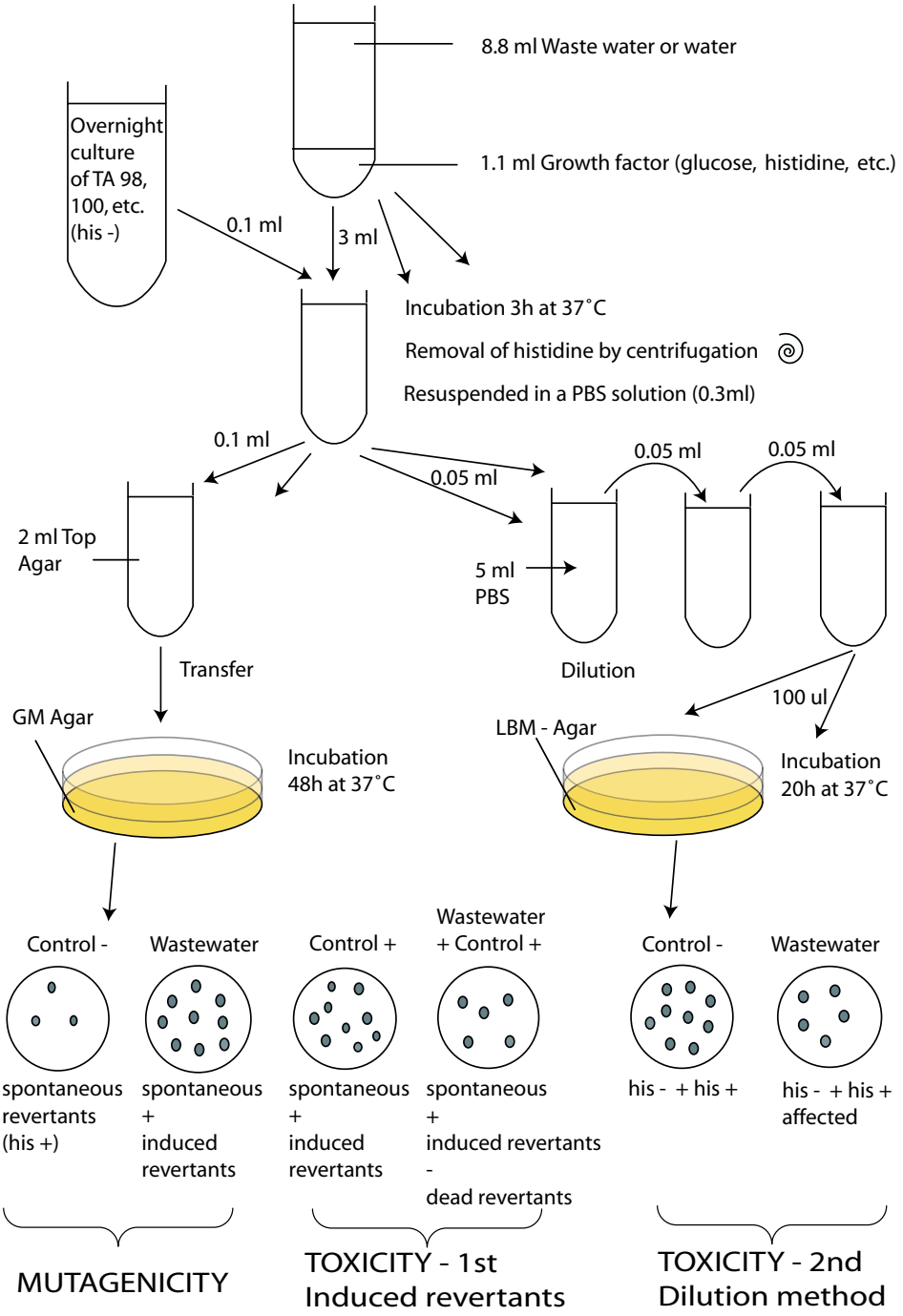

FIGURE 4.1: Ames test protocol

and has intact excision repair mechanisms. The plasmid pKM101 is present in the TA98 strain, that enhances chemical and UV-induced via an increase in the error-prone recombination DNA repair pathway [Mortelmans & Zeiger, 2000].

4.2.4 *Salmonella* toxicity assay using induced revertants - First method

The positive controls in DMSO were diluted to their respective concentrations specified in the tests in sterile bi-distilled water. To measure the toxicity of the wastewater samples, the appropriate positive control for each of the strains was added to wastewater samples (in mixture A) and the number of revertants obtained under these conditions was determined (see Figure 4.1). The toxicity of the water sample was obtained by comparing the number of revertants observed in the presence and absence of wastewater. Since the positive control for TA102 (Mitomycin C) is very toxic and needs a lot of precautionary work methods, the toxicity on this bacterial stain was not evaluated with this method.

4.2.5 *Salmonella* toxicity assay by dilution - Second method

0.05 ml of mixture B was diluted to 10^{-4} to 10^{-6} with PBS (see Figure 4.1). 0.1 ml of this dilution was used to inoculate LBM-Agar plate (12 g of Agar and 20 g of LB broth in 1 l of bi-distilled water, autoclave and dispensed 20 to 25 ml in sterile petri plates). The plates were inverted and placed in a 37°C incubator for 20 h.

4.2.6 Data and statistical analysis

For mutagenicity and toxicity (induced revertants), experiments were repeated 3 times with 2 plates per experiment. We obtained six replicate plates per sample and per strain. For the determination of toxicity by the dilution method, experiments were repeated only 2 times with 2 plates per experiment.

All raw data are available in the Appendix C.7 (pages 221 to 230).

In the reversion assays, the data obtained were the average of six replicates and were expressed as Mutation Ratio (MR), dividing the number of revertants per plate in presence of the sample (spontaneous + induced revertants) by the control number of revertant (spontaneous revertants).

For toxicity, the data obtained were expressed as survival (in %). With the first method of evaluation of the toxicity, the survival is the percentage of the number of his^+ colonies in the presence of sample and control positive (spontaneous + induced revertants - revertants killed by sample) in comparison to number of his^+ colonies in the positive control (spontaneous + induced revertants). With the second method of evaluation of the toxicity, the survival is the percentage of colonies in presence of sample in comparison with the number of colonies in bi-distilled water.

Instead of using the twofold rule [Ames *et al.*, 1975] according to which a sample is mutagenic when an increase of the revertant number by a factor of two relative to the negative control is observed, the results have been evaluated by the Mann-Whitney-Wilconxon U test as proposed by several authors [Perez *et al.*, 2003; Edler, 1992]. Differences with a probability (p) of 0.05 or less were considered as statistically significant. This non-parametric test was used instead of parametric test (t-test), because most of mutagenicity data do not meet assumptions of normality and homogeneous variance [Callahan & Short, 1995]. Some authors have reported Ames colony counts to be distributed according to Poisson statistics [Stead *et al.*, 1981; Hamada *et al.*, 1994] and others have reported them to be more variable than would be expected from the Poisson [Margolin *et al.*, 1981; Callahan & Short, 1995].

For the toxicity, Mann-Whitney-Wilconxon U test was also used to evaluate if the number of colonies observed in the samples and in the control were different at $\alpha<0.05$. If the toxic effect in the samples compared to the control was significant, the reversion frequency was adjusted according to the survival. The corrected number of revertants in the sample and the number in the negative control were compared using the Mann-Whitney-Wilconxon U test. The calculation of an adjusted mutagenic potency value has been proposed by several authors [Kargalioglu *et al.*, 2002]. The data of mutagenicity corrected with toxicity were expressed as adjusted Mutagenicity Ratio; MR_{adj1} corrected with the first method of toxic evaluation (induced revertants) and MR_{adj2} corrected with the second method (see Appendix B.6 on page 206). No adjustment was done when survival values were below 20%. The correction of MR with very low survival led to a very high Mutagenicity Ratio that was easily significant and increased the risk of type I error (false positive).

4.3 Results and discussion

4.3.1 Selection of strains

The aim of this first section was to select the strains that would be most informative for our samples. The toxicity and the mutagenicity (Figure 4.2) were first evaluated with several strains on samples of a hospital (CHUV) and of a sewage treatment plant (STP).

Both methods that were used to evaluate the toxicity indicated that most of the samples were very toxic. Comparison of the results from the two methods (Figure 4.2.A and B) showed lower toxicities when toxicity was determined with the first method (evaluation with induced revertants), the second method (by dilution) often yielding survival of less than 5%. The latter method could overestimate the toxicity due to the experimental protocol, where dilutions are made in order to be able to count the number of colonies. The lower cell density of bacteria could result in less efficient colony forming abilities.

The use of several Ames strains showed that TA98 was most sensitive to the toxic effects of hospital samples (Figure 4.2.A). TA1538 was slightly less sensitive to the toxic activity (20-50% of survival), and two samples were not toxic to this strain. TA100 exhibited a survival above 50%. Using the second method (dilution) to determine toxicity, TA102 was found to be the least sensitive (Figure 4.2.B). Based on these results, we analysed all samples using TA102, TA100 and TA1538 since they showed relative resistance to the wastewater samples.

Most of the hospital samples were mutagenic on TA102, even without correction for toxicity (Figure 4.2.C). Two samples were also mutagenic on TA1538. The wastewater of Monday was mutagenic on TA1538, but highly toxic on TA102 and TA100 (significant decrease of number of revertants, $p<0.05$). The hospital samples of Friday and Sunday were also toxic on TA100 (Figure 4.2.C), but the toxic effects were eliminated when S9 was added. Thus, transformation by a liver extract decreased the toxicity of both samples. Those appeared to be examples of toxic compounds that are metabolized predominately to nontoxic substances. Inactivation of mutagens by liver enzymes has been reported previously [Grabow *et al.*, 1980; Pelon *et al.*, 1977].

The adjusted Mutagenicity Ratio using the toxicity on induced revertants (MR_{adj1}) showed that most of the samples of hospital were mutagenic on at least one strain (Figure 4.2.D). TA98 was the less sensitive, only one sample was mutagenic on this strain. Contradictory results have been reported in the literature. Most of the studies showed that TA98 was more sensitive

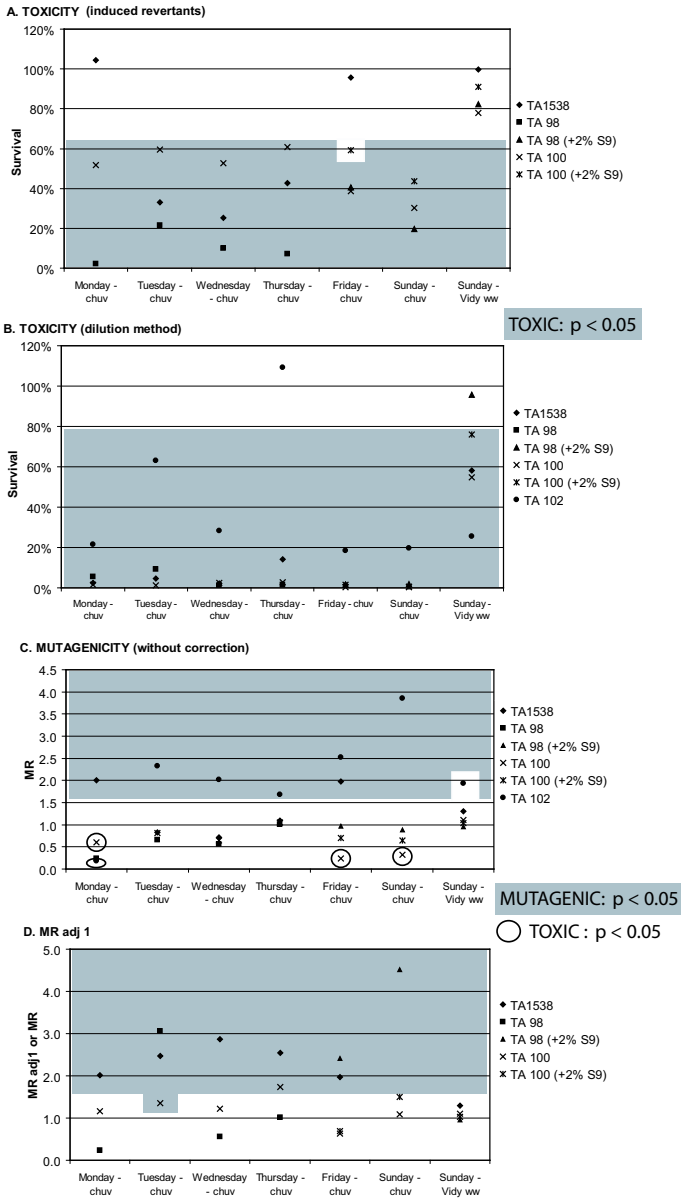

FIGURE 4.2: Sensitivity of different strains to wastewater - Toxicity with induced revertants (A), Toxicity with the second method (B), Mutation ratio: without correction (C) and corrected with toxicity first method (D). A sample is considered toxic or mutagenic when the number of colonies observed in the sample and in the control are different at α<0.05 (Mann-Whitney-Wilconxon U test) as described in the Section 4.2.6 (page 63)

than TA100, when sludge [Perez *et al.*, 2003], concentrated lake water samples [Monarca *et al.*, 1998], wastewater concentrates (XAD-2 and XAD-7) [Rappaport *et al.*, 1979], surface water (concentrated or not, 20-year survey) [Umbuzeiro *et al.*, 2001] were studied. However, few studies found than TA100 was more sensitive than TA98, for sludge [Babish *et al.*, 1983], hospital laboratories wastewater [Gartiser *et al.*, 1996] and drinking water [Park *et al.*, 2000].

Only two samples of hospital (Friday and Sunday) were tested on TA100 with metabolic activation (S9), but none were mutagenic. The results from TA98 in the presence of S9 were the same order of magnitude as without metabolic activation. S9 metabolic activation reduced genotoxic (SOS Chromotest) and mutagenic (TA98 and sometimes TA100) potency in several studies of water or extract samples [Umbuzeiro *et al.*, 2001; White *et al.*, 1996; Monarca *et al.*, 1998; Park *et al.*, 2000], suggesting a prevalence of direct-acting mutagens in the sample analyzed. Nevertheless, mutagenicity on TA100 was higher with metabolic activation in the first study [Umbuzeiro *et al.*, 2001], suggesting that there are some indirect-acting mutagens that could induce base pair substitution mutations. In our study, the use of metabolic activation (S9-mix) did not seem to influence our results (Figure 4.2), all other samples were analysed without metabolic activation.

The Mutagenicity Ratio was not adjusted for toxicity by the second method (MR_{adj2}). The correction of MR with so low survival values (often below 5% or even below 1%) would yield a high and significant Mutagenicity Ratio, increasing the risk of type I error (false positive). Thus, adjustment with survival values below 20% was not appropriate.

4.3.2 TA 102

The results of toxicity (dilution method) on TA102 showed that 70% (29 out of 41) of samples tested were toxic (see Figure 4.3.A). Wastewaters of hospital (six out of seven) were as toxic as those of residential area (seven out of eight). Samples from STP of Morges (five out of six) were more toxic than samples from Lausanne (three out of seven). For both STPs, the quantity of effluents showing toxic activity was similar to the influents indicating that the toxicity of influent was not removed by treatments of STPs. It is interesting to note that some influents that did not exhibit toxicity became toxic after treatment of STPs, as was observed in Thursday's sample of Morges and Lausanne and Saturday's sample of Lausanne. One wastewater of Morges and two of Lausanne showed a decrease of toxicity with treatment. A decrease in toxicity in effluent compared to influent sampled from different plants and measured with a bioluminescence assay, *Daphnia magna*, Microtox® and/or

ToxAlert® have been observed by several authors [Castillo & Schafer, 2000; Monarca *et al.*, 2000; Castillo *et al.*, 2001; Dizer *et al.*, 2002]. The plant treatment studied by these authors were successful in respect to the toxicity measured. Nevertheless, toxic activity were still measured in STP effluents for most of these studies.

Most of the samples that were mutagenic on TA102 were from the hospital (see Figure 4.3.B). Only two wastewaters of the residential area and of Lausanne STP were mutagenic. After the biological treatment of the STP, none of the wastewaters of Lausanne were mutagenic. Toxicity was, however, observed in two wastewaters of Lausanne (Thursday and Saturday) and could have masked their mutagenicity. When toxicity of the samples was taken into consideration, the two samples were found to be mutagenic (see MR_{adj2} on Figure 3.C).

All samples showing a significant decrease in the number of revertants in comparison with the control (○ in Figure 4.3.B) were also found to be toxic with the second method of toxicity evaluation (Figure 4.3.A). This implies that the method is reliable for the evaluation of survival of this bacterial strain.

The three mutagenic effluents of Lausanne were slightly more mutagenic than before the treatment, showing that the treatments were not able to remove the mutagenic substances and probably responsible for an increase of mutagenic activity (Figure 4.3.C). An increase in the mutagenicity of the wastewater following secondary treatment has also been observed in several studies [Meier *et al.*, 1987; Helma *et al.*, 1996]. Several possible explanations have been proposed for this observation. Cytotoxic interferences with the mutagenic response of the raw wastewaters may be a contributing factor, since the toxicity of raw wastewater could be higher than the toxicity of secondary effluents. This hypothesis could not be verified as the toxicity was not evaluated. Another explanation could be the formation of direct-acting mutagens during activated sludge treatment. As our results are corrected with the toxicity, they are consistent with the second hypothesis.

Nevertheless, treatments of Lausanne and Morges STPs were efficient for two days (Wednesday and Sunday) and for one day (Monday), respectively (Figure 4.3.C). During those days, the mutagenic activity found in the influent was not detected in the effluent. Several authors had reported removal of mutagens by biological treatment, a Pilot-Scale Integrated biological treatment process was found to reduce TA98 mutagenicity of water samples by 50% [Hu *et al.*, 2003]. In addition, the genotoxicity measured with the *umu*-test (with S9) cannot be completely removed by biological treatment [Ono *et al.*, 1996]. According to several studies [Babish *et al.*, 1983; Perez *et al.*, 2003], most of the sludge samples analysed showed mutagenic activity, sug-

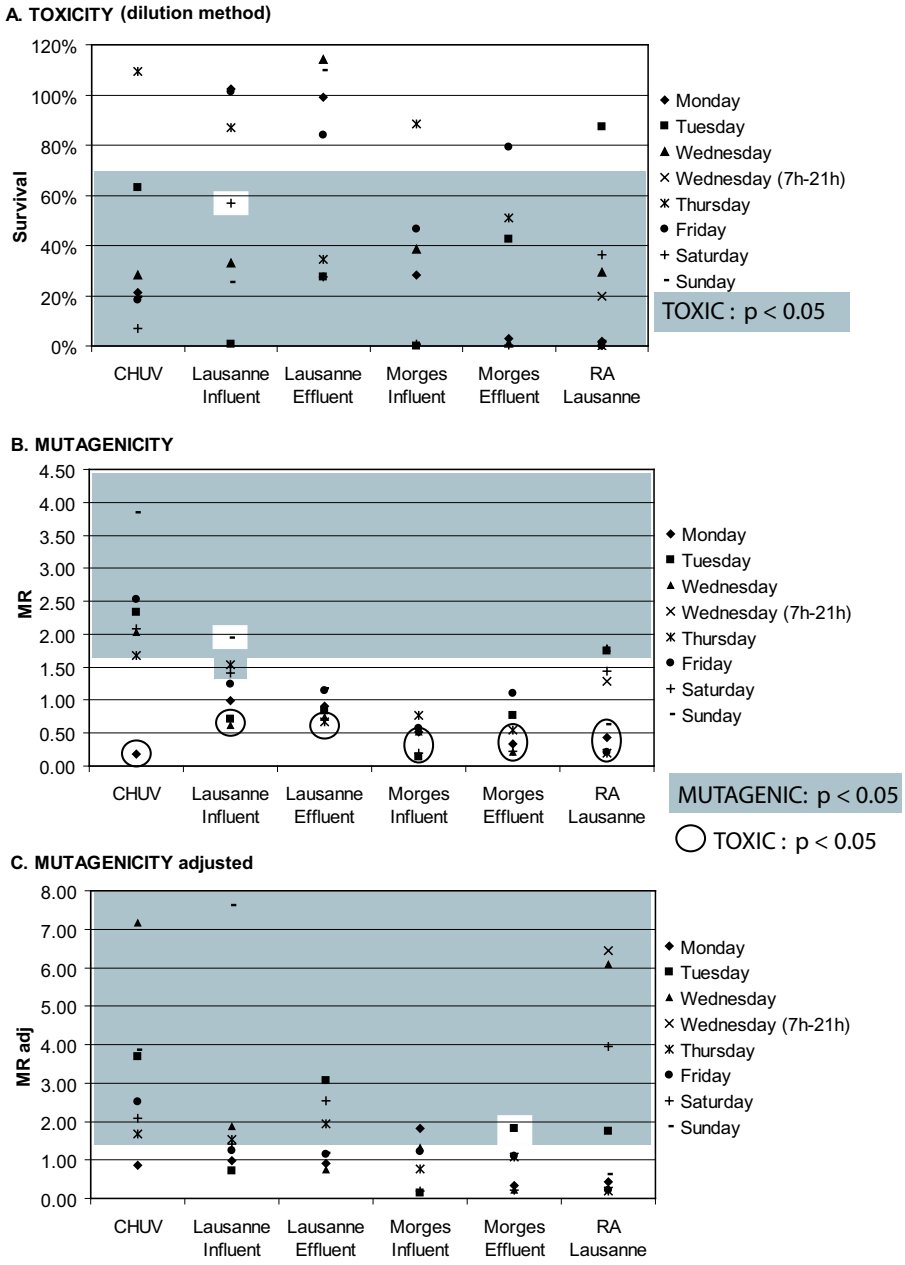

FIGURE 4.3: TA102 - Toxicity with the second method (A), Mutation ratio: without correction (B) and corrected with toxicity (C). RA: Residential area. Mutagenicity or toxicity are as described in the Section 4.2.6 (page 63)

gesting a transfer of a portion of mutagenicity from wastewater to sludge.

TA102 was very sensitive to mutagenicity of wastewater (MR significative: 10 out of 41 samples; MR_{adj2}: 18 out of 41 samples). This strain was also found to be very sensitive in another study [Malik & Ahmad, 1995]. Thus TA102 is a good indicator of potential risk of contamination by mutagenic substances in wastewater samples.

4.3.3 TA 1538

28% of samples tested (11 out of 40) were found to be toxic (induced revertant method) on TA1538 (see Figure 4.4.A). The dilution method of survival evaluation (Figure 4.4.B) showed a higher toxicity (48%). The bacterial survivals of hospital wastewaters were below 20% with the second method and between 25% to 65% with the induced revertant method. Two samples of hospital (Monday and Friday) and five influents of Lausanne and Morges were classified as toxic with the dilution method but not with the induced revertant method. And five effluents of Lausanne were toxic with the induced revertant method but only one with the dilution method. Because different results were obtained with the induced revertant and dilution methods, the mutagenicity of samples expressed without correction for toxicity were the only one with no ambiguity.

An increase in the number of revertant colonies was found following treatment with several water samples under conditions to measure toxicity (induced revertant method), (Figure 4.4.A). Most of these samples, except for one influent of Morges and two samples of the RA of Lausanne (Figure 4.4.C-E), did not by themselves induce an increase in the number of revertants. This result can be due to an increase in colony forming ability or interaction between the positive control and waste water possibly leading to a potentiation of the mutagenic effect of the positive control.

The results of mutagenicity without correction for toxicity showed that 18% of samples (7 out of 40) were mutagenic (Figure 4.4.C). A higher percentage (40%) was found for Mississipi River water samples with this strain [Pelon *et al.*, 1977], indicating that Swiss wastewaters from 2004 were less mutagenic than river water from thirty years ago (1974).

Mutagenic activity was observed in Thursday and Wednesday influents of Lausanne. During these two days, the samples from the hospital and the residential area were not mutagenic, indicating that another source of release contaminated the influents of Lausanne (Figure 4.4.C).

Lausanne effluents did not show mutagenic activity on TA1538, suggesting a removal of mutagenic substances for both mutagenic influents. Morges effluents were also not mutagenic on TA1538. The absence of mutagenic ac-

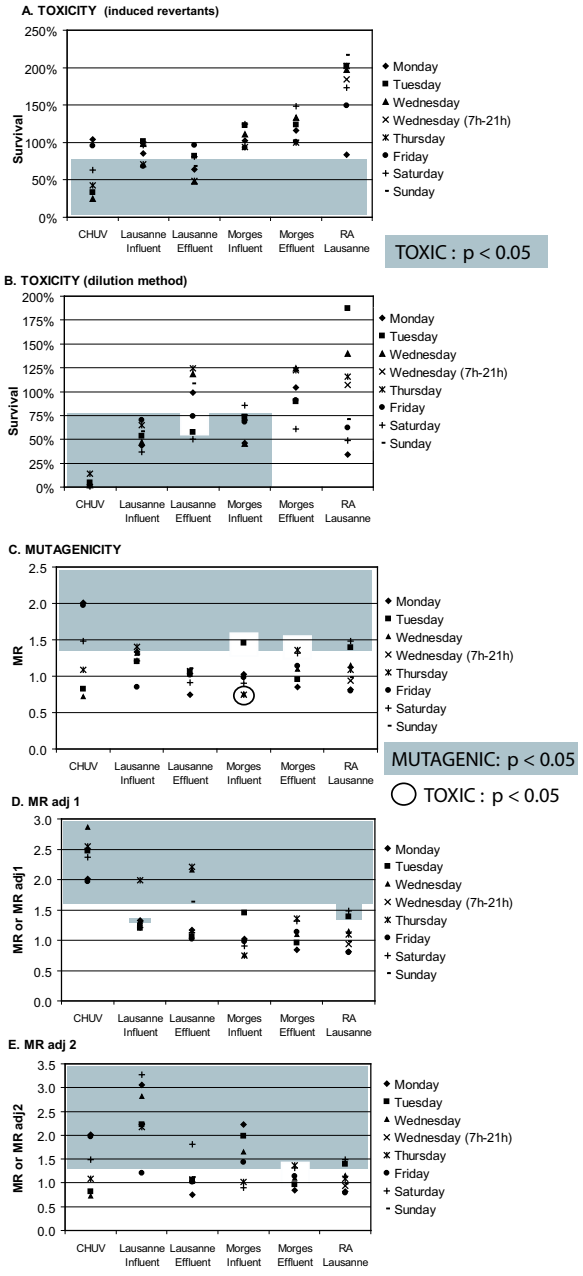

FIGURE 4.4: TA1538 - Toxicity with induced revertants (A), Toxicity with the second method (B), Mutation ratio: without correction (C) and corrected with toxicity first method (D) and second method (E). RA: Residential area. Mutagenicity or toxicity are as described in the Section 4.2.6 (page 63)

tivity in concentrated effluents has already been described in several studies on TA98 and TA100 [Shishida *et al.*, 2000; Rappaport *et al.*, 1979; Monarca *et al.*, 2000]. As we have observed with TA102, other studies have found effluents to be mutagenic on TA98 and/or on TA100, but these effluents contain a high proportion of industrial wastewater [Grabow *et al.*, 1980; Rappaport *et al.*, 1979].

Morges wastewaters were less mutagenic than Lausanne wastewaters. Morges being a smaller city than Lausanne with less contaminants could account for this variation. Other authors [White & Rasmussen, 1998] have shown a correlation between surface water mutagenicity and population.

4.3.4 TA 100

The results from the studies of wastewaters on TA100 showed that 57% (8 out of 14) of samples tested with induced revertants and that 64% (18 out of 28) of wastewater tested with dilution method were toxic (see Figure 4.5.A and B). All of the hospital samples were toxic with both methods of evaluations (Figure 4.5.A and B). One out of seven wastewaters of Lausanne STP was toxic with the induced revertants evaluation, but six were classified as toxic with the second method. Three out of six wastewaters of Morges were toxic with the second method. Toxicities were higher with the second method, as shown above.

Comparison of the results using the dilution method with samples from Morges and Lausanne showed a lower number of toxic samples from Morges (Figure 4.5.B). Only one sample from the residential area was toxic. The wastewater of this area did not appear to contribute to the toxic activity of Lausanne STP wastewaters. A significantly higher number of bacterial colonies was observed in the Wednesday sample of the residential area. We postulate that this sample contained growth nutrients that could have increased the growth efficiency of the bacteria. This artifact can induce a false positive result in the mutagenicity evaluation. For this reason, we corrected the mutagenicity ratio by this significant growth increase (MR_{adj1}).

The results of mutagenicity (without correction: Figure 4.5.C) also showed that samples of the hospital were toxic to this strain (four out of seven samples showed a decrease of revertants). After correction for the toxicity (induced revertant method), two samples could be classified as mutagenic (Figure 4.5.D). With adjustment (MR_{adj1}), our method improves the sensitivity of the Ames test. Without correction, none of hospital wastewater were mutagenic on TA100, as has been reported in another study [Gartiser *et al.*, 1996] on TA98 and TA100.

Three wastewaters of Lausanne were mutagenic on TA100. Mutagenic

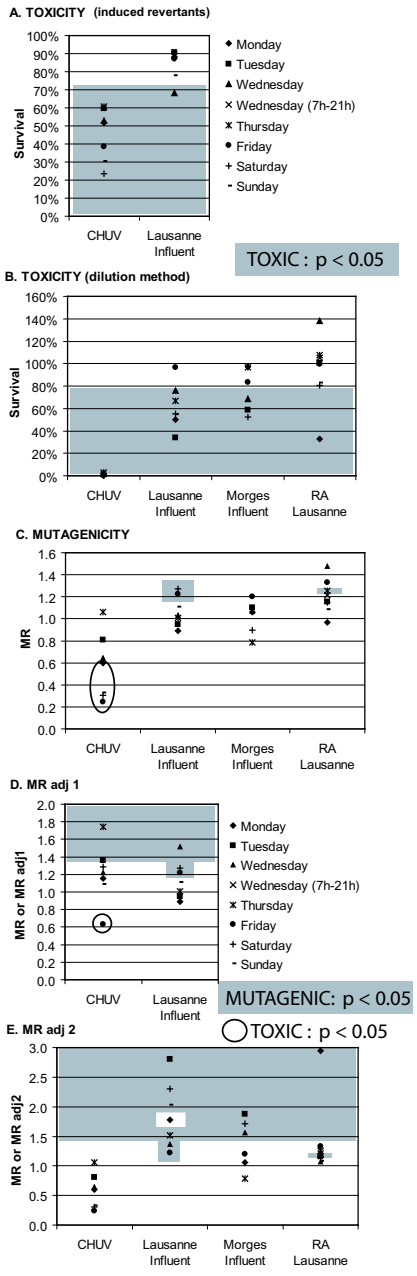

FIGURE 4.5: TA100 - Toxicity with induced revertants (A), Toxicity with the second method (B), Mutation ratio: without correction (C) and corrected with toxicity first method (D) and second method (E). RA: Residential area. Mutagenicity or toxicity are as described in the Section 4.2.6 (page 63)

samples from the STP and the hospital were not found to be from the same days (Figure 4.5.D), suggesting that the hospital mutagenic activity was diluted in wastewater of the city and that other sources contributed to the mutagenic activity of STP.

The adjustment with the toxicity (dilution method) of the MR (see MR_{adj2} on Figure 4.5.E) indicated that more wastewaters showed mutagenic activity (six out of seven Lausanne STP; three out of six Morges STP; two out of eight RA). For several samples, this activity was very low, but significant. Indeed, the standard deviations were very low. Another study did not show mutagenic activity on TA100 for wastewater of a residential area [Grabow *et al.*, 1980].

Figure 4.5.E shows that less Morges wastewaters (three out of six) were mutagenic than Lausanne wastewaters (six out of seven). These results were in accordance with non-adjusted results (Figure 4.5.C). The same result was found for the mutagenic activity of these both STPs and for TA1538.

Several studies pointed out the mutagenic activity on TA100 of wastewaters or surface waters. For instance, a not-concentrated influent and corresponding effluent of the Central municipal wastewater treatment plant in Domzale (Slovenia) were mutagenic on TA100 (+/-S9) [Filipic & Toman, 1996a].

4.3.5 General discussion

85% (35 out of 41) of samples analysed were toxic in at least one strain with the second method of evaluation (see Table 4.1). Though more samples exhibited toxicity on TA102, the survival observed in the different samples was however lower in TA98.

37% (15 out of 41) of samples were significantly mutagenic (without adjustment) in at least one strain (Table 4.1). Nevertheless, 54% (22 out of 41) of samples showed a decrease in the number of revertants in the mutagenicity assays. With the adjustment with toxicity as measured by induced revertants (MR_{adj1}), a small increase in the number of mutagenic samples was observed (44%; 18 out of 41). However, after adjustment with the dilution method to measure toxicity (MR_{adj2}), 71% (29 out of 41) of samples could be classified as mutagens. These percentages were the same order of magnitude as those observed from a French hospital, where 55% (10 out of 18) were genotoxic either on TA98/TA100 or on SOS chromotest [Jolibois *et al.*, 2003]. Nevertheless, our percentages were high in comparison to most other studies. On a study conducted by Giuliani *et al.* [1996], the UmuC assay detected genotoxic activity in 13% of samples of a hospital wastewater . As in our work, these authors did not concentrate the samples. Two samples (8%, n=25) of

TABLE 4.1: Number of toxic[a] and mutagenic[a] samples on TA102, TA1538 or TA100 evaluated with the various methods described in our study

Samples (Total) \ Methods	Toxicity 1^{st} [b]	Toxicity 2^{nd} [c]	MR	MR_{adj1}	MR_{adj2}
CHUV (7)	7	7	7	6	7
Lausanne Influent (7)	3	7	4	4	7
Lausanne Effluent (7)	5	3	0	3	4
Morges Influent (6)	0	6	0	0	5
Morges Effluent (6)	0	5	0	0	2
RA Lausanne (8)	0	7	4	2	4

[a] A sample is toxic or mutagenic when the number of colonies observed in the sample and in the control are different at $\alpha<0.05$ (Mann-Whitney-Wilconxon U test) as described in the Section 4.2.6 (page 63)
[b] Induced revertant method
[c] Dilution method

German Hospital wastewaters were mutagenic on TA98 or TA100, but 40% of these samples were genotoxic with the umuC test [Hartmann *et al.*, 1999]. This difference could have resulted from toxicity of hospital samples which masked the mutagenic activity in TA98. In a 20-year survey of water quality with the *Salmonella* mutagenicity assay, 14% (137/1007) of surface water and 18% of source water showed mutagenic activity [Umbuzeiro *et al.*, 2001].

Strain TA102 was the most sensitive, 10 out of 15 (67%) of all the mutagenic samples were found to be mutagenic on this strain without adjustment with toxicity. When toxicity was taken into consideration, 18 out of 29 (62%) of MR_{adj2} were mutagenic on TA102. TA1538 was more sensitive than TA100 with a MR significant for seven samples on TA1538 but only three samples on TA100 and with a MR_{adj2} significant for 16 samples on TA1538 but only 11 samples on TA100.

4.4 Conclusion

The two methods used to evaluate bacterial toxicity showed different sensitivities. Samples from the hospital were extremely toxic, survival was often below 20%. Influents of STPs were less toxic than hospital wastewaters. For

the mutagenicity assessment, TA102 strain was the most sensitive, followed by TA1538 and TA100. TA98 showed the lowest survival with the hospital samples, it is the reason why this strain is the less adapted for the evaluation of mutagenic activity of this kind of samples. Most of the samples from the hospital and from the larger STP studied showed mutagenic activities. More hospital wastewaters were mutagenic even without correction with toxicity than influents of STP, indicating a higher mutagenic activity in wastewater of hospital. Since less effluents were mutagenic than influents in both STPs, biological treatments were relatively efficient.

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Chapter 5

General conclusion and recommendations

5.1 Conclusion and outlook

The development of sensitive analytical methods allowed to evaluate the contamination of wastewaters by seven pharmaceutical substances, some are highly consumed and some are very toxic. The toxicity and genotoxicity assays permitted to assess the mixture effect of all contaminants in hospital and urban wastewaters.

5.1.1 Occurrence of pharmaceutical substances in the environment

The concentrations of the highly consumed pharmaceuticals (NSAIDs) were relatively important in the influents and effluents of STPs, and thus the substances in STP effluents contaminated the surface waters.

This thesis allowed the analysis of two of the most used anticancer drugs, that have never been detected. The concentrations of these toxic drugs were very low, below the limit of quantification or below the limit of detection. None of these drugs were detected in effluents of STPs, indicating that the surface water exposure was low.

A continue survey of the environmental concentrations of pharmaceutical substances would be necessary to evaluate the evolution of this contamination.

In addition, for lipophilic substances as Tamoxifen, the measurement of concentrations in sludge of STP would be interesting.

5.1.2 Fate

None of the NSAIDs were well removed by STPs, except Ibuprofen in only one STP. For Ibuprofen, the removal depends on the residence time of wastewater in biological treatment of the STP. The removals of Ibuprofen and Ketoprofen decreased during a raining period of winter, in comparison to dry periods. Diclofenac and Clofibric acid were resistant to all studied treatments, thus no removal was observed.

Due to its high lipophilic property, Tamoxifen was removed during the treatment of both studied STPs.

The removal of pharmaceutical substances were dependent on the STPs and on weather conditions, which can lead to increased aquatic exposures. Additional research are needed to know why there is such differences between STPs. A long-time survey (e.g. one year) would be useful to know the real exposure of aquatic environment, even in bad weather conditions.

The degradation pathway and resultant metabolites can differ from those observed in human metabolism [Winkler *et al.*, 2001]. In addition, only few research papers on the environmental degradability (e.g. photodegradation, ozonation) are available [Rey *et al.*, 1999; Andreozzi *et al.*, 2003; Zwiener & Frimmel, 2000; Werner *et al.*, 2005]. It is the reason why, there is an important need to elucidating the environmental transformation and fate of pharmaceutical products.

5.1.3 Risk assessment and effects data

Mefenamic acid seems to present the most important risk, followed by Ibuprofen, Clofibric acid, Diclofenac and Ketoprofen. But the risk ratio for surface water calculated with a dilution factor was above one only for Mefenamic acid.

To our knowledge, chronic ecotoxicity data are available only for Diclofenac and Clofibric acid. These kind of data are needed for the other chemicals to confirm our results. In addition, the risk evaluation was not possible for the two anticancer drugs, since no ecotoxicity data (even acute data) is available.

The knowledge on environmental toxicity of biologically active drugs is generally limited. Evaluation of the ecotoxicity of all pharmaceutical compounds and their metabolites have to be urgently performed, including pharmaceuticals not frequently detected in the environment.

In addition, ecotoxicity tests need to better accommodate subtle end points (e.g., genetic modifications, behavioral and endocrine effects) and to take into consideration the modes of action of pharmaceutical substances. Ac-

cording to Ferrari *et al.* [2004], the development of new endpoints related to the known mode of action of pharmaceuticals should improve the suitability of the acute ecotoxicity testing.

Mixture effects

Since the toxicity of a single drug might be enhanced by the occurrence of other pharmaceuticals with similar activity [Cleuvers, 2003], the overall risk of the NSAID drugs could be significant.

In the aquatic environment, most organisms are continually exposed to a range of toxic pollutants. When determining environmental risk of substances at low concentration, consideration must be given to both additive effects (drugs of like-mode of action) and to synergistic effects (interactions between drugs of different classes). Indeed, some compounds that have no inherent risk on their own may contribute to risk by increasing the toxicity of others.

Considerable combination effects of substances can occur. Since theses effects on aquatic life is little studied [see for instance Cleuvers, 2003; Brain *et al.*, 2004], further mixture toxicity testing is required to achieve a better assessment of the ecotoxicological potential impact of drug residues in the aquatic environment.

Adverse effect on human health

It is unknown what effects, if any, exposure to repeated doses of a mixture of subtherapeutic amounts of drug compounds could have on human health. Most likely they will be of little or no consequence in healthy people. Nevertheless, there is the possibility that the compounds may interact with other medications that an individual may be taking. For instance, Ibuprofen has been demonstrated to interfere with the cardioprotective properties of aspirin [MacDonald & Wei, 2003], while caffeine can enhance the effects of certain analgesics [Buerge *et al.*, 2003]. Although it is doubtful that concentrations would be high enough in drinking water to cause these effects.

According to several studies [Reddersen *et al.*, 2002; Webb *et al.*, 2003; Christensen, 1998], there are no substantial concerns with regards to exposure by pharmaceutical residues via drinking water. Nevertheless, further research in this area would be useful [Jones *et al.*, 2004].

5.1.4 Toxicity and genotoxicity of wastewaters

Samples from the hospital were extremely toxic. Influents of STPs were less toxic than hospital wastewaters. Most of the samples from the hospital

and from the larger STP studied showed mutagenic activities. More hospital wastewaters were mutagenic than influents of STP, indicating a higher mutagenic activity in wastewaters of hospital. Thus, hospitals are an important source of contamination by toxic and mutagenic substances, which could be pharmaceutical substances as anticancer drugs, but also disinfectants or cleaning products. The low contamination by anticancer drugs measured in this thesis showed that the (geno)toxic effect of a pollutant alone is unlikely, but the mixture effects of all pollutants from hospital were significant. Thus, it is important that hospital wastewaters are not released into the aquatic environment without an adequate treatment.

Toxicity or mutagenicity were often lower in effluents than in influents, indicating that biological treatments were relatively efficient. Nevertheless, several effluents were toxic or mutagenic, indicating that the surface waters were exposed to toxic and mutagenic compounds.

Evaluations of the toxicity/mutagenicity and chemical analyses are complementary. Toxicity/mutagenicity assays allow to evaluate the overall effect of the occurrence of pollutants. And analytical analyses identify and quantify compounds probably responsible for the genotoxicity and the toxicity measured.

A wastewater survey (especially with TA102) of several hospitals will be interesting to estimate if the dimension of hospital (number of beds) and the available treatments influence the toxicity and the mutagenicity of wastewaters.

A routine survey of the treated wastewaters of STPs will be useful to estimate the potential contamination of surface waters by toxic and mutagenic compounds.

5.2 Recommendations

Several recommendations are presented here to decrease the environmental risk of human pharmaceuticals in the environment.

5.2.1 Sewage treatment plants

Since sewage treatment plants are likely to be the most significant and continuous pathway of human medicinal compounds to surface water, an improvement of the efficiency of their treatments is needed. According to Larsen *et al.* [2004], from the technical point of view there is a choice between end-of-pipe technologies (e.g. ozonation of effluents) and more fundamental changes in wastewaters management (urine separation). In addition, the connection

to all the population at a STP and a capacity increase of the existing STPs, will prevent the direct discharge or the overflow of untreated sewage to the environment and will increase the residence time of waters in the STPs. These improvements would have a great impact on reducing the release of drugs.

Due to the high toxicity and mutagenicity of hospital wastewaters, it would be useful to add specific and efficient treatments at the end of hospitals.

5.2.2 Reduction at source

Nevertheless end-of-pipe technologies will never be able to solve the problem entirely, it is better to focus on reduction at source. But it is a ticklish task, due to the beneficial health effects and economic importance of these compounds. The reduction at source can be performed by different ways: (1) by decreasing the consumption and excretion of pharmaceutical substances, mainly the ecotoxic one and (2) by eliminating the discharge of unused drugs in the sewer or in the garbage. These changes would be possible with the following recommendations.

Environmental approval sign/label

An environmental information system should be created with simple and rapid accessibility. Such a system has to await the establishment of an environmental classification system for drugs. This classification should be tackled on an international platform (e.g. EU or OECD). When such a system is available, the doctors could be able to make an environmentally favorable selection between drug alternatives that are medically equivalent.

Environmentally friendly drugs should be given an environmental approval sign in the package and sheets of the product, catalogues, and other media in which the product is presented to doctors, pharmacists and patients. The approval sign should only be allowed to be used on products with the following characteristics which are favorable for the environment:

- The bio-availability, in terms of the percentage of the product that is taken up systemically in the patient, must be high (e.g. 75%), to avoid the direct elimination of the active compounds in urine and feces.

- The environmental adverse effects of the pharmaceutical compound and their metabolites must be minimal.

- The (bio)degradability of the pharmaceutical compound and their metabolites must be high.

- The package should also be environmentally friendly (e.g. recycling materials, optimisation of material quantities).
- Description on the package about the disposal of unwanted or expired drugs must be clearly present.

To meet those requirements, the producers will be encouraged to develop new or improved treatments, including the package, in accordance with the guidelines for sustainable development.

As already used for business contribution to sustainable development, the environmental approval sign/denomination could be "eco-efficiency", a term the World Business Council for Sustainable Development (WBCSD) invented in 1992.

Awareness campaign

An awareness campaign, promoting healthy style of live and alternative therapeutic treatments, would be efficient to reduce the consumption of several pharmaceutical substances and their release in the environment.

As already performed in Sweden [Wennmalm, 2003], carrying out a campaign informing patients about the environmental aspects of drugs is also proposed. The campaign goal is that patients should not dispose unused drugs in the garbage or in the sewer, but return it to the pharmacy. On that account, to avoid unused drugs, prescriptions should be better correspond to the duration of the treatment.

Education on the environmental aspects of pharmaceutical drugs should be given to the personnel of the public health care system. The prescribers should also be informed about the environmental consequences of released drugs residues.

In our society, it is sure that too many people underestimate the risk for human of drug consumption, maybe due to the pharmaceutical marketing and to the high trust in our doctors. "Advertising" about the potential toxicity of pharmaceuticals would be necessary to increase the awareness of adverse effects of drug consumption, even at therapeutic doses. Pharmacists should better inform the patients about the toxic effects of the products and about the potential dangerous interactions with others drugs. These advices should avoid a lot of drug poisoning and by this way decrease these emergency patients in hospital.

Another problem is that the purpose of most medical treatments is to control symptoms, but not actually to cure the patients. To be able to cure, it is necessary to diagnose the origin of the symptoms (e.g. skin diseases are often due to stress situations). Unfortunately, a lot of generalist practitioners

are not interested, have not the ability or have not enough time to diagnose the origin of the patient diseases, specially psychosomatic diseases. The formation of medical practitioners have to be improved in this way.

In summary, due to their beneficial health effects and economic importance, the actions taken to reduce inputs of drugs into the environment are much debated. The use of pharmaceutical compounds is expected to grow with the increasing age of the population. A solution for pollution control is to add sewage treatments in hospital and to avoid that municipal wastewaters are released without any treatment. Another solution is to focus on reduction at source, by developing a clear labelling on medicinal products, guidelines for the disposal and awareness campaign. These recommendations would have the potential benefit of improved consumer health (by minimizing the intake of active substances), as well as reduced health care spending.

Bibliography

Adams, M. S., Crumby, W. D., Greeley, Jr., M. S., Shugart, L. R., & Saylor, C. F. 1992. Responses of fish populations and communities to pulp mill effluents: A holistic assessment. *Ecotoxicology and Environmental Safety*, **24**(3), 347–360.

Adjei, A. A. 1999. A review of the pharmacology and clinical activity of new chemotherapy agents for the treatment of colorectal cancer. *British Journal of Clinical Pharmacology*, **48**(3), 265–277.

Adler, P., Steger-Hartmann, T., & Kalbfus, W. 2001. Distribution of natural and synthetic estrogenic steroid hormones in water samples from Southern and Middle Germany. *Acta Hydrochimica Et Hydrobiologica*, **29**(4), 227–241.

Aherne, G. W., English, J., & Marks, V. 1985. The role of immunoassay in the analysis of microcontaminants in water samples. *Ecotoxicology and Environmental Safety*, **9**, 79–83.

Ahrer, W., Scherwenk, E., & Buchberger, W. 2001. Determination of drug residues in water by the combination of liquid chromatography or capillary electrophoresis with electrospray mass spectrometry. *Journal of Chromatography A*, **910**(1), 69–78.

Al-Ahmad, A., Daschner, F. D., & Kümmerer, K. 1999. Biodegradability of cefotiam, ciprofloxacin, meropenem, penicillin G, and sulfamethoxazole and inhibition of waste water bacteria. *Archives of Environmental Contamination and Toxicology*, **37**(2), 158–163.

Ames, B. N., McCann, J., & Yamasaki, E. 1975. Methods for detecting carcinogens and mutagens with the salmonella/mammalian-microsome mutagenicity test. *Mutation Research/Environmental Mutagenesis and Related Subjects*, **31**(6), 347–363.

Anderson, D., Kerr, D. J., Blesing, C., & Seymour, L. W. 1997. Simultaneous gas chromatographic–mass spectrophotometric determination of [alpha]-fluoro-[beta]-alanine and 5-fluorouracil in plasma. *Journal of Chromatography B: Biomedical Sciences and Applications*, **688**(1), 87–93.

Andreozzi, R., Marotta, R., & Paxeus, N. 2003. Pharmaceuticals in STP effluents and their solar photodegradation in aquatic environment. *Chemosphere*, **50**(10), 1319–1330.

Anheden, M., Goswami, D. Y., & Svedberg, G. 1996. Photocatalytic treatment of wastewater from 5-fluorouracil manufacturing. *Journal of Solar Energy Engineering-Transactions of the Asme*, **118**(1), 2–8.

Ashton, D., Hilton, M., & Thomas, K. V. 2004. Investigating the environmental transport of human pharmaceuticals to streams in the United Kingdom. *Science of the Total Environment*, **333**(1-3), 167–184.

Atkins, J. H., & Gershell, L. J. 2002. Selective anticancer drugs. *Nature Reviews Drug Discovery*, **1**(7), 491–492.

Attrassi, B., Saghi, M., & Flatau, G. 1993. Multiple Antibiotic-Resistance of Bacteria in Atlantic Coast (Morocco). *Environmental Technology*, **14**(12), 1179–1186.

Ayscough, N.J., Fawell, J., Franklin, G., & Young, W. 2000. *Review of Human Pharmaceuticlas in the Environment*. Tech. rept. R&D Technical Report P390. Environment Agency.

Babish, J. G., Johnson, B. E., & Lisk, D. J. 1983. Mutagenicity of Municipal Sewage Sludges of American-Cities. *Environmental Science and Technology*, **17**(5), 272–277.

Barja, G. 2002. Rate of generation of oxidative stress-related damage and animal longevity. *Free Radical Biology and Medicine*, **33**(9), 1167–1172.

Baronti, C., Curini, R., D'Ascenzo, G., Di Corcia, A., Gentili, A., & Samperi, R. 2000. Monitoring natural and synthetic estrogens at activated sludge sewage treatment plants and in a receiving river water. *Environmental Science and Technology*, **34**(24), 5059–5066.

Baun, A., Andersen, J. S., & Nyholm, N. 1999. Correcting for toxic inhibition in quantification of genotoxic response in the umuC test. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, **441**(2), 171–180.

Bauza, R., Rios, A., & Valcarcel, M. 2001. Supercritical fluid extraction with in situ chiral derivatization for the enantiospecific determination of ibuprofen in urine samples. *Analytica Chimica Acta*, **450**(1-2), 1–11.

Brain, R. A., Johnson, D. J., Richards, S. M., Hanson, M. L., Sanderson, H., Lam, M. W., Young, C., Mabury, S. A., Sibley, P. K., & Solomon, K. R. 2004. Microcosm evaluation of the effects of an eight pharmaceutical mixture to the aquatic macrophytes Lemna gibba and Myriophyllum sibiricum. *Aquatic Toxicology*, **70**(1), 23–40.

Bruin, E. A. De, Tjaden, U. R., Van Oosterom, A. T., Leeflang, P., & Leclercq, P. A. 1983. Determination of the underivatized antineoplastic drugs cyclophosphamide and 5-fluorouracil and some their metabolites by capillary gas chromatography combined with electron-capture and nitrogen phosphorus selective detection. *Journal of Chromatography A*, **279**, 603–608.

Buerge, I. J., Poiger, T., Muller, M. D., & Buser, H. R. 2003. Caffeine, an anthropogenic marker for wastewater contamination of surface waters. *Environmental Science and Technology*, **37**(4), 691–700.

Buser, H. R., Poiger, T., & Muller, M. D. 1998a. Occurrence and fate of the pharmaceutical drug diclofenac in surface waters: Rapid photodegradation in a lake. *Environmental Science and Technology*, **32**(22), 3449–3456.

Buser, H. R., Muller, M. D., & Theobald, N. 1998b. Occurrence of the pharmaceutical drug clofibric acid and the herbicide mecoprop in various Swiss lakes and in the North Sea. *Environmental Science and Technology*, **32**(1), 188–192.

Buser, H. R., Poiger, T., & Muller, M. D. 1999. Occurrence and environmental behavior of the chiral pharmaceutical drug ibuprofen in surface waters and in wastewater. *Environmental Science and Technology*, **33**(15), 2529–2535.

Calamari, D., Zuccato, E., Castiglioni, S., Bagnati, R., & Fanelli, R. 2003. Strategic survey of therapeutic drugs in the rivers Po and Lambro in northern Italy. *Environmental Science and Technology*, **37**(7), 1241–1248.

Callahan, J. D., & Short, J. M. 1995. Transgenic Lambda/Laci Mutagenicity Assay - Statistical Determination of Sample-Size. *Mutation*

Research-Fundamental and Molecular Mechanisms of Mutagenesis, **327**(1-2), 201–208.

Cariello, N. F., & Piegorsch, W. W. 1996. The Ames test: The two-fold rule revisited. *Mutation Research/Genetic Toxicology*, **369**(1-2), 23–31.

Cash, G. G. 1998. Prediction of chemical toxicity to aquatic organisms: ECOSAR vs. Microtox (R) assay. *Environmental Toxicology and Water Quality*, **13**(3), 211–216.

Castillo, G., & Schafer, L. 2000. Evaluation of a bioassay battery for water toxicity testing: A Chilean experience. *Environmental Toxicology*, **15**(4), 331–337.

Castillo, M., & Smith, P. C. 1993. Direct determination of ibuprofen and ibuprofen acyl glucuronide in plasma by high-performance liquid chromatography using solid-phase extraction. *Journal of Chromatography: Biomedical Applications*, **614**(1), 109–116.

Castillo, M., Alonso, M. C., Riu, J., Reinke, M., Kloter, G., Dizer, H., Fischer, B., Hansen, P. D., & Barcelo, D. 2001. Identification of cytotoxic compounds in European wastewaters during a field experiment. *Analytica Chimica Acta*, **426**(2), 265–277.

Cerna, M., Pastorkova, A., mid, J., Bavorova, H., Oadlikova, D., Rossner, P., & Zavadil, J. 1996. Genotoxicity of industrial effluents, river waters, and their fractions using the Ames test and in vitro cytogenetic assay. *Toxicology Letters*, **88**(1-3), 191–197.

Christensen, F. M. 1998. Pharmaceuticals in the Environment-A Human Risk? *Regulatory toxicology and pharmacology*, **28**, 212–221.

Chu, K. C., Patel, K. M., Lin, A. H., Tarone, R. E., Linhart, M. S., & Dunkel, V. C. 1981. Evaluating statistical analyses and reproducibility of microbial mutagenicity assays. *Mutation Research/Environmental Mutagenesis and Related Subjects*, **85**(3), 119–132.

Cleuvers, M. 2003. Aquatic ecotoxicity of pharmaceuticals including the assessment of combination effects. *Toxicology Letters*, **142**(3), 185–194.

Daughton, C. G., & Ternes, T. A. 1999. Pharmaceuticals and personal care products in the environment: Agents of subtle change? *Environmental Health Perspectives*, **107**, 907–938.

Davidov, Y., Rozen, R., Smulski, D. R., Van Dyk, T. K., Vollmer, A. C., Elsemore, D. A., LaRossa, R. A., & Belkin, S. 2000. Improved bacterial SOS promoter:lux fusions for genotoxicity detection. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, **466**(1), 97–107.

De Flora, S., Bagnasco, M., & Zanacchi, P. 1991. Genotoxic, carcinogenic, and teratogenic hazards in the marine environment, with special reference to the Mediterranean Sea. *Mutation Research/Reviews in Genetic Toxicology*, **258**(3), 285–320.

Debruijn, E. A., Remeyer, L., Tjaden, U. R., Erkelens, C., Debrauw, L. M., & Vandevelde, C. J. H. 1986. Nonlinear Pharmacokinetics of 5-Fluorouracil as Described by Invivo Behavior of 5,6 Dihydro-5-Fluorouracil. *Biochemical Pharmacology*, **35**(15), 2461–2465.

Diasio, R. B. 1999. Improving fluorouracil chemotherapy with novel orally administered fluoropyrimidines. *Drugs*, **58**, 119–126.

Dizer, H., Wittekindt, E., Fischer, B., & Hansen, P. D. 2002. The cytotoxic and genotoxic potential of surface water and wastewater effluents as determined by bioluminescence, umu-assays and selected biomarkers. *Chemosphere*, **46**(2), 225–233.

EC. 2003. *Technical Guidance Document on Risk Assessment. TGD Part II.* Tech. rept. Institute for Health and Consumer Protection, European Chemicals Bureau, European Commission (EC).

Edler, L. 1992. Statistical methods for short-term tests in genetic toxicology: The first fifteen years. *Mutation Research/Reviews in Genetic Toxicology*, **277**(1), 11–33.

Ehlhardt, W. J., Beaulieu, Jr., B. B., & Goldman, P. 1988. Mammalian cell toxicity and bacterial mutagenicity of nitrosoimidazoles. *Biochemical Pharmacology*, **37**(13), 2603–2606.

El Haj, B. M., Al Ainri, A. M., Hassan, M. H., Bin Khadem, R. K., & Marzouq, M. S. 1999. The GC/MS analysis of some commonly used non-steriodal anti-inflammatory drugs (NSAIDs) in pharmaceutical dosage forms and in urine. *Forensic Science International*, **105**(3), 141–153.

Elyazigi, A., & Alrawithi, S. 1992. Analysis of Hydroxyurea in Capsules and Aqueous-Solution and Stability Study with Capillary Gas-Chromatography and Thermionic (N-P) Specific Detection. *Pharmaceutical Research*, **9**(1), 115–118.

EMEA. 2001 (2001). *Draft CPMP discussion paper on environmental risk assessement of non-genetically modified organism (non-GMO) containing medicinal products for human use.* Tech. rept. Report CPMP/SWP/4447/00. European Agency for the Evaluation of Medicinal Proucts.

Farré, M., & Barceló, D. 2003. Toxicity testing of wastewater and sewage sludge by biosensors, bioassays and chemical analysis. *Trac-Trends in Analytical Chemistry*, **22**(5), 299–310.

Farré, M., Ferrer, I., Ginebreda, A., Figueras, M., Olivella, L., Tirapu, L., Vilanova, M., & Barceló, D. 2001. Determination of drugs in surface water and wastewater samples by liquid chromatography-mass spectrometry: methods and preliminary results including toxicity studies with Vibrio fischeri. *Journal of Chromatography A*, **938**(1-2), 187–197.

Ferrari, B, Paxéus, N., Giudice, R L, Pollio, A, & Garric, J. 2003. Ecotoxicological impact of pharmaceuticals found in treated wastewaters: study of carbamazepine, clofibric acid, and diclofenac. *Ecotoxicology and Environmental Safety*, **55**(3), 359–370.

Ferrari, B., Mons, R., Vollat, B., Fraysse, B., Paxeus, N., Lo Giudice, R., Pollio, A., & Garric, J. 2004. Environmental risk assessment of six human pharmaceuticals: Are the current environmental risk assessment procedures sufficient for the protection of the aquatic environment? *Environmental Toxicology and Chemistry*, **23**(5), 1344–1354.

Filipic, M. 1995. Mutagenicity and Toxicity of Water Extracts from the Sora River Area. *Mutation Research-Genetic Toxicology*, **342**(1-2), 1–8.

Filipic, M., & Toman, M. J. 1996a. Ecotoxicological studies using modified ames bioassay. *Water Science and Technology*, **34**(7-8), 1–7.

Filipic, M., & Toman, M. J. 1996b. Genotoxicity of influents and effluents of the wastewater treatment plant. *Water Science and Technology*, **34**(7-8), 9–14.

Fisher, B., Costantino, J. P., Redmond, C. K., Fisher, E. R., Wickerham, D. L., Cronin, W. M., Bowman, D., Couture, J., Dimitrov, N. V., Evans, J., Farrar, W., Kavanah, M., Lickley, H. L., Margolese, R., Paterson, A. H. G., Robidoux, A., Shibata, H., & Terz, J. 1994. Endometrial Cancer in Tamoxifen-Treated

Breast-Cancer Patients - Findings from the National Surgical Adjuvant Breast and Bowel Project (Nsabp) B-14. *Journal of the National Cancer Institute*, **86**(7), 527–537.

Floridia, L., Pietropaolo, A. M., Tavazzani, M., Rubino, F. M., & Colombi, A. 1999. High-performance liquid chromatography of methotrexate for environmental monitoring of surface contamination in hospital departments and assessment of occupational exposure. *Journal of Chromatography B*, **726**, 95–103.

Fried, K. M., & Wainer, I. W. 1994. Direct Determination of Tamoxifen and Its 4 Major Metabolites in Plasma Using Coupled-Column High-Performance Liquid- Chromatography. *Journal of Chromatography B-Biomedical Applications*, **655**(2), 261–268.

Gartiser, S., Brinker, L., Erbe, T., Kümmerer, K., & Willmund, R. 1996. Contamination of hospital wastewater with hazardous compounds as defined by 7a WHG. *Acta Hydrochimica Et Hydrobiologica*, **24**(2), 90–97.

Geisslinger, G., Dietzel, K., Loew, D., Schuster, O., Rau, G., Lachmann, G., & Brune, K. 1989. High-performance liquid chromatographic determination of ibuprofen, its metabolites and enantiomers in biological fluids. *Journal of Chromatography B: Biomedical Sciences and Applications*, **491**, 139–149.

Giger, W., Alder, A. C., Golet, E. M., Kohler, H. P. E., McArdell, C. S., Molnar, E., Siegrist, H., & Suter, M. J. F. 2003. Occurrence and fate of antibiotics as trace contaminants in wastewaters, sewage sludges, and surface waters. *Chimia*, **57**(9), 485–491.

Giuliani, F, Koller, T, Wurgler, F E, & Widmer, R M. 1996. Detection of genotoxic activity in native hospital waste water by the umuC test. *Mutation Research*, **368**(1), 49–57.

Golet, E M, Alder, A C, Hartmann, A, Ternes, T A, & Giger, W. 2001. Trace determination of fluoroquinolone antibacterial agents in urban wastewater by solid-phase extraction and liquid chromatography with fluorescence detection. *Analytical Chemistry*, **73**(15), 3632–3638.

Golet, E. M., Alder, A. C., & Giger, W. 2002. Environmental exposure and risk assessment of fluoroquinolone antibacterial agents in wastewater and river water of the Glatt Valley Watershed, Switzerland. *Environmental Science and Technology*, **36**(17), 3645–3651.

Grabow, W. O. K., Denkhaus, R., & Vanrossum, P. G. 1980. Detection of Mutagens in Waste-Water, a Polluted River and Drinking-Water by Means of the Ames Salmonella-Microsome Assay. *South African Journal of Science*, **76**(3), 118–123.

Greaves, P., Goonetilleke, R., Nunn, G., Topham, J., & Orton, T. 1993. 2-Year Carcinogenicity Study of Tamoxifen in Alderley-Park Wistar-Derived Rats. *Cancer Research*, **53**(17), 3919–3924.

Griffith, J., Duncan, R. C., Riggan, W. B., & Pellom, A. C. 1989. Cancer Mortality in United-States Counties with Hazardous-Waste Sites and Ground-Water Pollution. *Archives of Environmental Health*, **44**(2), 69–74.

Guerrieri, A., Palmisano, F., Zambonin, C. G., & Delena, M. 1994. Simultaneous Determination of 5'-Deoxy-5-Fluorouridine, 5- Fluorouracil and 5,6-Dihydro-5-Fluorouracil in Serum by Liquid- Chromatography with Diode-Array Uv Detection. *Analytica Chimica Acta*, **296**(1), 43–50.

Halling-Sorensen, B, Nors Nielsen, S, Lanzky, P F, Ingerslev, F, Holten Lutzhoft, H C, & Jorgensen, S E. 1998. Occurrence, fate and effects of pharmaceutical substances in the environment–a review. *Chemosphere*, **36**(2), 357–393.

Hamada, C., Wada, T., & Sakamoto, Y. 1994. Statistical Characterization of Negative Control Data in the Ames Salmonella/Microsome Test. *Environmental Health Perspectives*, **102**, 115–119.

Harrington, T. R., Nestmann, E. R., & Kowbel, D. J. 1983. Suitability of the modified fluctuation assay for evaluating the mutagenicity of unconcentrated drinking water. *Mutation Research Letters*, **120**(2-3), 97–103.

Hartmann, A., Alder, A. C., Koller, T., & Widmer, R. M. 1998. Identification of fluoroquinolone antibiotics as the main source of umuC genotoxicity in native hospital wastewater. *Environmental Toxicology and Chemistry*, **17**(3), 377–382.

Hartmann, A., Golet, E. M., Gartiser, S., Alder, A. C., Koller, T., & Widmer, R. M. 1999. Primary DNA damage but not mutagenicity correlates with ciprofloxacin concentrations in German hospital wastewaters. *Archives of Environmental Contamination and Toxicology*, **36**(2), 115–119.

Haseman, J. K., Zeiger, E., Shelby, M. D., Margolin, B. H., & Tennant, R. W. 1990. Predicting Rodent Carcinogenicity from 4 In-vitro Genetic Toxicity Assays - an Evaluation of 114 Chemicals Studied by the National Toxicology Program. *Journal of the American Statistical Association*, **85**(412), 964–971.

Havard, J., Grygiel, J., & Sampson, D. 1992. Determination by high-performance liquid chromatography of hydroxyurea in human plasma. *Journal of Chromatography: Biomedical Applications*, **584**(2), 270–274.

Heberer, T. 2002a. Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: a review of recent research data. *Toxicology Letters*, **131**(1-2), 5–17.

Heberer, T. 2002b. Tracking persistent pharmaceutical residues from municipal sewage to drinking water. *Journal of Hydrology*, **266**(3-4), 175–189.

Heberer, T, Butz, S, & Stan, H-J. 1994. Detection of 30 acidic herbicides and related compounds as their pentafluorobenzylic derivatives using gas chromatography/mass spectrometry. *Journal of AOAC International*, **77**(6), 1587–1604.

Heberer, T., Dünnbier, U., Reilich, C., & Stan, H.-J. 1997. Detection of drugs and drug metabolites in ground water samples of a drinking water treatment plant. *Fresenius Environmental Bulletin*, **6**, 438–443.

Heberer, T, Fuhrmann, B., Schmidt-Baumler, K., Tsipi, D., Koutsouba, V., & Hiskia, A. 2001. Occurrence of pharmaceutical residues in sewage, river, ground and drinking water in Greece and Berlin (Germany). *Pages 70–83 of:* Daughton, C. G., & Jones-Lepp, T. L. (eds), *Pharmaceuticals and Personal Care Products in the Environment*. Washington: American chemical society.

Heberer, T., Reddersen, K., & Mechlinski, A. 2002. From municipal sewage to drinking water: fate and removal of pharmaceutical residues in the aquatic environment in urban areas. *Water Science and Technology*, **46**(3), 81–88.

Helma, C., MerschSundermann, V., Houk, V. S., Glasbrenner, U., Klein, C., Lu, W. Q., Kassie, F., SchulteHermann, R., & Knasmuller, S. 1996. Comparative evaluation of four bacterial assays for the detection of genotoxic effects in the dissolved water phases of aqueous matrices. *Environmental Science and Technology*, **30**(3), 897–907.

Henschel, K. P., Wenzel, A., Diedrich, M., & Fliedner, A. 1997. Environmental hazard assessment of pharmaceuticals. *Regulatory Toxicology and Pharmacology*, **25**(3), 220–225.

Hignite, C., & Azarnoff, D. L. 1977. Drugs and Drug Metabolites as Environmental Contaminants - Chlorophenoxyisobutyrate and Salicylic-Acid in Sewage Water Effluent. *Life Sciences*, **20**(2), 337–341.

Hirai, T., Matsumoto, S., & Kishi, I. 1997. Simultaneous analysis of several non-steroidal anti-inflammatory drugs in human urine by high-performance liquid chromatography with normal solid-phase extraction. *Journal of Chromatography B: Biomedical Sciences and Applications*, **692**(2), 375–388.

Hirsch, R., Ternes, T. A., Haberer, K., & Kratz, K.-L. 1996. Determination of betablockers and b-sympathomimetics in the aquatic environment. *Vom Wasser*, **87**, 263–274.

Hirsch, R., Ternes, T., Haberer, K., & Kratz, K-L. 1999. Occurrence of antibiotics in the aquatic environment. *Science of the Total Environment*, **225**, 109–118.

Hohenblum, P., Gans, O., Moche, W., Scharf, S., & Lorbeer, G. 2004. Monitoring of selected estrogenic hormones and industrial chemicals in groundwaters and surface waters in Austria. *Science of the Total Environment*, **333**(1-3), 185–193.

Houk, V. S. 1992. The Genotoxicity of Industrial-Wastes and Effluents. *Mutation Research*, **277**(2), 91–138.

Hu, J. Y., Ong, S. L., Ng, W. J., He, B. P., Liu, W. J., Fang, Z. D., Zhang, X. H., & Wang, Z. S. 2003. Mutagenicity of polluted reservoir water and its reduction by a pilot-scale integrated biological treatment process. *Bulletin of Environmental Contamination and Toxicology*, **71**(1), 204–211.

Huber, M. M., Canonica, S., Park, G. Y., & Von Gunten, U. 2003. Oxidation of pharmaceuticals during ozonation and advanced oxidation processes. *Environmental Science and Technology*, **37**(5), 1016–1024.

Iyamu, E. W., Roa, P. D., Kopsombut, P., Aguinaga, M. d. P., & Turner, E. A. 1998. New isocratic high-performance liquid chromatographic procedure to assay the anti-sickling compound hydroxyurea in

plasma with ultraviolet detection. *Journal of Chromatography B: Biomedical Sciences and Applications*, **709**(1), 119–126.

Jalonen, H. G. 1988. Simultaneous Determination of Tamoxifen Citrate and Its E- Isomer Impurity in Bulk Drug and Tablets by High-Performance Liquid-Chromatography. *Journal of Pharmaceutical Sciences*, **77**(9), 810–813.

Jolibois, B., & Guerbet, M. 2005. Evaluation of industrial, hospital and domestic wastewater genotoxicity with the Salmonella fluctuation test and the SOS chromotest. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, **565**(2), 151–162.

Jolibois, B., Guerbet, M., & Vassal, S. 2003. Detection of hospital wastewater genotoxicity with the SOS chromotest and Ames fluctuation test. *Chemosphere*, **51**(6), 539–543.

Jones, A. H., Voulvoulis, N., & Lester, J. N. 2004. Potential ecological and human health risks associated with the presence of pharmaceutically active compounds in the aquatic environment. *Critical Reviews in Toxicology*, **34**(4), 335–350.

Jones, O. A. H., Voulvoulis, N., & Lester, J. N. 2001. Human pharmaceuticals in the aquatic environment - A review. *Environmental Technology*, **22**(12), 1383–1394.

Jones, O. A. H., Voulvoulis, N., & Lester, J. N. 2002. Aquatic environmental assessment of the top 25 English prescription pharmaceuticals. *Water Research*, **36**(20), 5013–5022.

Jordan, V. C. 1995. Tamoxifen - Toxicities and Drug-Resistance During the Treatment and Prevention of Breast-Cancer. *Annual Review of Pharmacology and Toxicology*, **35**, 195–211.

Jorgensen, S. E., & Halling-Sorensen, B. 2000. Drugs in the environment. *Chemosphere*, **40**(7), 691–699.

Kaiser, K. L. E., Dearden, J. C., Klein, W., & Schultz, T. W. 1999. A note of caution to users of ECOSAR. *Water Quality Research Journal of Canada*, **34**(1), 179–182.

Kargalioglu, Y., McMillan, B. J., Minear, R. A., & Plewa, M. J. 2002. Analysis of the cytotoxicity and mutagenicity of drinking water disinfection by-products in Salmonella typhimurium. *Teratogenesis Carcinogenesis and Mutagenesis*, **22**(2), 113–128.

Kedar, R., Bourne, T. H., Collins, W. P., Campbell, S., Powles, T. J., Ashley, S., & Cosgrove, D. O. 1994. Effects of tamoxifen on uterus and ovaries of postmenopausal women in a randomised breast cancer prevention trial. *The Lancet*, **343**(8909), 1318–1321.

Khan, S.J., & Ongerth, J.E. 2004. Modelling of pharmaceutical residues in Australian sewage by quantities of use and fugacity calculations. *Chemosphere*, **54**(3), 355–367.

Kiffmeyer, T., Gotze, H. J., Jursch, M., & Luders, U. 1998. Trace enrichment, chromatographic separation and biodegradation of cytostatic compounds in surface water. *Fresenius Journal of Analytical Chemistry*, **361**(2), 185–191.

Kümmerer, K. 2001a. Drugs in the environment: emission of drugs, diagnostic aids and disinfectants into wastewater by hospitals in relation to other sources - a review. *Chemosphere*, **45**(6-7), 957–969.

Kümmerer, K. 2004. Resistance in the Environment. *Pages 223–231 of:* Kümmerer, K (ed), *Pharmaceuticals in the Environment, sources, fate, effects and risks*, second edition edn. Springer.

Kümmerer, K., & Al-Ahmad, A. 1997. Biodegradability of the antitumour agents 5-fluorouracil, cytarabine, and gemcitabine: Impact of the chemical structure and synergistic toxicity with hospital effluent. *Acta Hydrochimica et Hydrobiologica*, **25**(4), 166–172.

Kümmerer, K., & Henninger, A. 2003. Promoting resistance by the emission of antibiotics from hospitals and households into effluent. *Clinical Microbiology and Infection*, **9**(12), 1203–1214.

Kümmerer, K., StegerHartmann, T., Baranyai, A., & Burhaus, I. 1996. Examination of the biodegradation of the antineoplastics cyclophosphamide and ifosfamide with the closed bottle test (OECD 301 D). *Zentralblatt Für Hygiene Und Umweltmedizin*, **198**(3), 215–225.

Kümmerer, K., StegerHartmann, T., & Meyer, M. 1997. Biodegradability of the anti-tumour agent ifosfamide and its occurrence in hospital effluents and communal sewage. *Water Research*, **31**(11), 2705–2710.

Kümmerer, K., Helmers, E., Hubner, P., Mascart, G., Milandri, M., Reinthaler, F., & Zwakenberg, M. 1999. European hospitals as a source for platinum in the environment in comparison with other sources. *Science of the Total Environment*, **225**, 155–165.

Kümmerer, K., Al-Ahmad, A., & Mersch-Sundermann, V. 2000. Biodegradability of some antibiotics, elimination of the genotoxicity and affection of wastewater bacteria in a simple test. *Chemosphere*, **40**(7), 701–710.

Kümmerer, K (Ed.). 2001b. *Pharmaceuticals in the environment: Sources, fate, effects and risks*. Springer.

Koivusalo, M., Jaakkola, J. J. K., Vartiainen, T., Hakulinen, T., Karjalainen, S., Pukkala, E., & Tuomisto, J. 1994. Drinking-Water Mutagenicity and Gastrointestinal and Urinary-Tract Cancers - an Ecological Study in Finland. *American Journal of Public Health*, **84**(8), 1223–1228.

Kok, R. M., De Jong, A. P. J. M., Van Groeningen, C. J., Peters, G. J., & Lankelma, J. 1985. Highly sensitive determination of 5-fluorouracil in human plasma by capillary gas chromatography and negative ion chemical ionization mass spectrometry. *Journal of Chromatography B: Biomedical Sciences and Applications*, **343**, 59–66.

Kolpin, D. W., Furlong, E. T., Meyer, M. T., Thurman, E. M., Zaugg, S. D., Barber, L. B., & Buxton, H. T. 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams, 1999-2000: A national reconnaissance. *Environmental Science and Technology*, **36**(6), 1202–1211.

Koutsouba, V., Heberer, T., Fuhrmann, B., Schmidt-Baumler, K., Tsipi, D., & Hiskia, A. 2003. Determination of polar pharmaceuticals in sewage water of Greece by gas chromatography-mass spectrometry. *Chemosphere*, **51**(2), 69–75.

Kuspis, D. A., & Krenzelok, E. P. 1996. What happens to expired medications? A survey of community medication disposal. *Veterinary and Human Toxicology*, **38**(1), 48–49.

Kutlu, M., Aydogan, G., Susuz, F., & Ozata, A. 2004. The Salmonella mutagenicity of water and sediments from the Porsuk River in Turkey. *Environmental Toxicology and Pharmacology*, **17**(2), 111–116.

Larsen, T.A., Lienert, J., Joss, A., & Siegrist, H. 2004. How to avoid pharmaceuticals in the aquatic environment. *Journal of Biotechnology*, **113**(1-3), 295–304.

Lee, K.-H., Ward, B. A., Desta, Z., Flockhart, D. A., & Jones, D. R. 2003. Quantification of tamoxifen and three metabolites in plasma by high-performance liquid chromatography with fluorescence detection: application to a clinical trial. *Journal of Chromatography B*, **791**(1-2), 245–253.

Leff, L. G., Dana, J. R., McArthur, J. V., & Shimkets, L. J. 1993. Detection of Tn5-Like Sequences in Kanamycin-Resistant Stream Bacteria and Environmental DNA. *Applied and Environmental Microbiology*, **59**(2), 417–421.

Öllers, S., Singer, H. P., Fassler, P., & Müller, S. R. 2001. Simultaneous quantification of neutral and acidic pharmaceuticals and pesticides at the low-ng/l level in surface and waste water. *Journal of Chromatography. A*, **911**(2), 225–234.

Loos, W. J., de Bruijn, P., van Zuylen, L., Verweij, J., Nooter, K., Stoter, G., & Sparreboom, A. 1999. Determination of 5-fluorouracil in microvolumes of human plasma by solvent extraction and high-performance liquid chromatography. *Journal of Chromatography B: Biomedical Sciences and Applications*, **735**(2), 293–297.

MacCallum, J., Cummings, J., Dixon, J. M., & Miller, W. R. 1997. Solid-phase extraction and high-performance liquid chromatographic determination of tamoxifen and its major metabolites in breast tumour tissues. *Journal of Chromatography B: Biomedical Sciences and Applications*, **698**(1-2), 269–275.

MacDonald, T. M., & Wei, L. 2003. Effect of ibuprofen on cardioprotective effect of aspirin. *Lancet*, **361**(9357), 573–574.

Mahnik, S. N., Rizovski, B., Fuerhacker, M., & Mader, R. M. 2004. Determination of 5-fluorouracil in hospital effluents. *Analytical and Bioanalytical Chemistry*, **380**(1), 31–35.

Mahon, G. A. T., Middleton, B., Robinson, W.D., Green, M. H. L., Mitchell, I. de G., & Tweats, D. J. 1989. Analysis of data from microbial colony assays. *In:* Kirkland, D. J. (ed), *Statistical evaluation of mutagenicity test data*. Cambridge University Press.

Malik, A., & Ahmad, M. 1995. Genotoxicity of Some Wastewaters in India. *Environmental Toxicology and Water Quality*, **10**(4), 287–293.

Manns, J.E., Hanks, S., & Brown, J.E. 1998. Optimised separation of E- and Z- isomers of tamoxifen, and its principal metabolites using reversed-phase high performance liquid chromatography. *Journal of Pharmaceutical and Biomedical Analysis*, **16**(5), 847–852.

Margolin, B. H., Kaplan, N., & Zeiger, E. 1981. Statistical-Analysis of the Ames Salmonella-Microsome Test. *Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences*, **78**(6), 3779–3783.

Maron, D. M., & Ames, B. N. 1983. Revised methods for the Salmonella mutagenicity test. *Mutation Research/Environmental Mutagenesis and Related Subjects*, **113**(3-4), 173–215.

Matsushima, E., Yoshida, K., & Kitamura, R. 1997. Determination of S-1 (combined drug of tegafur, 5-chloro-2,4- dihydroxypyridine and potassium oxonate) and 5-fluorouracil in human plasma and urine using high-performance liquid chromatography and gas chromatography-negative ion chemical ionization mass spectrometry. *Journal of Chromatography B*, **691**(1), 95–104.

McGurk, K. A., Remmel, R. P., Hosagrahara, V. P., Tosh, D., & Burcherll, B. 1996. Reactivity of mefenamic acid 1-0-acyl glucuronide with proteins in vitro and ex vivo. *Drug Metabolism and Disposition*, **24**(8), 842–849.

Meier, J. R., Blazak, W. F., Riccio, E. S., Stewart, B. E., Bishop, D. F., & Condie, L. W. 1987. Genotoxic Properties of Municipal Wastewaters in Ohio. *Archives of Environmental Contamination and Toxicology*, **16**(6), 671–680.

Meylan, W. M., & Howard, P. H. 1995. Atom Fragment Contribution Method for Estimating Octanol-Water Partition-Coefficients. *Journal of Pharmaceutical Sciences*, **84**(1), 83–92.

Micoli, G., Turci, R., Arpellini, M., & Minoia, C. 2001. Determination of 5-fluorouracil in environmental samples by solid-phase extraction and high-performance liquid chromatography with ultraviolet detection. *Journal of Chromatography B*, **750**(1), 25–32.

Mihailescu, R., Aboul-Enein, H. Y., & Efstatide, M. D. 2000. Identification of tamoxifen and metabolites in human male urine by GC/MS. *Biomedical Chromatography*, **14**(3), 180–183.

Monarca, S., Zanardini, A., Feretti, D., Dalmiglio, A., Falistocco, E., Manica, P., & Nardi, G. 1998. Mutagenicity of extracts of lake drinking water treated with different disinfectants in bacterial and plant tests. *Water Research*, **32**(9), 2689–2695.

Monarca, S., Feretti, D., Collivignarelli, C., Guzzella, L., Zerbini, I., Bertanza, G., & Pedrazzani, R. 2000. The influence of different disinfectants on mutagenicity and toxicity of urban wastewater. *Water Research*, **34**(17), 4261–4269.

Morris, R. D., Audet, A. M., Angelillo, I. F., Chalmers, T. C., & Mosteller, F. 1992. Chlorination, Chlorination by-Products, and Cancer - a Metaanalysis. *American Journal of Public Health*, **82**(7), 955–963.

Mortelmans, K., & Zeiger, E. 2000. The Ames Salmonella/microsome mutagenicity assay. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, **455**(1-2), 29–60.

Murphy, C., Fotsis, T., Pantzar, P., Adlercreutz, H., & Martin, F. 1987. Analysis of Tamoxifen and Its Metabolites in Human-Plasma by Gas-Chromatography Mass-Spectrometry (Gc Ms) Using Selected Ion Monitoring (Sim). *Journal of Steroid Biochemistry and Molecular Biology*, **26**(5), 547–555.

Naudin, S., Garric, J., Vindimian, E., Bray, M., Migeon, B., Vollat, B., & Lenon, G. 1995. Influence of the Sample Preservation Mode to Assess the Chronic Toxicity of an Industrial Effluent. *Ecotoxicology and Environmental Safety*, **30**(1), 54–62.

Oaks, J. L., Gilbert, M., Virani, M. Z., Watson, R. T., Meteyer, C. U., Rideout, B. A., Shivaprasad, H. L., Ahmed, S., Chaudhry, M. J. I., Arshad, M., Mahmood, S., Ali, A., & Khan, A. A. 2004. Diclofenac residues as the cause of vulture population decline in Pakistan. *Nature*, **427**(6975), 630–633.

Ono, Y., Somiya, I., Kawaguchi, T., & Mohri, S. 1996. Evaluation of toxic substances in effluents from a wastewater treatment plant. *Desalination*, **106**(1-3), 255–261.

Park, J. H., Lee, B. J., Lee, S. K, Kim, K., Lee, K. H., Che, J. H., Kang, K. S., & Lee, Y. S. 2000. Genotoxicity of drinking water from three Korean cities. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, **466**(2), 173–178.

Pelon, W., Whitman, B. F., & Beasley, T. W. 1977. Reversion of Histidine-Dependent Mutant Strains of Salmonella-Typhimurium by Mississippi River Water Samples. *Environmental Science and Technology*, **11**(6), 619–623.

Perez, S., Reifferscheid, G., Eichhorn, P., & Barcelo, D. 2003. Assessment of the mutagenic potency of sewage sludges contaminated with polycyclic aromatic hydrocarbons by an ames fluctuation assay. *Environmental Toxicology and Chemistry*, **22**(11), 2576–2584.

Plagellat, C., Kupper, T., de Alencastro, L. F., Grandjean, D., & Tarradellas, J. 2004. Biocides in sewage sludge: Quantitative determination in some Swiss wastewater treatment plants. *Bulletin of Environmental Contamination and Toxicology*, **73**(5), 794–801.

Pratt, W. B., Ruddon, R. W., Ensminger, W. D., & Maybaum, J. 1994. *The Anticancer Drugs*. New York: Oxford University Press.

Rappaport, S. M., Richard, M. G., Hollstein, M. C., & Talcott, R. E. 1979. Mutagenic Activity in Organic Wastewater Concentrates. *Environmental Science and Technology*, **13**(8), 957–961.

Reddersen, K., Heberer, T., & Dunnbier, U. 2002. Identification and significance of phenazone drugs and their metabolites in ground- and drinking water. *Chemosphere*, **49**(6), 539–544.

Reifferscheid, G., & Hell, J. 1996. Validation of the SOS/umu test using test results of 486 chemicals and comparison with the Ames test and carcinogenicity data. *Mutation Research-Genetic Toxicology*, **369**(3-4), 129–145.

Rey, R. P., Padron, A. S., Leon, L. G., Pozo, M. M., & Baluja, C. 1999. Ozonation of cytostatics in water medium. Nitrogen bases. *Ozone-Science and Engineering*, **21**(1), 69–77.

Richardson, M. L., & Bowron, J. M. 1985. The Fate of Pharmaceutical Chemicals in the Aquatic Environment. *Journal of Pharmacy and Pharmacology*, **37**(1), 1–12.

Robin, P., & Soulet, B. 1999. *Analyse de médicaments acides dans les eaux usées et les eaux de surface suisse - Tests écotoxicologiques sur les produits purs*. Tech. rept. Cycle Postgrade en Sciences de l'Environnement-Ecole polytechnique fédérale de Lausanne.

Rodriguez, I., Quintana, J. B., Carpinteiro, J., Carro, A. M., Lorenzo, R. A., & Cela, R. 2003a. Determination of acidic drugs in sewage water by gas chromatography-mass spectrometry as tert.-butyldimethylsilyl derivatives. *Journal of Chromatography A*, **985**(1-2), 265–274.

Rodriguez, J., Berzas, J. J., Contento, A. M., & Cabello, M. P. 2003b. Capillary gas chromatographic determination of Tamoxifen in the presence of a number of antidepressants in urine. *Journal of Separation Science*, **26**(9-10), 915–922.

Sacher, F., Lange, F. T., Brauch, H.-J., & Blankenhorn, I. 2001. Pharmaceuticals in groundwaters; Analytical methods and results of a monitoring program in Baden-Wurttemberg, Germany. *Journal of Chromatography A*, **938**(1-2), 199–210.

Salvito, D. T., Senna, R. J., & Federle, T. W. 2002. A framework for prioritizing fragrance materials for aquatic risk assessment. *Environmental Toxicology and Chemistry*, **21**(6), 1301–1308.

Sanderson, H., Johnson, D. J., Wilson, C. J., Brain, R. A., & Solomon, K. R. 2003. Probabilistic hazard assessment of environmentally occurring pharmaceuticals toxicity to fish, daphnids and algae by ECOSAR screening. *Toxicology Letters*, **144**(3), 383–395.

Sebastine, I. M., & Wakeman, R. J. 2003. Consumption and environmental hazards of pharmaceutical substances in the UK. *Process Safety and Environmental Protection*, **81**(B4), 229–235.

Shelby, M. D., & Zeiger, E. 1990. Activity of Human Carcinogens in the Salmonella and Rodent Bone-Marrow Cytogenetics Tests. *Mutation Research*, **234**(3-4), 257–261.

Shelby, M. D., Newbold, R. R., Tully, D. B., Chae, K., & Davis, V. L. 1996. Assessing environmental chemicals for estrogenicity using a combination of in vitro and in vivo assays. *Environmental Health Perspectives*, **104**(12), 1296–1300.

Shishida, K., Echigo, S., Kosaka, K., Tabasaki, M., Matsuda, T., Takigami, H., Yamada, H., Shimizu, Y., & Matsui, S. 2000. Evaluation of advanced sewage treatment processes for reuse of wastewater using bioassays. *Environmental Technology*, **21**(5), 553–560.

Shugart, L., & Theodorakis, C. 1994. Environmental Genotoxicity - Probing the Underlying Mechanisms. *Environmental Health Perspectives*, **102**, 13–17.

Sorsa, M., & Anderson, D. 1996. Monitoring of occupational exposure to cytostatics anticancer agents. *Mutation Research*, **355**, 253–261.

Sorsa, M., Hemminki, K., & Vainio, H. 1985. Occupational exposure to anticancer drugs - Potential and real hazards. *Mutation Research*, **154**, 135–149.

Soulet, B., Tauxe, A., & Tarradellas, J. 2002. Analysis of acidic drugs in swiss wastewaters. *International Journal of Environmental Analytical Chemistry*, **82**(10), 659–667.

Stahl, R. G. 1991. The Genetic Toxicology of Organic-Compounds in Natural-Waters and Wastewaters. *Ecotoxicology and Environmental Safety*, **22**(1), 94–125.

Stan, H.-J., & Heberer, T. 1997. Pharmaceuticals in the aquatic environment. *Analusis Magazine*, **25**(7), M20–M23.

Stan, H.-J., Heberer, T., & Linkerhägner, M. 1994. Occurrence of clofibric acid in the aquatic system - Is the use in human medical care the source of the contamination of surface, ground and drinking water? *Vom Wasser*, **83**, 57–68.

Stead, A. G., Hasselblad, V., Creason, J. P., & Claxton, L. D. 1981. Modeling the Ames test. *Mutation Research/Environmental Mutagenesis and Related Subjects*, **85**(1), 13–27.

Steger-Hartmann, T., Kümmerer, K., & Schecker, J. 1996. Trace analysis of the antineoplastics ifosfamide and cyclophosphamide in sewage water by two-step solid-phase extraction and gas chromatography-mass spectrometry. *Journal of Chromatography A*, **726**(1-2), 179–184.

Steger-Hartmann, T., Kümmerer, K., & Hartmann, A. 1997. Biological degradation of cyclophosphamide and its occurrence in sewage water. *Ecotoxicology and Environmental Safety*, **36**(2), 174–179.

Steinert, S. A., Montee, R. S., & Sastre, M. P. 1998. Influence of sunlight on DNA damage in mussels exposed to polycyclic aromatic hydrocarbons. *Marine Environmental Research*, **46**(1-5), 355–358.

Stuer-Lauridsen, F., Birkved, M., Hansen, L. P., Lützhøft, H. C. H., & Halling-Sørensen, B. 2000. Environmental risk assessment of human pharmaceuticals in Denmark after normal therapeutic use. *Chemosphere*, **40**(7), 783–793.

Stumpf, M., Ternes, T. A., Haberer, K., Seel, P., & Baumann, W. 1996. Determination of pharmaceutics in sewage plants and river water. *Vom Wasser*, **86**, 291–303.

Stumpf, M., Ternes, T. A., Wilken, R. D., Rodrigues, S. V., & Baumann, W. 1999. Polar drug residues in sewage and natural waters in the state of Rio de Janeiro, Brazil. *The Science of the Total Environment*, **225**(1-2), 135–141.

Swissmedic. 2003. *Business Report 2002*. Tech. rept. Swiss Agency for Therapeutic Products.

Takanashi, H., & Urano, K. 1998. Statistical procedures for estimating the detection limit and determination limit of the Ames Salmonella mutagenicity assay. *Science of the Total Environment*, **221**(1), 31–42.

Tennant, R. W., Margolin, B. H., Shelby, M. D., Zeiger, E., Haseman, J. K., Spalding, J., Caspary, W., Resnick, M., Stasiewicz, S., Anderson, B., & Minor, R. 1987. Prediction of Chemical Carcinogenicity in Rodents from Invitro Genetic Toxicity Assays. *Science*, **236**(4804), 933–941.

Ternes, T. 2001a. Pharmaceuticals and metabolites as contaminants of the aquatic environment. *Pages 39–54 of:* Daughton, C. G., & Jones-Lepp, T. L. (eds), *Pharmaceuticals and Personal Care Products in the Environment*. Washington: American chemical society.

Ternes, T., Bonerz, M., & Schmidt, T. 2001. Determination of neutral pharmaceuticals in wastewater and rivers by liquid chromatography-electrospray tandem mass spectrometry. *Journal of Chromatography A*, **938**(1-2), 175–185.

Ternes, T. A. 1998. Occurrence of drugs in german sewage treatment plants and rivers. *Water Research*, **32**(11), 3245–3260.

Ternes, T. A. 2001b. Analytical methods for the determination of pharmaceuticals in aqueous environmental samples. *Trends in Analytical Chemistry*, **20**(8), 419–434.

Ternes, T. A., Stumpf, M., Mueller, J., Haberer, K., Wilken, R.-D., & Servos, M. 1999a. Behavior and occurrence of estrogens in municipal sewage treatment plants – I. Investigations in Germany, Canada and Brazil. *The Science of The Total Environment*, **225**(1-2), 81–90.

Ternes, T. A., Kreckel, P., & Mueller, J. 1999b. Behaviour and occurrence of estrogens in municipal sewage treatment plants – II. Aerobic batch experiments with activated sludge. *The Science of The Total Environment*, **225**(1-2), 91–99.

Ternes, T. A., Meisenheimer, M., McDowell, D., Sacher, F., Brauch, H. J., Gulde, B. H., Preuss, G., Wilme, U., & Seibert, N. Z. 2002. Removal of pharmaceuticals during drinking water treatment. *Environmental Science and Technology*, **36**(17), 3855–3863.

Teutenberg, T., Lerch, O., Gotze, H. J., & Zinn, P. 2001. Separation of selected anticancer drugs using superheated water as the mobile phase. *Analytical Chemistry*, **73**(16), 3896–3899.

Theodorakis, C.W., Swartz, C.D., Bickham, J.W., Rogers, W.J., Donnelly, K.C., & Adams, S.M. 2000. Relationship between genotoxicity, mutagenicity, and fish community structure in a contaminated stream. *Journal of Aquatic Ecosystem Stress and Recovery*, **7**(2), 131–143.

Thiele-Bruhn, S. 2003. Pharmaceutical antibiotic compounds in soils - a review. *Journal of Plant Nutrition and Soil Science-Zeitschrift Fur Pflanzenernahrung Und Bodenkunde*, **166**(2), 145–167.

Thomas, K. V., & Hilton, M. J. 2004. The occurrence of selected human pharmaceutical compounds in UK estuaries. *Marine Pollution Bulletin*, **49**(5-6), 436–444.

Tixier, C., Singer, H. P., Oellers, S., & Muller, S. R. 2003. Occurrence and fate of carbamazepine, clofibric acid, diclofenac, ibuprofen, ketoprofen, and naproxen in surface waters. *Environmental Science and Technology*, **37**(6), 1061–1068.

Tjaden, U. R., & De Bruijn, E. A. 1990. Chromatographic analysis of anticancer drugs. *Journal of Chromatography B: Biomedical Sciences and Applications*, **531**, 235–294.

Umbuzeiro, G. D., Roubicek, D. A., Sanchez, P. S., & Sato, M. I. Z. 2001. The Salmonella mutagenicity assay in a surface water quality monitoring program based on a 20-year survey. *Mutation Research - Genetic Toxicology and Environmental Mutagenesis*, **491**(1-2), 119–126.

Webb, S., Ternes, T., Gibert, M., & Olejniczak, K. 2003. Indirect human exposure to pharmaceuticals via drinking water. *Toxicology Letters*, **142**(3), 157–167.

Webb, S. F. 2001a. A Data-based Perspective on the environmental Risk Assessment of Human Pharmaceuticals I - Collation of Available Ecotoxicity Data. *Pages 175–201 of:* Kümmerer, K (ed), *Pharmaceutical in the Environment: Sources, fate, effects and risks*. Berlin Heidelberger New York: Springer.

Webb, S. F. 2001b. A Data-based Perspective on the environmental Risk Assessment of Human Pharmaceuticals II - Aquatic Risk Characterisation. *Pages 203–219 of:* Kümmerer, K (ed), *Pharmaceutical in the Environment: Sources, fate, effects and risks*. Berlin Heidelberger New York: Springer.

Wennmalm, A. 2003. Management of pharmaceutical drug residues from public health care in Stockholm conty conuncil. *Page 101 of: ENVIR-PHARMA, european conference on Human and Veterinaty Pharmaceuticals in the Environment*.

Werner, J. J., McNeill, K., & Arnold, W. A. 2005. Environmental photodegradation of mefenamic acid. *Chemosphere*, **58**(10), 1339–1346.

White, P. A., & Rasmussen, J. B. 1998. The genotoxic hazards of domestic wastes in surface waters. *Mutation Research/Reviews in Mutation Research*, **410**(3), 223–236.

White, P. A., Rasmussen, J. B., & Blaise, C. 1996. Comparing the presence, potency, and potential hazard of genotoxins extracted from a broad range of industrial effluents. *Environmental and Molecular Mutagenesis*, **27**(2), 116–139.

Winkler, M., Lawrence, J. R., & Neu, T. R. 2001. Selective degradation of ibuprofen and clofibric acid in two model river biofilm systems. *Water Research*, **35**(13), 3197–3205.

Zambonin, C. G., Guerrieri, A., & Palmisano, F. 1996. Simultaneous determination of 5'-deoxy-5-fluorouridine, 5- fluorouracil and 5,6-dihydro-5-fluorouracil in plasma by gas chromatography mass spectrometry. *Analytica Chimica Acta*, **329**(1-2), 143–152.

Zeiger, E. 1998. Identification of rodent carcinogens and noncarcinogens using genetic toxicity tests: Premises, promises, and performance. *Regulatory Toxicology and Pharmacology*, **28**(2), 85–95.

Zeiger, E., Haseman, J. K., Shelby, M. D., Margolin, B. H., & Tennant, R. W. 1990. Evaluation of 4 Invitro Genetic Toxicity Tests for Predicting Rodent Carcinogenicity - Confirmation of Earlier Results with 41 Additional Chemicals. *Environmental and Molecular Mutagenesis*, **16**, 1–14.

Zufia, L., Aldaz, A., & Giraldez, J. 2004. Simple determination of capecitabine and its metabolites by liquid chromatography with ultraviolet detection in a single injection. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences*, **809**(1), 51–58.

Zwiener, C., & Frimmel, F. H. 2000. Oxidative treatment of pharmaceuticals in water. *Water Research*, **34**(6), 1881–1885.

Appendices

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Appendix A

Pharmaceutical substances

Most of the information in the Appendices A.1 to A.15 are from the following databases:

- TOXNET, a cluster of databases on toxicology, hazardous chemicals, and related areas (http://toxnet.nlm.nih.gov/). Hazardous Substances Data Bank (HSDB) was specially used
- National Toxicology Program (NTP) Database (http://ntp-apps.niehs.nih.gov/ntp_tox/index.cfm)
- The Physical Properties Database (PHYSPROP) of Syracuse Research Corporation (http://www.syrres.com/esc/physdemo.htm)
- RxList, the internet drug index (http://www.rxlist.com)
- Compendium of drugs, a Swiss database on drugs (http://www.kompendium.ch)
- ChemFinder.com, a scientific databases

A.1 Clofibric acid

Generality

Name	2-(4-chlorophenoxy)-2-methyl propionic acid
CAS RN	882-09-7
Molecular weight	214.6 ($C_{10}H_{11}ClO_3$)
Melting point	120-122°C
λ_{max} (absorption)	225 nm in MeOH
Solubility in water (25°C)	583 mg/l (estimation)
Solubility in methanol (22°C)	1-5 mg/ml
Log Kow	2.57

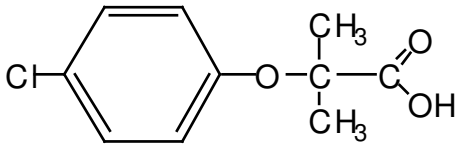

FIGURE A.1: Structure of Clofibric acid

Clofibric acid is an active metabolite of clofibrate, etofibrate, etofyllinclofibrate wich are drugs used as blood lipid regulators. These substances are used to decrease the plasmatic concentration of cholesterol and triglycerides. The mechanism of action has not been established definitively.

Occurrence in the environment

Clofibric acid has already been detected in STP effluents at concentrations up to 1600 ng/l [Stumpf *et al.*, 1996; Heberer *et al.*, 2002]. In the aquatic environment, it was detected in German river waters at concentrations up to 550 ng/l [Ternes, 1998] and in Swiss lakes at concentrations up to 9 ng/l [Buser *et al.*, 1998b]. It was even found in samples of ground water wells at a drinking water treatment plant (up to 7 300 ng/l) [Heberer *et al.*, 1997].

Dosage

Patients ingest one capsule (500 mg) of clofibrate one to four times per day.

Metabolism and Elimination

After an oral administration, Clofibrate is quickly and completely reduced of the gastro-intestinal tract and is degraded by hydrolysis in clofibric acid. Between 95% and 99% of an oral dose of clofibrate is excreted in the urine as free and conjugated clofibric acid.

Etofyllinclofibrate is well reduced after oral administration. It is not found in the blood under unchanged shape, but under the shape of its two main metabolites: etofylline and clofibric acid. The half-life of elimination is about 12-18 hours. Eight hours after the administration, 8.5% of the dose are eliminated in urine in the form of clofibric acid.

Toxicity

Ecotoxicity

Organisms	Toxicity	Ref.
Daphnia magna (Invertebrates)	EC_{50} = 106 mg/l	[Webb, 2001a]
Brachyderio rerio (Fish embryos)	EC_{50} (48h) = 86 mg/l	
Scenedesmus subspicatus (Algae)	EC_{50} (72h) = 89 mg/l	
Daphnia magna (Invert.)	EC_{50} (48h) > 200 000 μg/l	[Ferrari *et al.*, 2003]
Ceriodaphnia dubia (Invert.)	EC_{50} (48h) > 200 000 μg/l	
P. subcapitata (Algae)	NOEC (96h) = 75 000 μg/l	
B. Calyciflorus (Invert.)	NOEC (48h) = 246 μg/l	
Ceriodaphnia dubia (Invert.)	NOEC (7d) = 640 μg/l	
Brachyderio rerio (Fish embryos)	NOEC (10d) = 70 000 μg/l	

EC_{50}: median Effect Concentration or 50% effective concentration
NOEC: No Observed Effect Concentration

Carcinogenesis

Administration of clofibrate to mice and rats in long-term studies at 1 to 2 times the maximum recommended human dose (based on surface area, mg/m^2), resulted in a higher incidence of benign and malignant liver tumors than in controls. There was an increase in benign Leydig cell tumors in male rats treated at 400 mg/kg/day or 2 times the maximum recommended human

dose in one study. A comparative carcinogenicity study was also done in rats comparing three drugs in this class: fenofibrate (10 and 60 mg/kg; 0.3 and 1.6 times the human dose), clofibrate (400 mg/kg; 1.6 times the human dose), and gemfibrozil (250 mg/kg; 1.7 times the human dose). Pancreatic acinar adenomas were increased in males and females on fenofibrate; hepatocellular carcinoma and pancreatic acinar adenomas were increased in males and hepatic neoplastic nodules in females treated with clofibrate; hepatic neoplastic nodules were increased in males and females treated with gemfibrozil while testicular interstitial cell tumors were increased in males on all three drugs.

Impairment of Fertility

Arrest of spermatogenesis has been seen in both dogs and monkeys at doses approximately two times the maximum recommended human dose (based on surface area).

Adverse effect on human

The treatment with these drugs can result in some gastro-intestinal confusions, such as sickness, vomit and diarrhea. Very rare cases of fall of hair, impotence and muscular pains were reported. Diseases of bile tract can arise following a long treatment with blood lipid regulators.

Medicines on sale in Switzerland

Clofibrat Tripharma®, Lipo-Merz®retard, Duolip®

A.2 Ibuprofen

Generality

Name	2-(4-isobutylphenyl)propionic acid
CAS RN	15687-27-1
Molecular weight	206.3 ($C_{13}H_{18}O_2$)
Melting point	75-77°C
λ_{max} (absorption)	223 nm in MeOH
Solubility in water (25°C)	21 mg/l
Solubility in MeOH:water (1:99)	83 mg/l
Log Kow	3.97
pKa	4.91

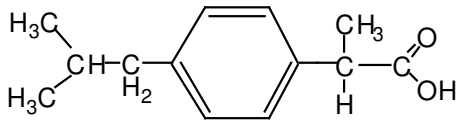

FIGURE A.2: Structure of Ibuprofen

Ibuprofen is a nonsteroidal anti-inflammatory drug (NSAID). Ibuprofen possesses analgesic and antipyretic activities. Its mode of action, like that of other NSAIDs, is not completely understood, but may be related to prostaglandin synthetase inhibition.

Ibuprofen is indicated for relief of the signs and symptoms of rheumatoid arthritis and osteoarthritis. Ibuprofen is also indicated for relief of mild to moderate pain and for the treatment of primary dysmenorrhea.

It is on of the most important pharmaceuticals in term of quantities consumed.

Occurrence in the environment

Ibuprofen was already detected in surface waters and sewage treatment plant at concentrations up to 530 ng/l and 3400 ng/l, respectively [Buser *et al.*, 1998a; Stumpf *et al.*, 1999; Ternes, 1998].

Dosage

The suggested dosage is 1200-3200 mg daily, and do not exceed 3200 mg total daily dose.

Metabolism and Elimination

Ibuprofen is rapidly absorbed when administered orally. Studies have shown that following ingestion of the drug, 45% to 79% of the dose was recovered in the urine within 24 hours as metabolite A (25%), (+)-2-4 -(2-hydroxy-2-methylpropyl)-phenylpropionic acid and metabolite B (37%), (+)-2-4 -(2-carboxypropyl)-phenylpropionic acid; the percentages of free and conjugated ibuprofen were approximately 1% and 14%, respectively.

Toxicity

Ecotoxicity

Organisms	Toxicity	References
Daphnia magna (Invertebrates)	EC_{50} (48h) = 9.06 mg/l	[Webb, 2001a]
Lepomis macrochirus (Fish)	EC_{50} (96h) = 173 mg/l	
Skeletonema costatum (Algae)	EC_{50} (96h) = 7.1 mg/l	

EC_{50}: median Effect Concentration or 50% effective concentration

Animal toxicity

Animals and absorption	Toxicity	References
LD_{50} (oral, mice)	1255 mg/kg	Merck index
LD_{50} (i.p., mice)	495 mg/kg	
LD_{50} (oral, rat)	1050 mg/kg	
LD_{50} (i.p., rat)	626 mg/kg	

LD_{50}: lethal dose 50 percent kill; i.p: intraperitoneal.

Adverse effect on human

The most frequent type of adverse reaction occurring with ibuprofen is gastrointestinal (nausea, epigastric pain, heartburn, diarrhea, abdominal distress, nausea and vomiting, indigestion, constipation, abdominal cramps or pain). Adverse reactions observed during controlled clinical trials at an incidence greater than 1% are effects on central nervous system (dizziness, headache, nervousness) and dermatologic effect (rash, pruritus).

Because of the known effects of Ibuprofen on the fetal cardiovascular system (closure of ductus arteriosus), use during late pregnancy should be avoided.

Medicines on sale in Switzerland

Algifor®, Dolocyl®, Ecoprofen®, etc.

A.3 Ketoprofen

Generality

Name	2-(meta-Benzoylphenyl) propionic acid
CAS RN	22071-15-4
Molecular weight	254.3 ($C_{16}H_{14}O_3$)
Melting point	94°C
Max (absorption)	216 and 244 nm in MeOH
λ_{max}	260 nm in acid solution
Solubility in MeOH:water (1:99)	220 mg/l
Solubility in water (pH=7)	0.3 g/ml
Log Kow	3.12
pKa	4.45

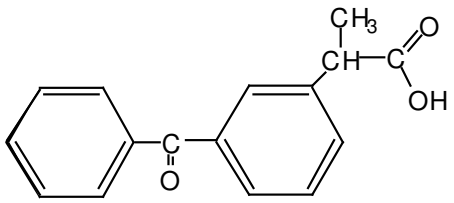

FIGURE A.3: Structure of Ketoprofen

Ketoprofen is a nonsteroidal anti-inflammatory drug (NSAID) with analgesic and antipyretic properties. In anti-inflammatory models ketoprofen has been shown to have inhibitory effects on prostaglandin and leukotriene synthesis, to have antibrady-kinin activity, as well as to have lysosomal membrane-stabilizing action. However, its mode of action, like that of other nonsteroidal anti-inflammatory drugs, is not fully understood.

Ketoprofen is indicated for the management of the signs and symptoms of rheumatoid arthritis and osteoarthritis. Ketoprofen is indicated for the management of pain and for treatment of primary dysmenorrhea.

Occurrence in the environment

Ketoprofen is detected in surface waters and sewage treatment plant at concentrations up to 120 ng/l and 380 ng/l, respectively [Stumpf *et al.*, 1996, 1999; Ternes, 1998]

Dosage

The recommended starting dose of ketoprofen in otherwise healthy patients is 75 mg three times or 50 mg four times a day, or 200 mg administered once a day. The recommended maximum daily dose of ketoprofen is 200-300 mg/day.

Metabolism and Elimination

The metabolic fate of ketoprofen is glucuronide conjugation to form an unstable acyl-glucuronide. The glucuronic acid moiety can be converted back to the parent compound. There are no known active metabolites of ketoprofen. Ketoprofen has been shown not to induce drug-metabolizing enzymes. In a 24-hour period, approximately 80% of an administered dose of ketoprofen is excreted in the urine, primarily as the glucuronide metabolite.

Toxicity

Ecotoxicity

Organisms	Toxicity	References
V. ficheri (ToxAlert 100)	EC_{50} = 15.6 μg/ml	[Farré & Barceló, 2003]
V. ficheri (Microtox®)	EC_{50} = 27 μg/ml	[Robin & Soulet, 1999]
Daphnia magna(Invert.)	EC_{50} (24h) = 101-138 μg/ml	
Daphnia magna(Invert.)	EC_{50} (48h) = 52-76 μg/ml	

EC_{50}: median Effect Concentration or 50% effective concentration

Animal toxicity

Animals and absorption	Toxicity	References
LD_{50} (oral, mice)	101 mg/kg	Merck index

LD_{50}: lethal dose 50 percent kill

Carcinogenesis, Mutagenesis, Impairment of Fertility

Chronic oral toxicity studies in mice (up to 32 mg/kg/day; 96 mg/m^2/day) did not indicate a carcinogenic potential for ketoprofen. A 2-year carcinogenicity study in rats, using doses up to 6.0 mg/kg/day (36 mg/m^2/day), showed no evidence of tumorigenic potential. Abnormal spermatogenesis or inhibition of spermatogenesis developed in rats and dogs at high doses, and a decrease in the weight of the testes occurred in dogs and baboons at high doses.

Adverse effect on human

The most frequent type of adverse reaction occurring with ketoprofen is gastrointestinal (nausea, abdominal pain, diarrhea, constipation, flatulence, anorexia, vomiting). Adverse reactions observed during controlled clinical trials at an incidence greater than 1% are effects on central nervous system (dizziness, headache, excitation) and dermatologic effect (rash).

Medicines on sale in Switzerland

Fastum®

A.4 Mefenamic acid

Generality

Name	N-(2,3-Xylyl)anthranilic acid
CAS RN	61-68-7
Molecular weight	241.29 ($C_{15}H_{15}NO_2$)
Melting point	230-231°C
λ_{max} (absorption)	285, 340 nm in NaOH 0.1N
Solubility in water (pH=7.1; 25°C)	0.004 g/100ml
Solubility in water (pH=7.1; 37°C)	0.008 g/100ml
Log Kow	5.12
pKa	4.2

Few soluble in chloroform and in ether. Slightly soluble in ethanol.

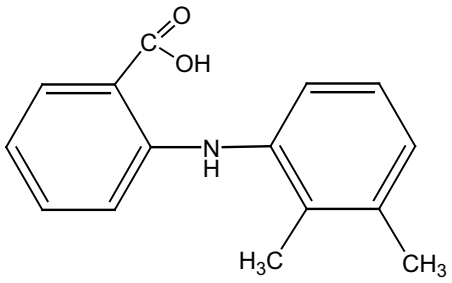

FIGURE A.4: Structure of Mefenamic acid

Mefenamic acid is a nonsteroidal anti-inflammatory drug (NSAID) that exhibits anti-inflammatory, analgesic, and antipyretic activities. The mechanism of action of mefenamic acid, like that of other NSAIDs, is not completely understood but may be related to prostaglandin synthetase inhibition.

Mefenamic acid is indicated for relief of mild to moderate pain, when therapy will not exceed one week (7 days) and for treatment of primary dysmenorrhea.

Occurrence in the environment

To our knowledge, no study has been conducted to detect mefenamic acid in influent of sewage treatment plant. Mefenamic acid was measured in river (with a very low recovery rate of 43.1%) [Ahrer *et al.*, 2001] and in effluent of sewage treatment plants [Ashton *et al.*, 2004].

Dosage

The recommended dose is 500 mg as an initial dose followed by 250 mg every 6 hours as needed, usually not to exceed one week.

Metabolism and Elimination

Mefenamic acid is metabolised by cytochrome P450 enzyme CYP2C9 to 3-hydroxymethyl mefenamic acid (Metabolite I). Further oxidation to a 3-carboxymefenamic acid (Metabolite II) may occur. The activity of these metabolites has not been studied. The metabolites may undergo glucuronidation and mefenamic acid is also glucuronidated directly. Approximately 52 percent of a mefenamic acid dose is excreted into the urine primarily as glucuronides of mefenamic acid (6%), 3-hydroxymefenamic acid (25%) and 3-carboxymefenamic acid (21%). The proportion of free mefenamic acid in the urine is lower than 5%. The fecal route of elimination accounts for up to 20% of the dose, mainly in the form of unconjugated 3-carboxymefenamic acid.

Toxicity

Ecotoxicity

None

Animal toxicity

Animals and absorption	Toxicity	References
LD_{50} (oral, mice)	630 mg/kg	Merck index
LD_{50} (oral, rat)	790 mg/kg	

LD_{50}: lethal dose 50 percent kill

Adverse effect on human

The most frequently reported adverse experiences occurring in approximately 1-10% of patients are abdominal pain, constipation, diarrhea, dyspepsia, flatulence, gross bleeding/perforation, heartburn, nausea, GI ulcers (gastric/duodenal), vomiting, abnormal renal function, anemia, dizziness, edema, elevated liver enzymes, headaches, increased bleeding time, pruritus, rashes, tinnitus.

Medicines on sale in Switzerland

Méfé-basan, Ponstan®, Spiralgin®, etc.

A.5 Diclofenac

Generality

Name	2-[(2,6-dichlorophenyl)amino] benzeneacetic acid
CAS RN	15307-86-5
Molecular weight	318,14 ($C_{14}H_{10}Cl_2NNaO_2$)
Melting point	283-285°C
λ_{max} (absorption)	221 nm and 280 nm in MeOH
Solubility in MeOH/water (1/99)	$\sim$ 7 760 mg/l
Log Kow	4.51
pKa	4.15

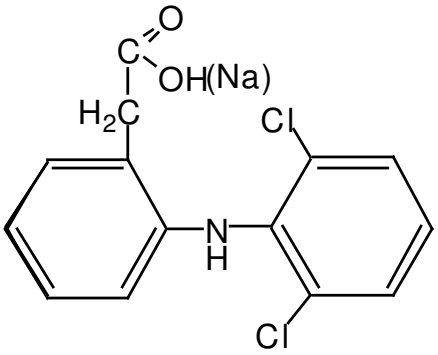

FIGURE A.5: Structure of Diclofenac

Diclofenac is a nonsteroidal anti-inflammatory drug (NSAID). In pharmacologic studies, diclofenac has shown anti-inflammatory, analgesic, and antipyretic activity.

Diclofenac is indicated for the acute and chronic treatment of signs and symptoms of osteoarthritis and rheumatoid arthritis. In addition, diclofenac are indicated for the treatment of ankylosing spondylitis and for the management of pain and primary dysmenorrhea.

As with other NSAIDs, its mode of action is not known; its ability to inhibit prostaglandin synthesis, however, may be involved in its anti-inflammatory activity, as well as contribute to its efficacy in relieving pain related to inflammation and primary dysmenorrhea.

Occurrence in the environment

Diclofenac was already detected in surface waters and sewage treatment plant at concentrations up to 1200 ng/l and 2100, ng/l, respectively [Buser *et al.*, 1998a; Stumpf *et al.*, 1999; Ternes, 1998].

Dosage

Diclofenac may be administered from 25 mg to 75 mg.

Metabolism and Elimination

Diclofenac is eliminated through metabolism and subsequent urinary and biliary excretion of the glucuronide and the sulfate conjugates of the metabolites. Approximately 65% of the dose is excreted in the urine, and approximately 35% in the bile. Conjugates of unchanged diclofenac account for 5%-10% of the dose excreted in the urine and for less than 5% excreted in the bile. Little or no unchanged unconjugated drug is excreted. Conjugates of the principal metabolite account for 20%-30% of the dose excreted in the urine and for 10%-20% of the dose excreted in the bile. Conjugates of three other metabolites together account for 10%-20% of the dose excreted in the urine and for small amounts excreted in the bile. Some of the metabolites may have activity.

Toxicity

Animal toxicity

Animals and absorption	Toxicity	References
LD_{50} (oral, mice)	390 mg/kg	Merck index
LD_{50} (oral, rat)	150 mg/kg	
LD_{50} (oral, monkey)	3200 mg/kg	HSBD
LD_{50} (oral, dog)	500 mg/kg	

LD_{50}: lethal dose 50 percent kill

Ecotoxicity

Organisms	Toxicity	References
Daphnia magna	EC_{50} = 106 mg/l	[Webb, 2001a]
Brachydanio reria (embryos)	48h EC_{50} = 86 mg/l	
Scenedesmus subspicatus	72h EC_{50} = 89 mg/l	
Daphnia magna (Invert.)	EC_{50} (48h) = 22 430 μg/l	[Ferrari *et al.*, 2003]
Ceriodaphnia dubia (Invert.)	EC_{50} (48h) = 22 704 μg/l	
P. subcapitata (Algae)	NOEC (96h) = 10 000 μg/l	
B. Calyciflorus (Invert.)	NOEC (48h) = 12 500 μg/l	
Ceriodaphnia dubia (Invert.)	NOEC (7d) = 1 000 μg/l	
Brachyderio rerio (Fish embryos)	NOEC (10d) = 4 000 μg/l	

EC_{50}: median Effect Concentration or 50% effective concentration
NOEC: No Observed Effect Concentration

Adverse effect on human

Incidence greater than 1% : Abdominal pain or cramps, headache, fluid retention, abdominal distention. Digestive perturbation (diarrhea, indigestion, nausea, constipation, flatulence, liver test abnormalities, peptic ulcer, with or without bleeding and/or perforation, or bleeding without ulcer). Effect on nervous system (dizziness). Etc.

Medicines on sale in Switzerland

Inflama®, Voltaren, Olfen®, etc.

A.6 Hydroxycarbamide / Hydroxyurea

Generality

Name	
CAS RN	127-07-1
Molecular weight	76.06 ($CH_4N_2O_2$)
Melting point	141-146°C
Solubility in water (21°C)	≥100 mg/ml
Solubility in 95% ethanol, acetone (21°C)	<1 mg/ml
Log Kow	-1.80

This chemical is hygroscopic and decomposes in the presence of moisture. Solution of this chemical in water, DMSO, ethanol or acetone should be stable for 24 hours under normal laboratory conditions.
Distribution: pharmacy (the most cytostatics sold in pharmacy, for instance: ∼500g/year for one pharmacy in Vevey).

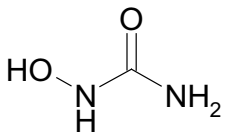

FIGURE A.6: Structure of Hydroxyurea

Hydroxyurea is used in the treatment of chronic myeloid leukemia, malignant melanomas and inoperable tumors of the ovary, and squamous cell carcinomas of the head and neck (with radiotherapy).

The precise mechanism by which produces its cytotoxic and cytoreductive effects is not really known. However, various studies support the hypothesis that hydroxyurea causes an immediate inhibition of DNA synthesis by acting as a ribonucleotide reductase inhibitor, without interfering with the synthesis of ribonucleic acid or of protein.

Biodegradability

To our knowledge, no study has been conducted to evaluate the biodegradability of hydroxyurea.

Occurrence in the environment

To our knowledge, no study has been conducted to detect hydroxyurea in wastewaters or in the environment.

Detection methods

According to Tjaden & De Bruijn [1990], data are limited. One study described an LC assay for the determination of hydroxyurea in pharmaceutical formulation.

Dosage

The initial dose of hydroxyurea is 15 mg/kg/day as a single dose. If blood counts are in an acceptable range, the dose may be increased by 5 mg/kg/day every 12 weeks until a maximum tolerated dose (the highest dose that does not produce toxic blood counts over 24 consecutive weeks), or 35 mg/kg/day, is reached.

Metabolism and Elimination

Up to 50% of an oral dose undergoes conversion through metabolic pathways that are not fully characterised. In one minor pathway, hydroxyurea may be degraded by urease found in intestinal bacteria. Acetohydroxamic acid was found in the serum of three leukemic patients receiving hydroxyurea and may be formed from hydroxylamine resulting from action of urease on hydroxyurea. Excretion of hydroxyurea in humans is a nonlinear process occurring through two pathways. One is saturable, probably hepatic metabolism; the other is first-order renal excretion. In adults, mean cumulative urinary hydroxyurea excretion was 62% of the administered dose at 8-hours.

Toxicity

Ecotoxicity

None

Animal toxicity

Animals and absorption	Toxicity	References
LD_{50} (ipr, mouse)	5800 mg/kg	NTP
LD_{10} (scu, mouse)	2400 mg/kg	
TD_{Lo} (oral, human)	80 mg/kg/d	
TD_{Lo} (iv, human)	86 mg/kg	

LD_{50}: lethal dose 50 percent kill; LD_{10}: lethal dose 10 percent kill; TD_{Lo}: lowest published toxic dose; iv: intravenous; ipr: intraperitoneal; scu: subcutaneous.

Carcinogenicity and mutagenesis

Hydroxyurea is mutagenic and clastogenic, and causes cellular transformation to a tumorigenic phenotype. Hydroxyurea is thus unequivocally genotoxic and a presumed transspecies carcinogen which implies a carcinogenic risk to humans. In patients receiving long-term hydroxyurea whether this leukemogenic effect is secondary to hydroxyurea or is associated for myeloproliferative disorders, such as polycythemia vera and thrombocythemia, secondary leukemias have been reported.

Impairment of Fertility

Hydroxyurea administered to male rats at 60 mg/kg/day (about 0.3 times the maximum recommended human daily dose on a mg/m^2 basis) produced testicular atrophy, decreased spermatogenesis, and significantly reduced their ability to impregnate females.

Adverse effect on human

Treatment of patients with hydroxyurea may be complicated by severe, sometimes life-threatening, adverse effects. Hematologic effects (neutropenia), gastrointestinal symptoms (stomatitis, anorexia, nausea, vomiting, diarrhea, and constipation), and dermatological reactions such as maculopapular rash, skin ulceration, dermatomyositis-like skin changes, peripheral erythema and facial erythema. Hyperpigmentation, atrophy of skin and nails, scaling and violet papules have been observed in some patients after several years of long-term daily maintenance therapy with hydroxyurea. Skin cancer has been reported. Dysuria and alopecia occur very rarely. Large doses may

produce moderate drowsiness. Neurological disturbances have occurred extremely rarely and were limited to headache, dizziness, disorientation, hallucinations, and convulsions. Hydroxyurea occasionally may cause temporary impairment of renal tubular function accompanied by elevations in serum uric acid and creatinine levels. Fever, chills, malaise, edema, asthenia, and elevation of hepatic enzymes have also been reported.

Medicines on sale in Switzerland

Litalir®

A.7 Tamoxifen / Tamoxifen citrate

Generality

Name	(Z)-2-[4-(1,2-diphenyl-1-butenyl) phenoxy]-N,N-dimethylethanamine
CAS RN	10540-29-1/ 54965-24-1
Molecular weight	371.53 ($C_{26}H_{29}NO$) / 563.62 ($C_{32}H_{37}NO_8$)
Melting point	97°C / 140-142°C
Solubility in water (37°C)	0.5 mg/ml (Tamoxifen citrate)
Log Kow	6.3

Tamoxifen citrate is soluble in ethanol, methanol and acetone. Hygroscopic at high relative humidity. Sensitive to UV light.
Distribution: pharmacy (Sold in most of the swiss pharmacies: 2-100 g per pharmacy and per year)

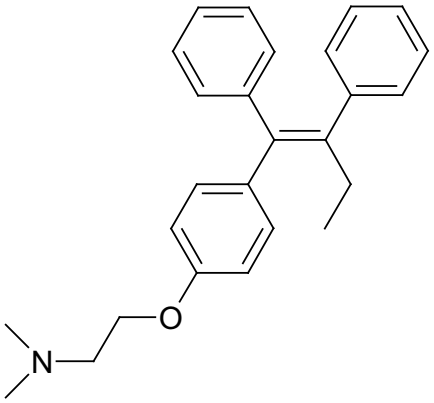

FIGURE A.7: Structure of Tamoxifen

Tamoxifen citrate is effective in the treatment of metastatic breast cancer in women and men. Available evidence indicates that patients whose tumors are estrogen receptor positive are more likely to benefit from tamoxifen citrate therapy.

Tamoxifen citrate is a nonsteroidal agent that has demonstrated potent antiestrogenic properties in animal test systems. The antiestrogenic effects may be related to its ability to compete with estrogen for binding sites in target tissues such as breast. Tamoxifen inhibits the induction of rat mammary

carcinoma induced by dimethylbenzanthracene (DMBA) and causes the regression of already established DMBA-induced tumors. In this rat model, tamoxifen appears to exert its antitumor effects by binding the estrogen receptors.

Biodegradability

To our knowledge, no study has been conducted to evaluate the biodegradability of tamoxifen.

Occurrence in the environment

Authors tried to detect this drug in wastewaters, but most of the samples showed concentrations below the limit of detection, excepted two samples [Ashton *et al.*, 2004].

Detection methods

Biological matrice GC, GC-MS or HPLC method were developed for the determination of tamoxifen and its metabolites in pharmaceuticals, human bile, rat uterine cytoplasm and in breast tumor from tamoxifen-treated patients [Tjaden & De Bruijn, 1990].

Dosage

For patients with breast cancer, the recommended daily dose is 20-40 mg. Dosages greater than 20 mg per day should be given in divided doses (morning and evening).

Metabolism and Elimination

Tamoxifen is extensively metabolized after oral administration. Studies in women receiving 20 mg of 14C tamoxifen have shown that approximately 65% of the administered dose is excreted from the body over a period of 2 weeks with fecal excretion as the primary route of elimination. The drug is excreted mainly as polar conjugates, with unchanged drug and unconjugated metabolites accounting for less than 30% of the total fecal radioactivity. N-desmethyl tamoxifen was the major metabolite found in patients' plasma. The biological activity of N-desmethyl tamoxifen appears to be similar to tamoxifen. 4-Hydroxytamoxifen and a side chain primary alcohol derivative of tamoxifen have been identified as minor metabolites in plasma.

Toxicity

Ecotoxicity

None

Animal toxicity (Tamoxifen citrate)

Animals and absorption	Toxicity	References
LD_{50} (iv, mice)	62.5 mg/kg	Merck index
LD_{50} (oral,mice)	3000-6000 mg/kg	
LD_{50} (iv, rats)	62.5 mg/kg	
LD_{50} (oral, rats)	1200-2500 mg/kg	

LD_{50}: lethal dose 50 percent kill; iv: intravenous

Carcinogenesis

A conventional carcinogenesis study in rats (doses of 5, 20, and 35 mg/kg/day for up to 2 years) revealed hepatocellular carcinoma at all doses, and the incidence of these tumors was significantly greater among rats given 20 or 35 mg/kg/day (69%) than those given 5 mg/kg/day (14%). The incidence of these tumors in rats given 5 mg/kg/day (29.5 mg/m^2) was significantly greater than in controls. As with other additive hormonal therapy (estrogens), an increased incidence of endometrial cancer has been reported in association with tamoxifen citrate treatment. In a large randomized trial in Sweden of adjuvant tamoxifen citrate 40 mg/day for 2 to 5 years, an increased incidence of uterine cancer was noted. Tamoxifen is classified as human carcinogen.

Mutagenesis

Although no genotoxic potential was found in a conventional battery of in vivo and in vitro tests with pro- and eukaryotic test systems with drug metabolizing systems present, increased levels of DNA adducts have been found in the livers of rats exposed to tamoxifen. Tamoxifen also has been found to increase levels of micronucleus formation in vitro in human lymphoblastoid cell line (MCL-5). Based on these findings, tamoxifen is genotoxic in rodent and human MCL-5 cells.

Impairment of Fertility

Fertility in female rats was decreased following administration of 0.04 mg/kg for two weeks prior to mating through day 7 of pregnancy. There was a decreased number of implantations, and all fetuses were found dead.

Adverse effect on human

Adverse reactions to tamoxifen citrate are relatively mild and rarely severe enough to require discontinuation of treatment in breast cancer patients. Increased bone and tumor pain and, also, local disease flare have occurred. The most frequent adverse reaction to tamoxifen citrate is hot flashes. Other adverse reactions which are seen infrequently are hypercalcemia, peripheral edema, distaste for food, pruritus vulvae, depression, dizziness, light-headedness, headache, hair thinning and/or partial hair loss, and vaginal dryness.

Medicines on sale in Switzerland

Nolvadex® - Tamec® - Kessar®

A.8 5-Fluorouracil

Generality

Name	5-fluoro-2,4(1H,3H)-Pyrimidinedione
CAS RN	51-21-8
Molecular weight	130.08 ($C_4H_3FN_2O_2$)
Melting point	283 °C
λ_{max} (absorption)	265-266 nm in NaOH 0.1N
Solubility in water (19°C)	<1 mg/ml
Solubility in methanol (22°C)	1-5 mg/ml
Log Kow	-0.89
pKa	8.02

This chemical may be sensitive to prolonged exposure to light. It undergoes hydrolysis in alkaline solution. Hydrolysis is enhanced by increased pH and temperature. The shelf-life of aqueous solutions is about 3 years at pH 9 and at room temperature.
Distribution: Hospital (3.2 kg in 2001 CHUV) and pharmacy (cream and capecitabin which is metabolized in 5-fluorouracil in the tumor).

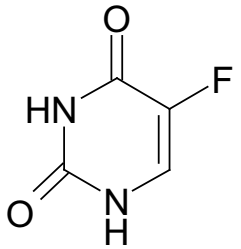

FIGURE A.8: Structure of 5-Fluorouracil

Fluorouracil is effective in the palliative management of carcinoma of the colon, rectum, breast, stomach and pancreas. Fluorouracil is an antineoplastic antimetabolite.

There is evidence that the metabolism of fluorouracil in the anabolic pathway blocks the methylation reaction of deoxyuridylic acid to thymidylic acid. In this manner 5-fluorouracil interferes with the synthesis of DNA and to a lesser extent inhibits the formation of ribonucleic acid (RNA). Since DNA and RNA are essential for cell division and growth, the effect of fluorouracil

may be to create a thymine deficiency which provokes unbalanced growth and death of the cell. The effects of DNA and RNA deprivation are most marked in those cells which grow more rapidly and which take up fluorouracil at a more rapid rate.

Biodegradability

5-fluorouracil was not biodegradable in the closed bottle test (CBT) nor in the Zahn-Wellens test (ZWT) [Kümmerer & Al-Ahmad, 1997]. According to Kiffmeyer *et al.* [1998], 5-Fluorouracil was completely biodegraded within a few days in a laboratory sewage plant. But the rate of biodegradation seems depending on the initial concentration: the higher the initial concentration the slower the degradation.

Occurrence in the environment

To our knowledge, no study has been conducted to detect 5-fluorouracil in sewage treatment plant or in the environment. Nevertheless, in wastewater from a 5-Fluorouracil plant, 900 ppm was measured [Anheden *et al.*, 1996].

Detection methods

Environmental matrice Solid phase extraction (SPE) followed by HPLC-UV was used by Kiffmeyer *et al.* [1998]. 5-Fluorouracil, methotrexate, 7-hydroxymethotrexate ant etoposide can be detected in one chromatographic run with HPLC-UV with a polystyrene-divinylbenzene (PS-DVB) column with buffered superheated water as the mobile phase [Teutenberg *et al.*, 2001].

Dosage and administration

Twelve mg/kg are given intravenously once daily for four successive days. The daily dose should not exceed 800 mg. If no toxicity is observed, 6 mg/kg are given on the 6th, 8th, 10th and 12th days unless toxicity occurs. No therapy is given on the 5th, 7th, 9th or 11th days. Therapy is to be discontinued at the end of the 12th day, even if no toxicity has become apparent. In instances where toxicity has not been a problem, it is recommended that therapy be continued using either of the following schedules: 1. Repeat dosage of first course every 30 days after the last day of the previous course of treatment. 2. When toxic signs resulting from the initial course of therapy have subsided,

administer a maintenance dosage of 10 to 15 mg/kg/week as a single dose. Do not exceed 1 g per week.

Metabolism and Elimination

Seven to twenty percent of the parent drug is excreted unchanged in the urine in six hours; of this over 90% is excreted in the first hour. The remaining percentage of the administered dose is metabolized, primarily in the liver. The catabolic metabolism of 5-fluorouracil results in degradation products (e.g., CO2 urea and -fluoro- -alanine) which are inactive. The inactive metabolites are excreted in the urine over the next 3 to 4 hours. No intact drug can be detected in the plasma three hours after an intravenous injection.

Toxicity

Ecotoxicity

None

Animal and human toxicity

Animals and absorption	Toxicity	References
LD_{50} (oral, rat)	230 mg/kg	NTP
LD_{50} (oral, mouse)	115 mg/kg	
TD_{Lo} (oral, human)	450 mg/kg/30d	
TD_{Lo} (iv, human)	6 mg/kg/3d	
TD_{Lo} (iv, man)	39 mg/kg/1d-I	

LD_{50}: lethal dose 50 percent kill; TD_{Lo}: lowest published toxic dose; I: intermittent; iv: intravenous.

Carcinogenesis

Longterm studies in animals to evaluate the carcinogenic potential of 5-fluorouracil have not been conducted. However, there was no evidence of carcinogenicity in small groups of rats given 5-fluorouracil orally at doses of 0.01, 0.3, 1 or 3 mg per rat 5 days per week for 52 weeks, followed by a six-month observation period. Also, in other studies, 33 mg/kg of 5-fluorouracil was administered intravenously to male rats once a week for 52 weeks followed by observation for the remainder of their lifetimes with no evidence of carcinogenicity. Female mice were given 1 mg of 5-fluorouracil intravenously

once a week for 16 weeks with no effect on the incidence of lung adenomas. On the basis of the available data, no evaluation can be made of the carcinogenic risk of 5-fluorouracil to humans.

Mutagenesis

Oncogenic transformation of fibroblasts from mouse embryo has been induced in vitro by 5-fluorouracil, but the relationship between oncogenicity and mutagenicity is not clear. 5-Fluorouracil has been shown to be mutagenic to several strains of Salmonella typhimurium, including TA1535, TA1537 and TA1538, and to *Saccharomyces cerevisiae*, although no evidence of mutagenicity was found with *Salmonella typhimurium* strains TA92, TA98 and TA100. In addition, a positive effect was observed in the micronucleus test on bone marrow cells of the mouse, and fluorouracil at very high concentrations produced chromosomal breaks in hamster fibroblasts in vitro.

Impairment of Fertility

5-Fluorouracil has not been adequately studied in animals to permit an evaluation of its effects on fertility and general reproductive performance. However, doses of 125 or 250 mg/kg administered intraperitoneally, have been shown to induce chromosomal aberrations and changes in chromosomal organization of spermatogonia in rats. Spermatogonial differentiation was also inhibited by 5-fluorouracil, resulting in transient infertility. However, in studies with a strain of mouse which is sensitive to the induction of sperm head abnormalities after exposure to a range of chemical mutagens and carcinogens, 5-fluorouracil did not produce any abnormalities at oral doses of up to 80 mg/kg/day. In female rats, 5-fluorouracil, administered intraperitoneally at weekly doses of 25 or 50 mg/kg for three weeks during the pre-ovulatory phase of oogenesis, significantly reduced the incidence of fertile matings, delayed the development of pre- and postimplantation embryos, increased the incidence of preimplantation lethality and induced chromosomal anomalies in these embryos. In a limited study in rabbits, a single 25 mg/kg dose of 5-fluorouracil or 5 daily doses of 5 mg/kg had no effect on ovulation, appeared not to affect implantation and had only a limited effect in producing zygote destruction. Compounds such as 5-fluorouracil, which interfere with DNA, RNA and protein synthesis, might be expected to have adverse effects on gametogenesis.

Adverse effect on human

Stomatitis and esophagopharyngitis (which may lead to sloughing and ulceration), diarrhea, anorexia, nausea and emesis are commonly seen during therapy. Leukopenia usually follows every course of adequate therapy with 5-fluorouracil. The lowest white blood cell counts are commonly observed between the 9th and 14th days after the first course of treatment, although uncommonly the maximal depression may be delayed for as long as 20 days. By the 30th day the count has usually returned to the normal range. Alopecia and dermatitis may be seen in a substantial number of cases. The dermatitis most often seen is a pruritic maculopapular rash usually appearing on the extremities and less frequently on the trunk. It is generally reversible and usually responsive to symptomatic treatment.

Medicines on sale in Switzerland

Efudix®, Fluoro-uracil ICN

A.9 Ifosfamide

Generality

Name	N,3-bis(2-chloroethyl)tetrahydro-2H,1,3,2-oxazaphospharin-2-amine,2-oxide (9CI)
CAS RN	3778-73-2
Molecular weight	261.09 ($C_7H_{15}Cl_2N_2O_2P$)
Melting point	39-41°C
Solubility in water (25°C)	3780 mg/l (estimation)
Log Kow	0.86
Distribution	Hospital (1.3 kg in 2001 CHUV)

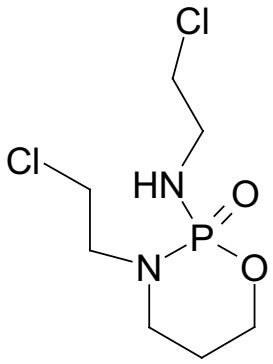

FIGURE A.9: Structure of Ifosfamide

Ifosfamide used in combination with certain other approved antineoplastic agents, is indicated for germ cell testicular, ovarian, bronchial, breast, liver cancer.

Ifosfamide is an alkylating agent. Ifosfamide has been shown to require metabolic activation by microsomal liver enzymes to produce biologically active metabolites. The alkylated metabolites of ifosfamide have been shown to interact with DNA.

Biodegradability

Ifosfamide was not biodegradable in the Zahn-Wellens test and the sewage treatment plant simulation test [Kümmerer *et al.*, 1997]. Ifosfamide was not biodegraded at a concentration of 5 mg/l in the Closed Bottle Test within 28

days ("not readily biodegradable") [Kümmerer *et al.*, 1996]. Ifosfamide was not adsorbed into the sewage sludge [Kümmerer *et al.*, 1997].

Occurrence in the environment

Ifosfamide was detected in hospital effluents and in communal sewage treatment plant up to 2.9 μg/l [Kümmerer *et al.*, 1997; Ternes, 1998].

Detection methods

Environmental matrice Solid phase extraction (SPE) followed by HPLC-MS-MS or GC-MS were used by several studies [Kümmerer *et al.*, 1997; Sacher *et al.*, 2001; Steger-Hartmann *et al.*, 1996; Ternes, 1998]. These study show recovery rates which are very low (<51%), excepted for groundwater.

Dosage

Ifosfamide should be administered intravenously at a dose of 1.2 g/m^2 per day for 5 consecutive days. Treatment is repeated every 3 weeks or after recovery from hematologic toxicity (Platelets = 100 000/μl, WBC = 4 000/μl).

Metabolism and Elimination

Ifosfamide has been shown to require metabolic activation by microsomal liver enzymes to produce biologically active metabolites. Activation occurs by hydroxylation at the ring carbon atom 4 to form the unstable intermediate 4-hydroxyifosfamide. This metabolite rapidly degrades to the stable urinary metabolite 4-ketoifosfamide. Opening of the ring results in formation of the stable urinary metabolite, 4-carboxyifosfamide. These urinary metabolites have not been found to be cytotoxic. N, N- bis (2-chloroethyl)-phosphoric acid diamide (ifosphoramide) and acrolein are also found. Enzymatic oxidation of the chloroethyl side chains and subsequent dealkylation produces the major urinary metabolites, dechloroethyl ifosfamide and dechloroethyl cyclophosphamide.

After administration of doses of 5 g/m^2 of 14 C-labeled ifosfamide, from 70% to 86% of the dosed radioactivity was recovered in the urine, with about 61% of the dose excreted as parent compound. At doses of 1.6-2.4 g/m^2 only 12% to 18% of the dose was excreted in the urine as unchanged

drug within 72 hours. Two different dechloroethylated derivatives of ifosfamide, 4-carboxyifosfamide, thiodiacetic acid and cysteine conjugates of chloroacetic acid have been identified as the major urinary metabolites of ifosfamide in humans and only small amounts of 4-hydroxyifosfamide and acrolein are present. Small quantities (nmole/ml) of ifosfamide mustard and 4-hydroxyifosfamide are detectable in human plasma. Metabolism of ifosfamide is required for the generation of the biologically active species and while metabolism is extensive, it is also quite variable among patients.

Toxicity

Ecotoxicity

None

Animal toxicity

Animals and absorption	Toxicity	References
LD_{50} (ip, rats)	150-160 mg/kg	Merck index
LD_{50} (oral, rats)	143 mg/kg	HSDB
LD_{50} (iv, rats)	190 mg/kg	
LD_{50} (oral, mouse)	1005 mg/kg	
LD_{50} (ip, mouse)	397 mg/kg	
LD_{50} (iv, mouse)	338 mg/kg	

LD_{50}: lethal dose 50 percent kill; ip: intraperitoneal; iv: intravenous;

Carcinogenesis

Ifosfamide has been shown to be carcinogenic in rats, with female rats showing a significant incidence of leiomyosarcomas and mammary fibroadenomas.

Mutagenesis

The mutagenic potential of ifosfamide has been documented in bacterial systems in vitro and mammalian cells in vivo. In vivo, ifosfamide has induced mutagenic effects in mice and Drosophila melanogaster germ cells, and has induced a significant increase in dominant lethal mutations in male mice as well as recessive sex-linked lethal mutations in Drosophila.

Impairment of Fertility

In pregnant mice, resorptions increased and anomalies were present at day 19 after a 30 mg/m^2 dose of ifosfamide was administered on day 11 of gestation. Embryolethal effects were observed in rats following the administration of 54 mg/m^2 doses of ifosfamide from the 6th through the 15th day of gestation and embryotoxic effects were apparent after dams received 18 mg/m^2 doses over the same dosing period. Ifosfamide is embryotoxic to rabbits receiving 88 mg/m^2/day doses from the 6th through the 18th day after mating. The number of anomalies was also significantly increased over the control group.

Adverse effect on human

In patients receiving ifosfamide as a single agent, the dose-limiting toxicities are myelosuppression and urotoxicity. Dose fractionation, vigorous hydration, and a protector such as mesna can significantly reduce the incidence of hematuria, especially gross hematuria, associated with hemorrhagic cystitis. At a dose of 1.2 g/m^2 daily for 5 consecutive days, leukopenia, when it occurs, is usually mild to moderate. Other significant side effects include alopecia, nausea, vomiting, and central nervous system toxicities.

Medicines on sale in Switzerland

Holoxan®

A.10 Cyclophosphamide

Generality

Name	1-(bis(2-chloroethyl)amino)-1-oxo-2-aza-5-oxaphosphoridine
CAS RN	50-18-0
Molecular weight	261.09 ($C_7H_{15}Cl_2N_2O_2P$)
Melting point	41-51°C
Solubility in water (23°C)	10-50 mg/ml
Solubility in acetone (23°C)	50-100 mg/ml
Log Kow	0.63
Distribution	Hospital (0.6 kg in 2001 CHUV) and pharmacy

This compound is sensitive to exposure to light. Hydrolysis occurs at temperatures above 30°C. Solution of this chemical in DMSO, 95% ethanol or acetone should be stable for 24 hours under lab conditions.

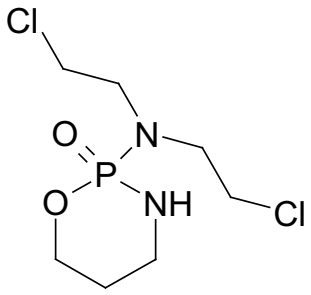

Figure A.10: Structure of Cyclophosphamide

Cyclophosphamide, although effective alone in susceptible malignancies, is more frequently used concurrently or sequentially with other antineoplastic drugs. The following malignancies are often susceptible to cyclophosphamide treatment: 1. Malignant lymphomas, Hodgkin's disease, lymphocytic lymphoma, mixed-cell type lymphoma, histiocytic lymphoma, Burkitt's lymphoma. 2. Multiple myeloma. 3. Leukemias. 4. Mycosis fungoides (advanced disease). 5. Neuroblastoma (disseminated disease). 6. Adenocarcinoma of the ovary. 7. Retinoblastoma. 8. Carcinoma of the breast.

Cyclophosphamide is an alkylating agents. Cyclophosphamide is biotransformed principally in the liver to active alkylating metabolites by a mined

function microsomal oxidase system. These metabolites interfere with the growth of susceptible rapidly proliferating malignant cells. The mechanism of action is thought to involve cross-linking of tumor cell DNA.

Biodegradability

Cyclophosphamide exhibited only poor degradability in the Zahn-Wellens test and the sewage treatment plant simulation test [Steger-Hartmann *et al.*, 1997]. Cyclophosphamide was not biodegraded at a concentration of 5 mg/l in the Closed Bottle Test within 28 days ("not readily biodegradable") [Kümmerer *et al.*, 1996].

Occurrence in the environment

Cyclophosphamide was detected in hospital effluents and in communal sewage treatment plant up to 4.5 μg/l [Steger-Hartmann *et al.*, 1996, 1997; Ternes, 1998].

Detection methods

Environmental matrice Solid phase extraction (SPE) followed by HPLC-MS-MS or GC-MS were used by several studies [Sacher *et al.*, 2001; Steger-Hartmann *et al.*, 1996, 1997; Ternes, 1998]. These studies show very low recovery rates (<60%), excepted for groundwater.

Dosage

When used as the only oncolytic drug therapy, the initial course of cyclophosphamide for patients with no hematologic deficiency usually consists of 40 to 50 mg/kg given intravenously in divided doses over a period of 2 to 5 days. Other intravenous regimens include 10 to 15 mg/kg given every 7 to 10 days or 3 to 5 mg/kg twice weekly. Oral cyclophosphamide dosing is usually in the range of 1 to 5 mg/kg/day for both initial and maintenance dosing. Many other regimens of intravenous and oral cyclophosphamide have been reported. Dosages must be adjusted in accord with evidence of anti-tumor activity and/or leukopenia.

Metabolism and Elimination

Cyclophosphamide is well absorbed after oral administration with a bioavailability greater than 75%. The unchanged drug has an elimination half-life of

3 to 12 hours. It is eliminated primarily in the form of metabolites, but from 5 to 25% of the dose is excreted in urine as unchanged drug. Several cytotoxic and noncytotoxic metabolites have been identified in urine and in plasma. Concentrations of metabolites reach a maximum in plasma 2 to 3 hours after an intravenous dose. Plasma protein binding of unchanged drug is low but some metabolites are bound to an extent greater than 60%. It has not been demonstrated that any single metabolite is responsible for either the therapeutic or toxic effects of cyclophosphamide. Although elevated levels of metabolites of cyclophosphamide have been observed in patients with renal failure, increased clinical toxicity in such patients has not been demonstrated.

Toxicity

Ecotoxicity

None

Animal or human toxicity

Animals and absorption	Toxicity	References
LD_{50} (oral, rat)	350 mg/kg	Merck index
LD_{50} (oral, mouse)	137 mg/kg	NTP
TD_{Lo} (oral, woman)	45 mg/kg	
TD_{Lo} (oral, human)	20 mg/kg	
LD_{Lo} (oral, woman)	16 mg/kg/4d-I	
TD_{Lo} (oral, man)	56 mg/kg/26d-I	
TD_{Lo} (oral, man)	56 mg/kg/4w-I	

LD_{50}: lethal dose 50 percent kill; TD_{Lo}: lowest published toxic dose; LD_{Lo}: lowest published lethal dose; I: intermittent.

Carcinogenesis

Second malignancies have developed in some patients treated with cyclophosphamide used alone or in association with other antineoplastic drugs and/or modalities. Most frequently, they have been urinary bladder, myeloproliferative, or lymphoproliferative malignancies. Second malignancies most frequently were detected in patients treated for primary myeloproliferative or lymphoproliferative malignancies or nonmalignant disease in which immune processes are believed to be involved pathologically. There is sufficient animal

and human evidence for this compound. Cyclophosphamide is classified as human caricnogen.

Impairment of Fertility

Cyclophosphamide can cause fetal harm when administered to a pregnant woman and such abnormalities have been reported following cyclophosphamide therapy in pregnant women. Cyclophosphamide interferes with oogenesis and spermatogenesis. It may cause sterility in both sexes.

Adverse effect on human

The adverse reactions are listed in order of decreasing incidence

- Reproductive System: impairment of fertility.
- Digestive System: nausea and vomiting commonly occur. Anorexia and, less frequently, abdominal discomfort or pain and diarrhea may occur.
- Skin and its Structures: alopecia. Skin rash occurs occasionally in patients receiving the drug.
- Hematopoietic System: leukopenia. Fever without documented infection has been reported in neutropenic patients. Thrombocytopenia or anemia.
- Urinary System: cystitis and urinary bladder fibrosis. Hemorrhagic ureteritis and renal tubular necrosis.
- Infections: reduced host resistance to infections.

Medicines on sale in Switzerland

Endoxan-Asta®

A.11 Carmustine

Generality

Name	Bis(chloroethyl)nitrosourea
CAS RN	154-93-8
Molecular weight	214.05 ($C_5H_9Cl_2N_3O_2$)
Melting point	31°C
Solubility in water (18°C)	<1 mg/ml
Solubility in 95% ethanol, acetone (18°C)	$\geqslant$ 100 mg/ml
Log Kow	1.53
Distribution	Hospital (15 g in 2001 CHUV)

This compound is stable under normal laboratory conditions. This compound decomposes rapidly in acid and in solutions above pH7; most stable in petroleum ether or aqueous solution at pH4.

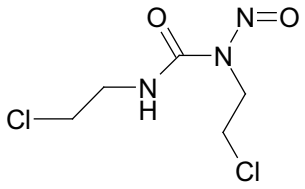

FIGURE A.11: Structure of Carmustine

Carmustine belongs to the group of medicines known as alkylating agents. It is used to treat cancer of the lymph system, cancerous brain tumors, and a certain type of cancer in the bone marrow.

Although it is generally agreed that carmustine alkylates DNA and RNA, it is not cross resistant with other alkylators. As with other nitrosoureas, it may also inhibit several key enzymatic processes by carbamoylation of amino acids in proteins.

Biodegradability

To our knowledge, no study has been conducted to evaluate the biodegradability of carmustine.

Occurrence in the environment

To our knowledge, no study has been conducted to detect carmustine in sewage treatment plant or in the environment.

Detection methods

Environmental matrice Solid phase extraction (SPE) followed by HPLC-UV is used by Kiffmeyer *et al.* [1998].

Dosage

The recommended dose of carmustine as a single agent in previously untreated patients is 150 to 200 mg/m^2 intravenously every 6 weeks.

Metabolism and Elimination

It is thought that the antineoplastic and toxic activities of carmustine may be due to metabolites. Approximately 60% to 70% of a total dose is excreted in the urine in 96 hours and about 10% as respiratory CO_2. The fate of the remainder is undetermined. Because of the high lipid solubility and the relative lack of ionization at physiological pH, carmustine crosses the blood-brain barrier quite effectively.

Toxicity

Ecotoxicity

None

Animal and human toxicity

Animals and absorption	Toxicity	References
LD_{50} (oral, mouse)	19 mg/kg	NTP
LD_{50} (oral, rat)	20 mg/kg	
TD_{Lo} (iv, human)	125 mg/kg	
TD_{Lo} (iv, human)	6 mg/kg	

LD_{50}: lethal dose 50 percent kill; TD_{Lo}: lowest published toxic dose; iv: intravenous.

Carcinogenesis, Mutagenesis, Impairment of Fertility

Carmustine is carcinogenic in rats and mice, producing a marked increase in tumor incidence in doses approximating those employed clinically. Nitrosourea therapy does have carcinogenic potential in humans. There is sufficient animal evidence, but human limited evidence of carcinogenicity. Carmustine is classified as probable human carcinogen (IARC: 2A).

Impairment of Fertility

Carmustine also affects fertility in male rats at doses somewhat higher than the human dose.

Adverse effect on human

Pulmonary Toxicity (characterized by pulmonary infiltrates and/or fibrosis). Hematologic toxicity (myelosuppression, thrombocytopenia, leukopenia, acute leukemia and bone marrow dysplasias, anemia), gastrointestinal toxicity (nausea and vomiting), nephrotoxicity (renal abnormalities consisting of progressive azotemia, decrease in kidney size and renal failure). Accidental contact of reconstituted carmustine with skin has caused burning and hyperpigmentation of the affected areas. Neuroretinitis, chest pain, headache, allergic reaction, hypotension and tachycardia have been reported as part of ongoing surveillance.

Medicines on sale in Switzerland

BiCNU®

A.12 Cisplatin

Generality

Name	2'-Deoxycytidine diphosphate
CAS RN	15663-27-1
Molecular weight	300.06 ($H_6Cl_2N_2Pt$)
Melting point	270°C
Solubility in water, 95% ethanol or acetone (19°C)	<1 mg/ml
Log Kow	-2.19
Distribution	Hospital (57 g in 2001 CHUV)

This compound is stable under normal laboratory conditions.

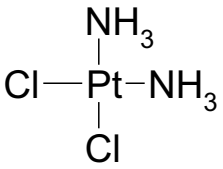

FIGURE A.12: Structure of Cisplatin

Cisplatin is widely prescribed for a variety of tumors (germ-cell, advanced bladder carcinoma, adrenal cortex carcinoma, breast cancer, head and neck carcinoma, lung carcinoma).

Cisplatin is believed to kill cancer cells by binding to DNA and interfering with its repair mechanism, eventually leading to cell death.

Biodegradability

Cisplatin is not biodegraded in a laboratory sewage plant [Kiffmeyer *et al.*, 1998].

Occurrence in the environment

The average daily concentrations in the hospital effluents were apporximately <10-601 ng/l Pt [Kümmerer *et al.*, 1999].

Detection methods

Environmental matrice Platin was detected with HPLC-ICP-MS [Kümmerer *et al.*, 1999]. Solid phase extraction (SPE) followed by HPLC-UV was used by Kiffmeyer *et al.* [1998].

Dosage and administration

Cisplatin is administered intravenously for one (50-120 mg/m^2) to 5 days (15-20 mg/m^2) in a row, followed by a rest period of 2-4 weeks.

Metabolism and Elimination

The metabolic fate of cisplatin has not been completely elucidated. There is no evidence to date that the drug undergoes enzymatic biotransformation; the chloride ligands of the cisplatin complex are believed to be displaced by water, forming positively charged platinum complexes that react with nucleophilic sites. The chemical identities of platinum-containing products of the drug that are formed in vivo have not been definitely determined. Intact cisplatin and its platinum-containing product(s)are excreted principally in urine (27-43% during 5 first days[1]); fecal elimination of platinum appears to be insignificant.

Toxicity

Ecotoxicity

None

[1] compendium

Animal toxicity

Animals and absorption	Toxicity	References
LD_{50} (oral, mouse)	32.7 mg/kg	NTP
LD_{50} (iv, mouse)	11 mg/kg	
LD_{50} (oral, rat)	25.8 mg/kg	
LD_{50} (iv, rat)	8 mg/kg	
TD_{Lo} (iv, human)	1.5 mg/kg/6d-I	
TD_{Lo} (iv, human)	0.5 mg/kg/13d-I	
TD_{Lo} (iv, human)	2.5 mg/kg	
TD_{Lo} (iv, human)	72 mg/kg/25d-I	

LD_{50}: lethal dose 50 percent kill; I: intermittent; iv: intravenous; TD_{Lo}: lowest published toxic dose.

Carcinogenesis

There is sufficient animal evidence, but human inadequate evidence. Cisplatin is classified as probable human carcinogen.

Adverse effect on human

There are serious side effects associated with cisplatin, notably renal toxicity, emesis, neurotoxicity, bone marrow suppression and hearing loss.

Medicines on sale in Switzerland

Platiblastin® - Platinol®

A.13 Doxorubicin / Adriamycin

Generality

Name	10-((3-Amino-2,3,6-trideoxy-alpha-L-lyso-hexopyranosyl)oxy) -7,8,9,10-tetrahydro-6,8,11-trihydroxy- 8-(hydroxyacetyl)-1-methoxy-5,12-naphthacenedione Adriamycin : hydrochlorid form ($C_{27}H_{30}ClNO_{11}$)
CAS RN	23214-92-8
Molecular weight	543.53 ($C_{27}H_{29}NO_{11}$)
Melting point	229-231°C
Solubility in water (25°C)	92.8 mg/l (estimation)
Log Kow	1.27
Distribution	Hospital (15 g in 2001 CHUV)

Adriamycin is pratically insoluble in acetone, benzene and ethyl ether. Aqueous solution unchanged after one month at 5°C, but unstable at higher temperature or at either acid or alkaline pHs.

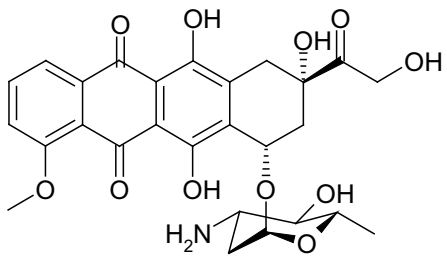

Figure A.13: Structure of Doxorubicin

Doxorubicin HCl is used for various tumor (breast cancer, pulmonary cancer, sarcomas of bones, genital cancers, cancer of the bladder, cancer of testicles, etc.).

Doxorubicin is a cytotoxic anthracycline antibiotic isolated from *Streptomyces peucetius* (var. *caesius*). The mechanism of action of doxorubicin HCl is thought to be related to its ability to bind DNA and inhibit nucleic acid synthesis. Cell structure studies have demonstrated rapid cell penetration and perinuclear chromatin binding, rapid inhibition of mitotic activity and nucleic acid synthesis, and induction of mutagenesis and chromosomal aberrations.

Biodegradability

To our knowledge, no study has been conducted to evaluate the biodegradability of doxorubicin.

Occurrence in the environment

To our knowledge, no study has been conducted to detect doxorubicin in sewage treatment plant or in the environment.

Detection methods

Biological matrice HPLC is generally accepted for anthracycline pharmacokinetic analysis, since it permits sufficient selectivity and sensitivity for the parent coumpounds and their metabolites. Derivatisation was required for GC analysis [Tjaden & De Bruijn, 1990].

Dosage

For ovarian cancer patients, doxorubicin HCl should be administered intravenously at a dose of 50 mg/m^2 (swiss compenium: 75 mg/m^2 every three week).

Metabolism and Elimination

Doxorubicine is quickly (in one hour) metabolised in the liver in its main active metabolite, the doxorubicinol. The biliary excretion represents the main way of elimination: 40-50% of the administered dose are eliminated in 7 days by biliary way. The renal excretion is unimportant: about 4-5% of the dose injected by way i.v. are eliminated by kidneys in 5 days.

Toxicity

Ecotoxicity

None

Animal toxicity

Animals and absorption	Toxicity	References
LD_{50} (iv, mouse)	21.1 mg/kg	Merk index - Adriamycine
LD_{50} (iv, rat)	12.6 mg/kg	HSDB
LD_{50} (iv, mouse)	9.4 mg/kg	
LD_{50} (iv, rabbit)	6 mg/kg	

LD_{50}: lethal dose 50 percent kill; iv: intravenous.

Carcinogenesis

There is sufficient animal evidence, but human inadequate evidence. Carmustine is classified as probable human carcinogen (IARC: 2A).

Mutagenesis

Although no studies have been conducted with doxorubicin HCl, but related compounds have been shown to have mutagenic and carcinogenic properties when tested in experimental models.

Impairment of Fertility

The possible adverse effects on fertility in males and females in humans or experimental animals have not been adequately evaluated. However, Doxorubicin resulted in mild to moderate ovarian and testicular atrophy in mice after a single dose of 36 mg/kg (about twice the 50 mg/m^2 human dose on a mg/m^2 basis). Decreased testicular weights and hypospermia were present in rats after repeat doses 0.25 mg/kg/day (about one thirtieth the 50 mg/m^2 human dose on a mg/m^2 basis), and diffuse degeneration of the seminiferous tubules and a marked decrease in spermatogenesis were observed in dogs after repeat doses of 1 mg/kg/day (about one half the 50 mg/m^2 human dose on a mg/m^2 basis).

Adverse effect on human

Irreversible myocardial toxicity leading to congestive heart failure often unresponsive to cardiac supportive therapy may be encountered as the total dosage of doxorubicin HCl approaches 550 mg/m^2. Acute infusion-associated reactions (flushing, shortness of breath, facial swelling, headache, chills, back

pain, tightness in the chest or throat, and/or hypotension) have occurred in about 5% to 10% of patients. Severe myelosuppression may occur.

Medicines on sale in Switzerland

Adriblastin® - Caelyx®

A.14 Melphalan

Generality

Name	4-[bis(2-chloroethyl)amino]- L- Phenylalanine
CAS RN	148-82-3
Molecular weight	305.21 ($C_{13}H_{18}Cl_2N_2O_2$)
Melting point	177-182.5 °C
Solubility in water, 95% ethanol, acetone (22°C)	<1 mg/ml (estimation)
Log Kow	-0.52
Distribution	Hospital (13 g in 2001 CHUV) and pharmacy

This chemical is sensitive to light. It decomposes on storage. It also hydrolyzes in water. Stability increases in acidic solutions and higher temperatures accelerate the hydrolysis. Solutions in methanol were found to retain full integrity for >12 hours at room temperature and for at least 4 weeks at 20°C.

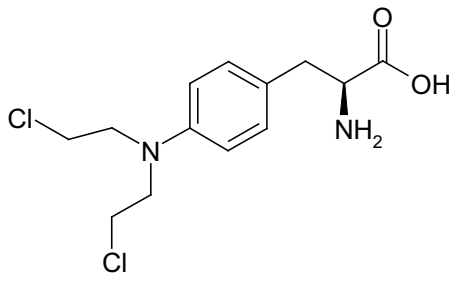

FIGURE A.14: Structure of Melphalan

Melphalan tablets are indicated for the palliative treatment of multiple myeloma and for the palliation of non-resectable epithelial carcinoma of the ovary.

Melphalan is an alkylating agent of the bischloroethylamine type. As a result, its cytotoxicity appears to be related to the extent of its interstrand cross-linking with DNA, probably by binding at the N 7 position of guanine. Like other bifunctional alkylating agents, it is active against both resting and rapidly dividing tumor cells.

Biodegradability

To our knowledge, no study has been conducted to evaluate the biodegradability of melphalan.

Occurrence in the environment

To our knowledge, no study has been conducted to detect melphalan in sewage treatment plant or in the environment.

Detection methods

Environmental matrice Solid phase extraction (SPE) followed by HPLC-fluorescence was used by Kiffmeyer *et al.* [1998].

Dosage

For multiple myeloma, the usual oral dose is 6 mg daily. The entire daily dose may be given at one time. One commonly employed regimen for the treatment of ovarian carcinoma has been to administer melphalan at a dose of 0.2 mg/kg daily for 5 days as a single course. Courses are repeated every 4 to 5 weeks depending upon hematologic tolerance.

Metabolism and Elimination

Melphalan is eliminated from plasma primarily by chemical hydrolysis to monohydroxymelphalan and dihydroxymelphalan. Aside from these hydrolysis products no other melphalan metabolites have been observed in humans. The 24-hour urinary excretion of parent drug was 10% ± 4.5%, suggesting that renal clearance is not a major route of elimination of parent drug. One study using universally labeled 14C melphalan, found substantially less radioactivity in the urine of patients given the drug by mouth (30% of administered dose in 9 days) than in the urine of those given it intravenously (35% to 65% in 7 days).

Toxicity

Ecotoxicity

None

Animal toxicity

Animals and absorption	Toxicity	References
LD_{50} (iv, mouse)	23 mg/kg	NTP
LD_{50} (oral, rat)	11.2 mg/kg	
LD_{50} (iv, rat)	4.1 mg/kg	
TD_{Lo} (oral, human)	1.2 mg/kg/5d-I	
TD_{Lo} (oral, human)	700 mg/kg/7d	
TD_{Lo} (iv, man)	8140 mg/kg	

LD_{50}: lethal dose 50 percent kill; TD_{Lo}: lowest published toxic dose; I: intermittent; iv: intravenous.

Carcinogenesis

Secondary malignancies, including acute nonlymphocytic leukemia, myeloproliferative syndrome, and carcinoma have been reported in patients with cancer treated with alkylating agents (including melphalan). Adequate and well controlled carcinogenicity studies have not been conducted in animals. However, intraperitoneal administration of melphalan in rats (5.4 to 10.8 mg/m^2) and in mice (2.25 to 4.5 mg/m^2) three times per week for 6 months followed by 12 months post dose observation produced peritoneal sarcoma and lung tumors respectively.

There is sufficient animal and human evidence of carcinogenicity. Melphalan is classified as human carcinogen (IARC: 1).

Mutagenesis

Melphalan has been shown to cause chromatid or chromosome damage in humans. Intramuscular administration of melphalan at 6 and 60 mg/m^2 produced structural aberrations of the chromatid and chromosomes in bone marrow cells of Wistar rats.

Impairment of Fertility

Melphalan causes suppression of ovarian function in premenopausal women, resulting in amenorrhea in a significant number of patients. Reversible and irreversible testicular suppression have also been reported.

Adverse effect on human

Hematologic (the most common side effect is bone marrow suppression). Gastrointestinal disturbances such as nausea and vomiting, diarrhea, and oral ulceration occur infrequently. Hepatic toxicity has been reported rarely. Other reported adverse reactions include: pulmonary fibrosis and interstitial pneumonitis, skin hypersensitivity, vasculitis, alopecia, and hemolytic anemia.

Medicines on sale in Switzerland

Alkeran®

A.15 Methotrexate

Generality

Name	(+)-4-Amino-10-methylfolic acid
CAS RN	59-05-2
Molecular weight	454.45 ($C_{20}H_{22}N_8O_5$)
Melting point	185-204°C
λ_{max} (absorption)	244 and 307 nm in NaOH 0.1N
Solubility in water, 95% ethanol, methanol, acetone or toluene (19°C)	<1 mg/ml
Log Kow	-1.85
pKa	4.7
Distribution	Hospital (0.6 kg in 2001 CHUV) and pharmacy

This compound is sensitive to hydrolysis, oxidation and light. Solution of this chemical in DMSO, 95% ethanol or acetone should be stable for 24hours at pH 7.

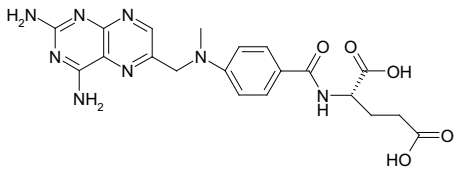

Figure A.15: Structure of Methotrexate

Methotrexate (formerly Amethopterin) is an antimetabolite used in the treatment of certain neoplastic diseases (gestational carcinoma, leukemia, breast cancer, epidermoid cancers of the head and neck, lung cancer), severe psoriasis, and adult rheumatoid arthritis.

Methotrexate inhibits dihydrofolic acid reductase. Dihydrofolates must be reduced to tetrahydrofolates by this enzyme before they can be utilised as carriers of one-carbon groups in the synthesis of nucleotides and thymidylate. Therefore, methotrexate interferes with DNA synthesis, repair, and cellular replication. Actively proliferating tissues such as malignant cells, bone marrow, fetal cells, buccal and intestinal mucosa, and cells of the urinary bladder are in general more sensitive to this effect of methotrexate. When cellular proliferation in malignant tissues is greater than in most normal tissues,

methotrexate may impair malignant growth without irreversible damage to normal tissues.

Biodegradability

The biodegradation of methotrexate increase continuously during the first week and reached a constant value of 95% after about eight days. Little dependence on the initial concentration was observed. On the second day of the biodegradation experiments a metabolite of methotrexate appeared: 7-hydroxymethotrexate. The concentration of this degradation product increases in the same rate as the methotrexate concentration decreases, indicating that the 7-hydroxymethotrexate does not undergo further biodegradation. [Kiffmeyer *et al.*, 1998]

Occurrence in the environment

A concentration of 1 μg/l of methotrexate was found in a sample of hospital effluent [Aherne *et al.*, 1985].

Detection methods

Environmental matrice Radioimmunoassay with or without concentration by lyophilization [Aherne *et al.*, 1985].

Laboratory surface contamination Solid phase exctraction (SPE) followed by HPLC-UV was used by Floridia *et al.* [1999].

Dosage

Methotrexate is administered orally or intramuscularly in doses of 15 to 30 mg daily for a five-day course. Such courses are usually repeated for 3 to 5 times as required, with rest periods of one or more weeks interposed between courses, until any manifesting toxic symptoms subside.

Metabolism and Elimination

After absorption, methotrexate undergoes hepatic and intracellular metabolism to polyglutamated forms which can be converted back to methotrexate by hydrolase enzymes. These polyglutamates act as inhibitors of dihydrofolate reductase and thymidylate synthetase. Small amounts of methotrexate polyglutamates may remain in tissues for extended periods. The retention and prolonged drug action of these active metabolites vary among different cells,

tissues and tumors. A small amount of metabolism to 7-hydroxymethotrexate may occur at doses commonly prescribed. Accumulation of this metabolite may become significant at the high doses used in osteogenic sarcoma. The aqueous solubility of 7-hydroxymethotrexate is 3 to 5 fold lower than the parent compound. Methotrexate is partially metabolized by intestinal flora after oral administration. Renal excretion is the primary route of elimination and is dependent upon dosage and route of administration. With intravenous administration, 80% to 90% of the administered dose is excreted unchanged in the urine within 24 hours. There is limited biliary excretion amounting to 10% or less of the administered dose.

Toxicity

Ecotoxicity

Organisms	Toxicity	References
Daphnia magna (Invert.)	$EC_{50} \geqslant 1000$ mg/l	[Webb, 2001a]
Brachydanio reria (Fish embryos)	48h $EC_{50} = 85$ mg/l	
Scenedesmus subspicatus (Algae)	72h $EC_{50} = 260$ mg/l	

EC_{50}: median Effect Concentration or 50% effective concentration

Animal toxicity

Animals and absorption	Toxicity	References
LD_{50} (iv, rat)	14 mg/kg	(Merck index)
LD_{50} (oral, mouse)	146 mg/kg	NTP
LD_{50} (iv, mouse)	50 mg/kg	
TD_{Lo} (oral, child)	2 mg/kg/12d	
TD_{Lo} (iv, child)	100 mg/kg/4h	
TD_{Lo} (oral, human)	43 mg/kg/5y	
TD_{Lo} (iv, human)	4.6 mg/kg/4w-I	
TD_{Lo} (im, human)	200 mg/kg/5y	

LD_{50}: lethal dose 50 percent kill; TD_{Lo}: lowest published toxic dose; I: intermittent; iv: intravenous; im: intramuscular.

Carcinogenesis and mutagenesis

No controlled human data exist regarding the risk of neoplasia with methotrexate. Methotrexate has been evaluated in a number of animal studies for carcinogenic potential with inconclusive results. Although there is evidence that methotrexate causes chromosomal damage to animal somatic cells and human bone marrow cells, the clinical significance remains uncertain.

Impairment of Fertility

Methotrexate causes embryotoxicity, abortion, and fetal defects in humans. It has also been reported to cause impairment of fertility, oligospermia and menstrual dysfunction in humans, during and for a short period after cessation of therapy.

Adverse effect on human

The most frequently reported adverse reactions include ulcerative stomatitis, leukopenia, nausea, and abdominal distress. Other frequently reported adverse effects are malaise, undue fatigue, chills and fever, dizziness and decreased resistance to infection.

Medicines on sale in Switzerland

Methotrexat Farmos, Méthotrexate “Ebewe”, Méthotrexate Lederle, Méthotrexate Proreo.

A.16 Consumption of the acidic drugs in Switzerland

TABLE A.1: Quantity of substances sold from July 2001 to June 2002 by hospitals, pharmacies and selfdispensing doctors

Substances	Amount sold [kg/year][a]			
	Total	Hospitals	Pharmacies	Doctors
Ibuprofen	17982	551	13028	4404
Mefenamic acid	17276	1652	10367	5257
Diclofenac	3884	204	2342	1338
Ketoprofen	254	2	157	96
Etofibrate	67	0.22	49	18
Clofibrate	41	0.25	35	6
Etofylline clofibrate	34	16	17	

[a] From Institut für Haushaltsanalysen (IHA) - IMS in Switzerland

A.17 Consumption and prediction of environmental concentrations (PECs) of anticancer drug in Switzerland

TABLE A.2: Quantity of anticancer drugs sold from July 2001 to June 2002 and PECs calculated as described in the Section 3.2.8. The data of consumption were kindly supplied by the Institut für Haushaltsanalysen (IHA) - IMS HEALTH, Switzerland

Substances	Total [kg]	$PEC_{inf\mathbf{a}}$ [ng/l]
Capecitabine	455.484	572.879
Hydroxycarbamide	352.245	443.031
Tamoxifen	155.734	195.872
5-Fluorouracil	81.137	102.049
Cyclophosphamide	34.364	43.221
Gemcitabine	32.411	40.765
Imatinib	22.973	28.894
Estramustine	21.744	27.349
Methotrexate	12.960	16.300
Ifosfamide	12.275	15.439
Cytarabine	8.454	10.634
Carboplatin	7.094	8.922
Mercaptopurine	5.992	7.537
Etoposide phosphate	3.503	4.405
Temozolomide	3.185	4.006
Rituximab	2.553	3.211
Paclitaxel	2.312	2.908
Irinotecan	1.973	2.481
Dacarbazine	1.914	2.407
Etoposide	1.845	2.321
Epirubicin	1.247	1.569
Trastuzumab	1.226	1.542
Mitotane	1.200	1.509
Cisplatin	1.092	1.373
Doxorubicin	0.909	1.143
Docetaxel	0.719	0.904
Procarbazine	0.700	0.880

continued

A.17. Consumption and prediction of environmental concentrations (PECs) of anticancer drug in Switzerland

Substances	Total [kg]	$PEC_{inf\mathbf{a}}$ [ng/l]
Vinorelbine	0.646	0.813
Oxaliplatin	0.618	0.777
Chlorambucil	0.487	0.613
Lomustine	0.364	0.458
Fludarabine	0.256	0.323
Melphalan	0.213	0.268
Tretinoin	0.201	0.253
Tioguanine	0.163	0.205
Bleomycin	0.142	0.178
Pancreatin	0.114	0.143
Pegylated liposomal doxorubicin	0.069	0.087
Amsacrine	0.069	0.087
Mitoxantrone	0.058	0.073
Carmustine	0.057	0.072
Thymus gland	0.057	0.072
Mitomycin	0.041	0.052
Daunorubicin	0.041	0.052
Papain	0.028	0.036
Vinblastine	0.025	0.031
Viscum album	0.022	0.028
Thiotepa	0.021	0.027
Idarubicin	0.020	0.025
Vincristine	0.019	0.023
Busulfan	0.017	0.022
Cladribine	0.012	0.015
Topotecan	0.010	0.012
Raltitrexed	0.004	0.004
Chlormethine	0.002	0.003
Vindesine	0.001	0.002

A.18 Consumption of anticancer drugs in CHUV

TABLE A.4: Quantity of anticancer drugs prepared by the pharmacy of CHUV (hospital in Lausanne) during one year (2001). These data were kindly supplied by Marc Voeffray (CHUV pharmacist)

Substances	Abbreviation	2001 [mg]
5-Fluorouracil	5-FU	3 243 595
Ifosfamide	IFOS	1 270 655
Cytarabine	ARAC	682 866
Cyclophosphamide	CYCL	638 937
Methotrexate	MTX	607 790
Etoposide phosphate	ETOP	162 101
Carboplatin	CBDCA	104 421
Gemcitabine	GEM	77 850
Cisplatin	CDDP	56 846
Taxol	TAX	49 875
Etoposide	VP16	21 029
Dacarbazine	DTIC	17 940
Taxotere	TXT	17 072
Mitoguazone	Me-GAG	15 870
Carmustine	BCNU	15 492
Adriblastine	ADM	14 916
Melphalan	L-PAM	13 388
Fotemustine	FOTE	6 040
Vinorelbine		4 955
Daunorubicin	DAUN	4 829
Busulfan		4 258
Thiotepa	THIO	3 995
Amsacrine	m-AMSA	3 855
Epirubicin	4-EPI	3 503
Fludarabine	FAMP	1 560
Idarubicin	IDA	1 204
Mitoxantrone	MITOX	976
Vincristine	VCR	554
Bleomycin	BLEO	514
Irinotecan	CPT11	373
Cladribine	2-CDA	177

continued

Substances	Abbreviation	2001 [mg]
Oxaliplatin		120
Vinblastine	VLB	84
Vindesine	VDS	81
Topotecan	TOPO	45
Actinomycine D	DACT	27
Mitomicine C	MMC	16

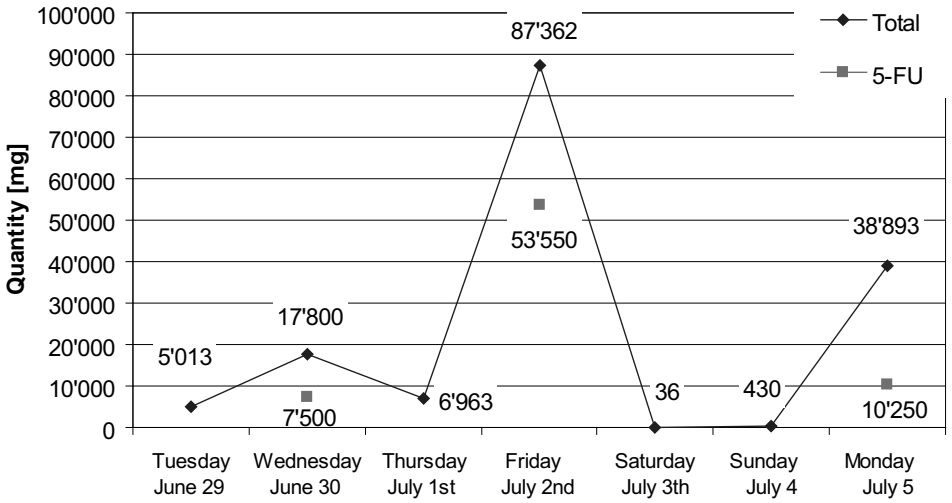

FIGURE A.16: Quantity of anticancer drugs (total of all substances and 5-Fluorouracil in mg) prepared each day by the pharmacy of CHUV during the sampling period (June 29 to July 5 2004). These data were kindly supplied by Grégory Podilsky (CHUV pharmacist)

TABLE A.6: Predicted Environmentally Concentrations in wastewaters of CHUV (PEC_{CHUV}) estimated with the quantity of drugs prepared for injection by the pharmacy of CHUV during the sampling period (June 29 to July 5 2004) and with the average of water consumption (703 m^3/d), comparison of several cytostatic drugs

Substances	PEC_{CHUV} [a] [ng/l]	PEC_{CHUV} [b] [ng/l]
5-Fluorouracil	0 - 76 000	0 - 15 000
Ifosfamide	0 - 17 000	0 - 2 000
Methotrexate	0 - 1 560	0 - 1 250
Cyclophosphamide	0 - 7 200	0 - 3 600

[a] Without metabolism
[b] With metabolism

Appendix B

Protocols of analysis

B.1 Protocol for the analysis of NSAIDs and Clofibric acid in wastewaters

B.1.1 Principle

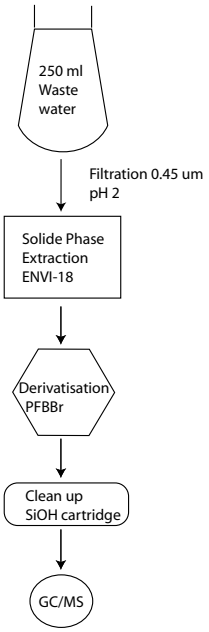

FIGURE B.1: Protocol principle of the acidic drugs analysis

B.1.2 Materials washed with acetone and hexane

1 funnel, n SPE tubes, n Erlenmeyer flasks of 300 ml, n+1 Becher flasks of 350 ml.

B.1.3 Materials

Membrane filters (ME 25, 0.45 μm) from Schleicher & Schuell (Dassel Germany); 6 ml Supelclean ENVI-18 from Supelco (Bellefonte, USA); 3 ml SiOH cartridges containing 500 mg of unmodified silica from Macherey-Nagel Chromabond (Düren, Germany).

B.1.4 Solutions

MilliQ water, acid water (pH2, with HCl), acetone, methanol, toluene, hydrochloric acid (32%), pentafluorobenzyl bromide solution (2% in toluene), standard solution for the 5 substances in methanol.

B.1.5 Equipment

Millipore Hazardous Waste Filtration System; Supelco Visiprep DL; stream of nitrogen; drying oven.

B.1.6 Filtation and Extraction

Weigh 250 ml of wastewater. Add the wastewater through the Millipore Hazardous Waste Filtration System previously well washed with water and alcohol. Add HCl to obtain a pH of 2.

Prepare the SPE Cartridge (ENVI-18) on the Visiprep. Conditionne the cartridge with 3 ml of acetone, 3 ml of methanol and 3 ml of acid water (pH2).

Pass the sample through the cartridge at a flow rate of approximately 3 ml/min by applying a low vacuum. Dry the solid phase for one hour under vacuum. Elute the analytes with 6 ml of methanol. Evaporate the methanol extract till dryness under a gentle stream of nitrogen.

For the sample of surface water, one liter of water was used. It was passed through four cartridges (250 ml per cartridge) and the methanol extract was collected together.

B.1.7 Derivatisation and purification

Add 400 μl of pentafluorobenzyl bromide (2% in toluene) and 4 μl of triethylamine. Incubate the solution at 90°C for one hour. The derivatised extract is passed through a SiOH cartridge conditioned with toluene. Elute the analytes with 15 ml of toluene. Reduce the eluate volume under a gentle stream of nitrogen to 100-1500 μl, to be inside the range of concentration tested in the calibration curve. If higher/smaller concentration were found, the samples were diluted/concentred and analysed a second time.

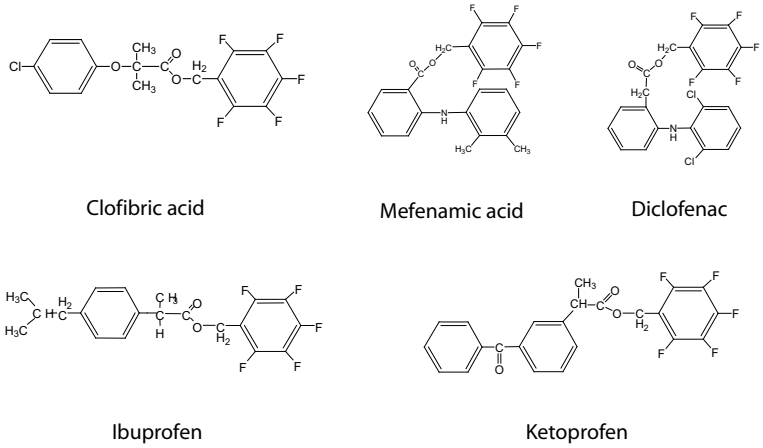

FIGURE B.2: Structures of derivatised substances

B.1.8 Detection

GC/MS system was used for quantitative analysis: a Varian CP 3800 gas chromatograph coupled with a Varian 1200L mass spectrometer.

GC Parameters

The gas chromatograph was equipped with a 60 m x 0.25 mm i.d. x 0.25 μm RTX-5 capillary column connected to a 5 m deactivated fused silica pre-column. Constant column flow mode was chosen (1 ml/min).

GC injection parameters: 1 μl with a SPI - Septum-equipped Programmable injector (on-column); injection port: 85°C for 0.2 min; 100°C/min to 250°C.

GC oven temperature programm: 80°C for 1 min; 30°C/min to 150°C; 3.5°C/min to 280°C; 280°C isothermal 30 min.

MS Parameters

Transfer line temperature: 250°C; EI or NCI mode, electron energy: 70 eV. For SIM mode, two to four characteristic ions were selected for each compound (see Table B.1 for EI mode and Table B.2 for NCI mode) and scanned using corresponding time windows.

TABLE B.1: GC/MS data of the PFB derivatives in EI mode

Substances	Retention time (min)	Characteristic ions (relative abundance)
Clofibric acid	24.9	130 (100), 169 (47),***394 (37)***
Ibuprofen	26.0	***118 (30)***,161 (100),***386 (15)***
Ketoprofen	40.3	***105 (32), 209 (100), 434 (6)***
Mefenamic acid	40.9	***194 (80), 223 (100), 421 (74)***
Diclofenac	42.5	***214 (100), 242 (22)***, 476 (19)

In italic and bold: ions chosen for the quantification

TABLE B.2: GC/MS data of the PFB derivatives in NCI mode

Substances	Characteristic ions (relative abundance)
Clofibric acid	213 (100), 214 (22), 215 (43), 286 (80)
Ibuprofen	181 (1), 205 (100), 206 (66)
Ketoprofen	209 (2), 210 (3), 253 (100), 254 (24)
Mefenamic acid	181 (1), 240 (100), 241 (33)
Diclofenac	258 (11), 260 (6), 294 (100), 296 (60), 439 (3)

B.1.8.1 Chromatograms

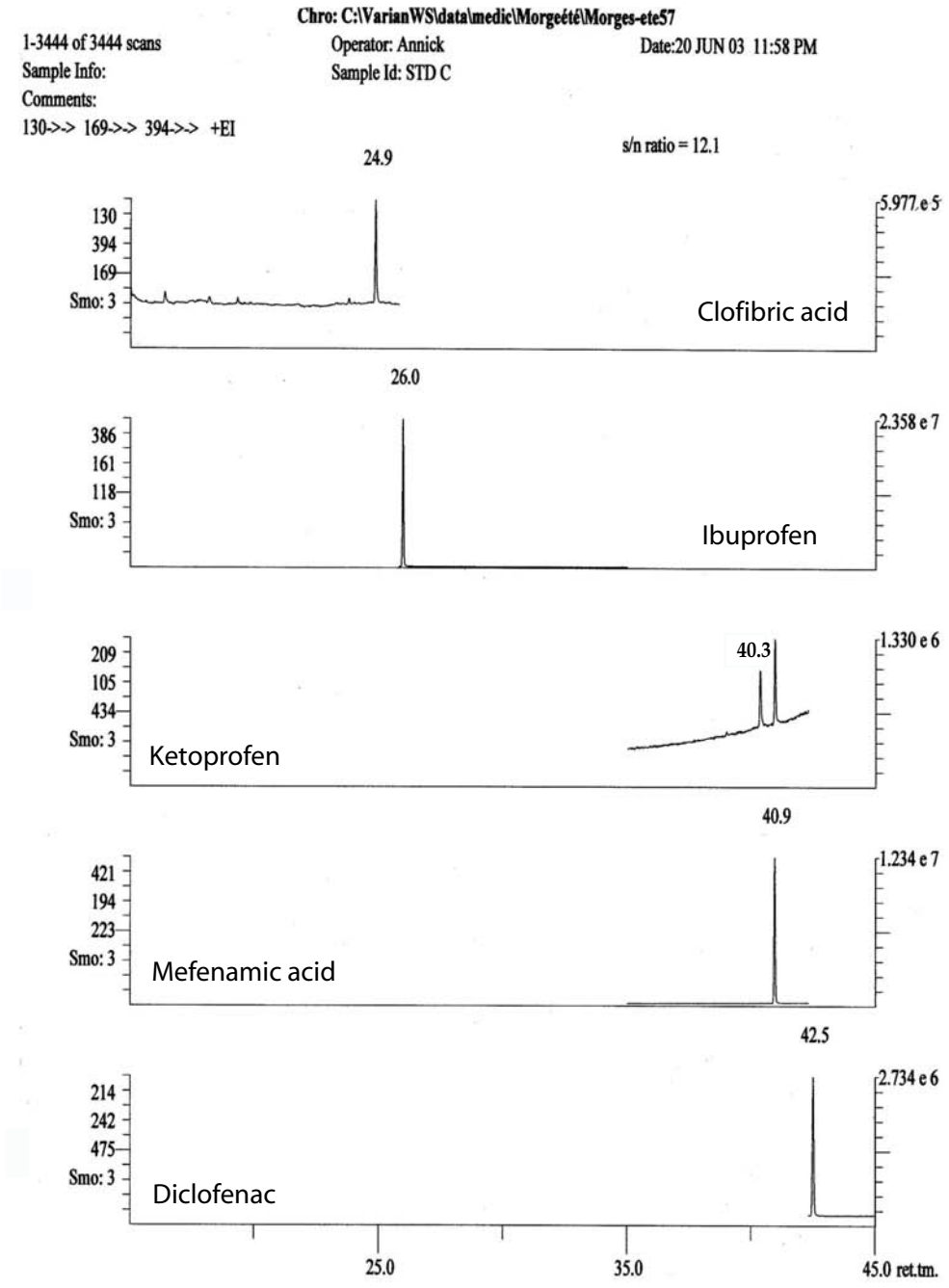

FIGURE B.3: Chromatogram of a standard solution injected in GC/MS

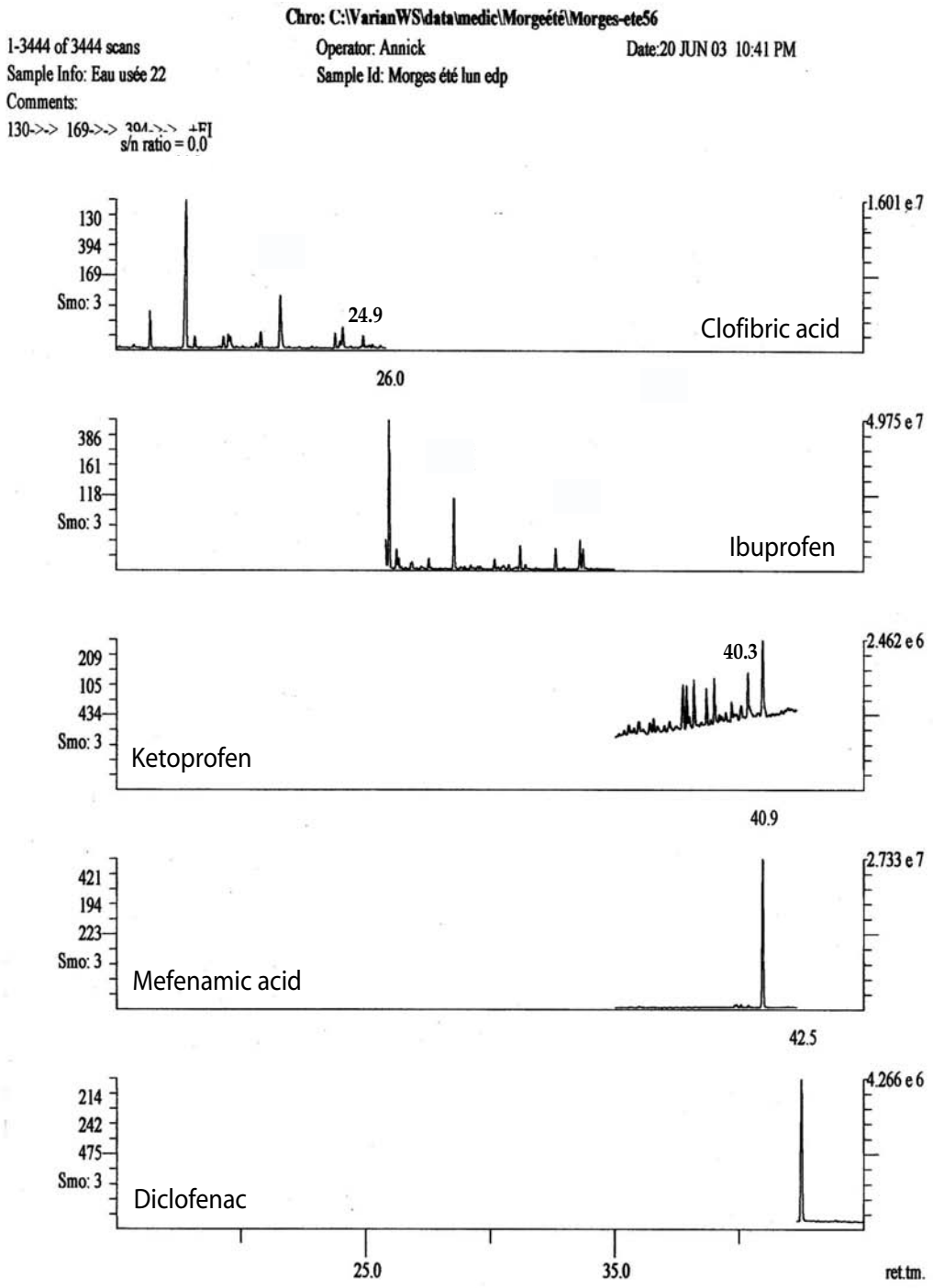

FIGURE B.4: Chromatogram of a wastewater extract injected in GC/MS

B.1.9 Method validation

B.1.9.1 Recovery evaluations: Linear regressions

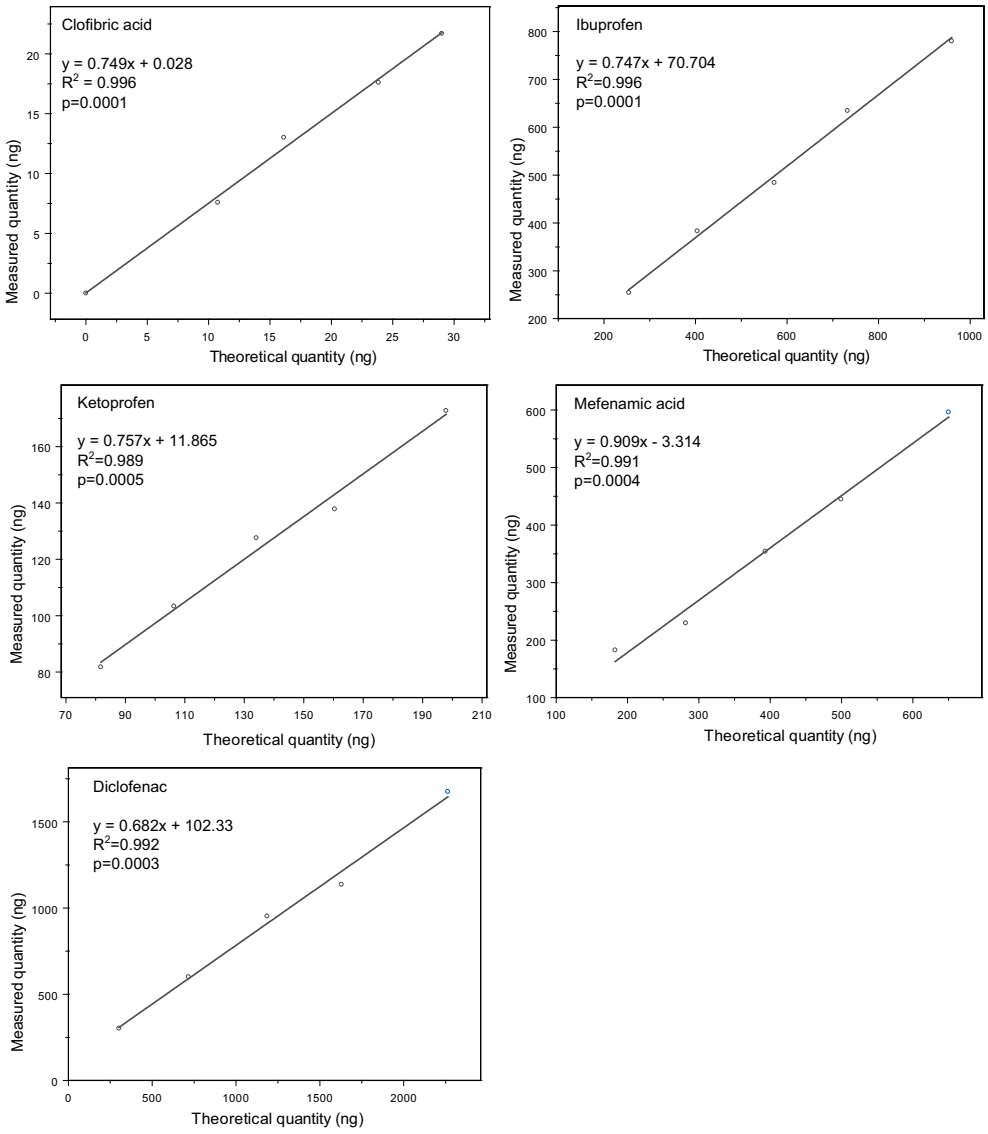

FIGURE B.5: Determination of the recoveries; samples of wastewaters were spiked with the pharmaceutical substances at four concentration. Linear regressions between the theoretical quantities and the measured quantities [ng]. The recovery rates were derived from the slopes. See Section 2.2.4 (page 24)

B.1.9.2 Limits of detection

Table B.3: Limits of detection (LOD) per liter of wastewater in EI mode and per liter of surface water in NCI mode

Substances	LOD in EI mode [ng/l] Wastewater (0.25 l)	LOD in NCI mode [ng/l] Surface water (1 l)
Clofibric acid	15	0.3
Ibuprofen	8	0.4
Ketoprofen	8	0.06
Mefenamic acid	5	0.03
Diclofenac	6	0.04

B.2 Protocol for the analysis of 5-Fluorouracil (5-FU) in wastewaters

B.2.1 Principle

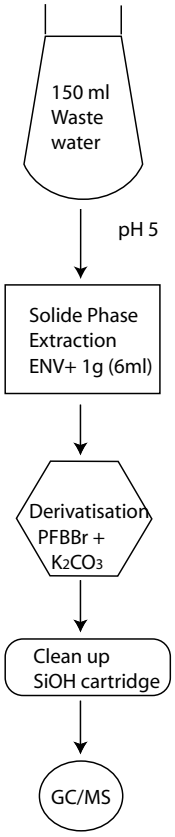

FIGURE B.6: Protocol principle of 5-Fluorouracil analysis

B.2.2 Materials

ENV+ cartridges: 1g, 6ml (Isolute); SiOH cartridges of 500 mg (Chromabond); 10 ml SPE tubes (previously washed with acetone and hexane); 150 ml Erlenmeyer flasks (washed with acetone and hexane).

B.2.3 Solutions

P3: phosphate buffer, 0.01 mol/l KH_2PO_4 (0.136 g in 100 ml) adjusted with 0.1 mol/l phosphoric acid to pH3,

P5: phosphate buffer, 0.01 mol/l KH_2PO_4 adjusted with 0.01 mol/l KOH solution to pH5,

PFBBr/ACN (20/80, v/v), 25% of K_2CO_3 in water (w/w), Hexane/Acetone (80/20), Toluene/Hexane (15/85), hexane, acetonitrile, pentofluoroBenzyl bromide, methanol, toluene.

B.2.4 Equipment

SPE Visiprep Vacuum Manifold (Supelco); stream of nitrogen; drying oven.

B.2.5 Extraction

Pre-treatment of 150 ml samples

Add one (or two) drop of HCl (32%) and adjust the pH to 5 with P3.

Extraction with cartridge ENV+ (1g, 6ml)

Conditione the cartridge with 12 ml MeOH and 12 ml P5.

Load the sample to a flow rate of 3-5 ml/min (30-50 minutes for 150 ml).

Dry the cartridge for 2 to 3 hours under vacuum.

Elute the analyte with 4 x 3 ml of MeOH. Lets soak during 4 minutes each 3 ml and then elute the analyte dropwise in a SPE tube.

B.2.6 Derivatisation and purification

- Evaporate till dryness under a stream of nitrogen.

Then add:

- 1 ml Acetonitrile
- 100 μl K_2CO_3 solution
- Vortex during 30 sec and add
- 100 μl PFBBr solution (20% in ACN)

- 1 hour at 80°C (incubation)

- Add 1 ml Toluene
- Evaporated to 200 μl under a stream of nitrogen and add
- 1 ml Isooctane

- In the SiOH cartridge, add 0.5 cm of Na_2SO_4. Then condition the cartridge with:
- 5 ml of Hexane/Acetone (80/20) and
- 5 ml of Hexane

- Add the extract in the SiOH cartridge.
- Wash the SPE tube with 3x1 ml of Toluene/Hexane (15/85), and pass through the cartridge
- And pass 5 ml of Toluene/Hexane (15/85) (with low vacuum) through the cartridge
- Dry the cartridge for 1 min under vacuum

Then pass through the cartridge:

- 2 ml Hexane/Acetone (80/20) (pass with low vacuum, do not let dry the cartridge), and
- 2 ml Hexane/Acetone (80/20) (pass dropwise, without vacuum) and collect in a SPE tube

- Add 0.7 ml of toluene in the SPE tube and evaporate to 200 μl under a stream of nitrogen
- Transfer into a vial for GC/MS and wash the SPE tube with 3 x 0.5 ml of toluene.

B.2.7 Detection

See Section 3.2.5 (page 45).

B.2.8 Method validation

B.2.8.1 Recovery evaluation: Linear regression

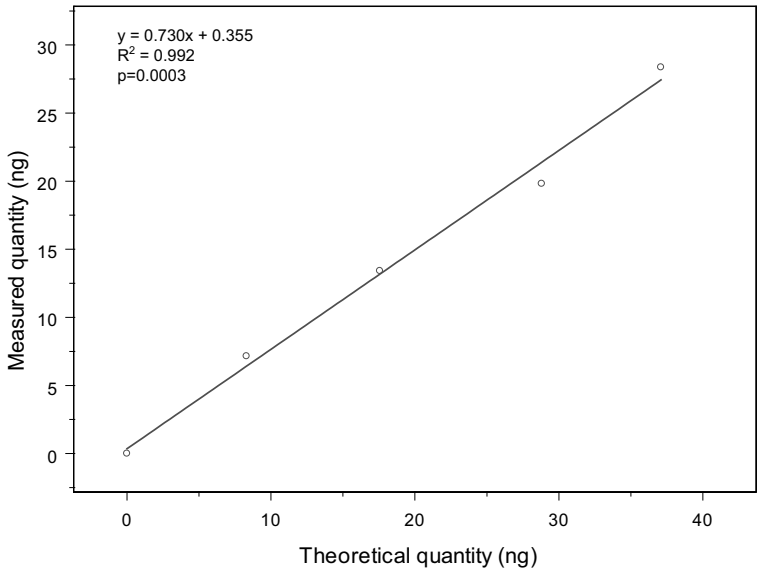

FIGURE B.7: Determination of the recoveries; samples of wastewaters were spiked with 5-Fluorouracil at four concentration. Linear regression between the theoretical quantities and the measured quantities [ng]. The recovery rate was derived from the slope. See Section 3.2.6 (page 46)

B.2.8.2 Limit of detection

See Section 3.2.6 (page 46).

B.3 Protocol for the analysis of Tamoxifen (TAM) in wastewaters

B.3.1 Principle

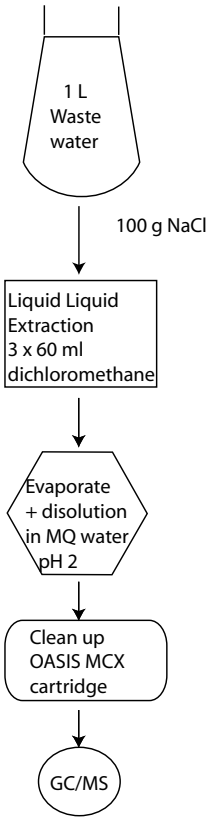

FIGURE B.8: Protocol principle of Tamoxifen analysis

B.3.2 Materials washed with acetone and hexane

n Erlenmeyer flasks of 1 l; n Balloons of 250 ml; n LLE separating funnel of 2 l; n small funnels fulled with sodium sulfate; n Becher flasks of 150 ml; 1 graduated cylinder of 100 ml; n 10 ml SPE tubes, n tubes (100-200 ml) for centrifuge.

B.3.3 Materials

n OASIS MCX cartridges: 150 mg, 6 ml, 30 μm (Waters).

B.3.4 Solutions

Sodium chloride, dichloromethane, MilliQ water, acid water (pH2, with HCl), hydrochloric acid (32%), methanol, 0.1 N HCl (10.6 g of HCl 32% in 100 ml of MQ water), Methanol/Acetonirile (30/70), Methanol/NH_4OH (95/5), toluene.

B.3.5 Equipment

SPE Visiprep Vacuum Manifold (Supelco); rotary evaporator system; stream of nitrogen.

B.3.6 Liquid-Liquid Extraction

Weigh 1 l of wastewater.
Add 100 g of sodium chloride.
Mix and pour in the LLE separating funnel.

To do three times:

- Add 60 ml of dichloromethane.
- Shake vigorously during 2 minutes.
- Wait 15 minutes (30 minutes the last extraction).
- Collect the dichloromethane emulsion in a tube for centrifuge.
- Centrifugation: 2500 t/min during 10 minutes.
- Pass the dichloromethane phase (bottom layer) in the funnel fulled with sodium sulfate and collect in a balloon.

Add 2 ml of Methanol.
Evaporate to 0.2-0.4 ml in a rotary evaporator system (850 mbar, 40°C).

B.3.7 Purification

Condition the cartridge (OASIS® MCX) with 6 ml MeOH and 1 ml MQ water.

Mix 10 ml of Acid water (pH2) with the extract and load it through the cartridge to a flow rate of 1 drop/sec (5-10 minutes for 10 ml).

Wash three times the balloon with 5 ml of acid water, and pass through the cartridge.

To wash the cartridge, pass 4 ml of 0.1N HCl through the cartridge.

Dry the cartridge during 2 minutes under vacuum.

Wash the cartridge with 4 ml of Methanol.

Wash the cartridge with 4 ml of Methanol/Acetonirile (30/70).

Elute with 3 ml of Methanol/NH_4OH (95/5): lets soak during 4 minutes and then collect the analyte dropwise in a SPE tube.

Evaporate to dryness under a stream of nitrogen.

Add 0.7 ml of Toluene. Transfer into a vial for GC/MS and wash the SPE tube with 3 x 0.5 ml of toluene.

B.3.8 Detection

See Section 3.2.5 (page 45).

B.3.9 Method validation

B.3.9.1 Recovery evaluation: Linear regression

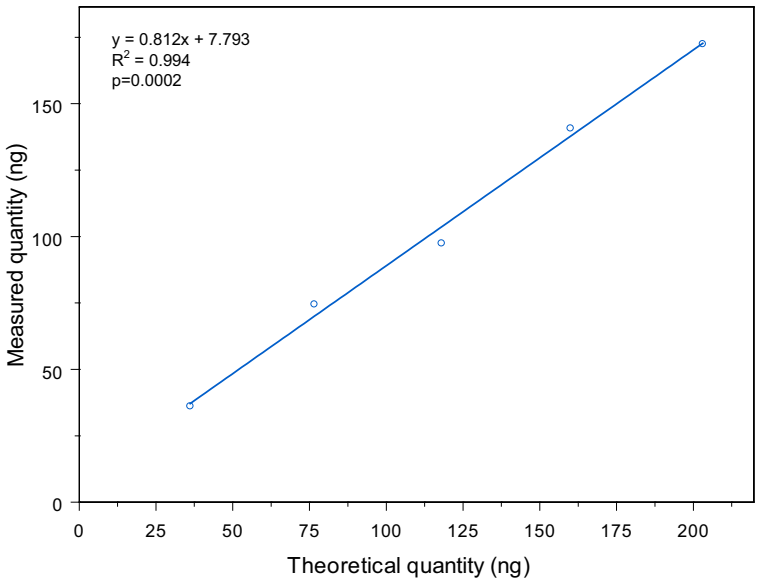

FIGURE B.9: Determination of the recoveries; samples of wastewaters were spiked with Tamoxifen at four concentration. Linear regression between the theoretical quantities and the measured quantities [ng]. The recovery rate was derived from the slope. See Section 3.2.6 (page 46)

B.3.9.2 Limit of detection

See Section 3.2.6 (page 46).

B.4 Preparation of the solutions for the Ames Test

B.4.1 Min A 10X

This is made by dissolving the following salts in bi-distilled water to a final volume of 800 ml. Autoclave the salt solution for 30 minutes at 121°C.

Salts	Quantitiy [g]
Na_2HPO_4-$7H_2O$	102.4
KH_2PO_4	24
NaCl	4
NH_4Cl	8

B.4.2 Growth supplement mixture

Dissolve the following substances in 20 ml of sterile MQ water, and sterilize by filtering using 0.22 μm sterile filter. Store at 4°C.

Substance	Quantitiy [mg]
L-Proline	200
L-Threonine	200
L-Arginine	200
L-Leucine	400
Thiamine HCL (stocked at 4°c)	10
Biotin	100

B.4.3 50% Glucose

Dissolve 15 g of glucose in 30 ml of sterile MilliQ Water, and sterilize by filtering using 0.22 μm sterile filter. Store at 4°C.

B.4.4 Histidine 20 mg/ml

Dissolve 0.2 g of histidine in 10 ml of sterile MilliQ Water, and sterilize by filtering using 0.22 μm sterile filter. Store at 4°C.

B.4.5 $CaCl_2$ 0.1M

Dissolve 0.73 g of $CaCl_2$-$2H_2O$ in 50 ml of sterile MilliQ Water, and sterilize by filtering using 0.22 μm sterile filter. Store at 4°C.

B.4.6 $MgSO_4$ 1M

Dissolve 7.38 g of $MgSO_4$-$7H_2O$ in 30 ml of sterile MilliQ Water, and sterilize by filtering using 0.22 μm sterile filter. Store at 4°C.

B.4.7 Phosphate-Buffered saline (PBS)

This is made by dissolving the following salts in bi-distilled water to a final volume of 800 ml. Adjust the pH to 7.4 with HCl (normally not necessary). Autoclave the salt solution for 30 minutes at 121°C.

Salts	Quantitiy [g]
NaCl	6.4
KCl	0.16
Na_2HPO_4	1.15
KH_2PO_4	0.19

B.4.8 Biotine/Histidine 5mM

Dissolve 50 mg of biotin and 50 mg of histidine in 50 ml of nearly boiling sterile MilliQ Water. Sterilize by filtering using 0.22 μm sterile filter. Store up to one year at 4°C.

B.4.9 LB

Dissolve 4 g of LB Broth in 200 ml of MilliQ Water, and autoclave for 30 minutes at 121°C.

B.4.10 Top Agar

This is made by dissolving the agar and salt in 396 ml of bi-distilled water. Autoclave the solution for 30 minutes at 121°C. Then, 4 ml of the biotin/histidine 5mM solution is added.

Salts	Quantitiy
Agar	2.4 g
NaCl	2.4 g
Distilled water	396 ml
Biotin/histidine 5mM	4 ml

B.4.11 Top Agar 10X

This is made by dissolving the agar and salt in 180 ml of bi-distilled water. Autoclave the solution for 30 minutes at 121°C. Then, 20 ml of the biotin/histidine 5 mM solution is added.

Salts	Quantitiy
Agar	12 g
NaCl	12 g
Distilled water	180 ml
Biotin/histidine 5mM	20 ml

B.4.12 LBM-Agar petri boxes

This is made by adding the following substances in 800 ml of bi-distilled water. Autoclave the solution for 30 minutes at 121°C. And directly flushed in the plates (about 30 ml per plate). Let dried the plates during 12-24 hours and store in a plastic bag up to several months at 4°C.

Salts	Quantitiy [g]
Agar	9,6
LB	16 g

B.4.13 Agar petri boxes

Dissolve 12 g of Agar in 800 ml of bi-distilled water. Autoclave the solution for 30 minutes at 121°C. Then the following solutions are added. The petri boxes are directly filled with the final solution (about 20-30 ml per plate). Let dried the plates during 12-24 hours and store in a plastic bag up to several months at 4°C.

Salts	Quantitiy
Mini a 10x	100 ml
Growth supplement mixture	2 ml
$CaCl_2$ 0.1M	1 ml
$MgSO_4$ 1M	1 ml
50% Glucose	4 ml

B.4.14 Overnight culture

Add about 1 ml of the fizzed bacteria culture or some bacteria of a LBM-Agar plate to 5 ml of LB. The culture is incubated at 37°C in a shaking incubator during the night before the Ames Test.

B.4.15 Bacteria conservation

Put 1 ml of the "overnight culture" in an ependorf tube. Add 150 μl of autoclaved glycerol. Store at -20°C.

B.4.16 S9 20%

Mix the following solutions and sterilize by filtering using 0.22 μm sterile filter. Store all the solutions in ice. Store the mix solution up to one year at -20°C.

Salts	Quantitiy	Stock concentration	Final conc
Mg-KCl	0.2 ml	400 mM - 1.65 M	8 mM - 33 mM
Phosphate buffer	5 ml	200 mM	100 mM
NADP	0.8 ml	50mM	4 mM
Glucose-6-Phosphate	0.8 ml	62.5 mM	5 mM
Milli-Q Water	1.4 ml		

Dissolve the S9 powder with 2 ml of Milli-Q water. And add S9 solution to the previous mix solution.

$MgCl_2$ 400 mM - KCl 1.65 M

Dissolve 0.81 g of $MgCl_2$-$6H_2O$ and 1.23 g of KCl in 10 ml of sterile MilliQ Water. Sterilize by filtering using 0.22 μm sterile filter. Store at ambiante temperature.

Phosphate buffer

Solution A: 0.2 M NaH_2PO_4-H_2O (1.05 g in 38 ml of MQ Water)
Solution B: 0.2 M Na_2HPO_4-$2H_20$ (5.77 g in 162 ml of MQ Water)
Mix 38 ml of solution A with 162 ml of Solution B, adjust to pH 7.4. Store at ambiante temperature.

NADP

Dissolve 0.3827 g of NADP in 10 ml of sterile MilliQ Water. Store up to one year at -20°C.

Glucose-6-Phosphate

Dissolve 0.19 g of Glucose-6-Phosphate in 10 ml of sterile MilliQ Water. Store up to one year at -20°C.

B.5 Protocol of preincubation Ames Test including toxicity

B.5.1 Principle

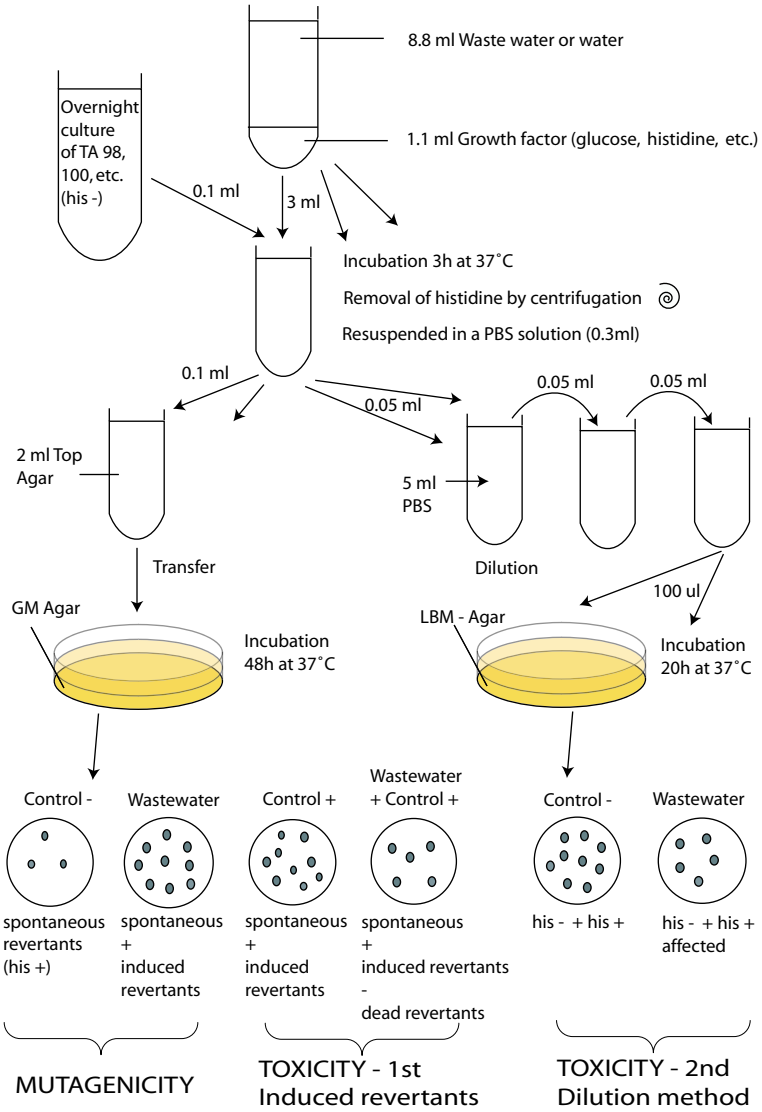

FIGURE B.10: Ames test principle

B.5.2 Sterile Materials

50 ml tubes, 13 ml tubes, 15 ml tubes, tips for pipettes, serological pipettes (5 ml, 10 ml and 25 ml), etc.

B.5.3 Solutions

Min A 10X, Growth supplement mixture, 50% Glucose, Histidine 20mg/ml, $CaCl_2$ 0.1M, $MgSO_4$ 1M, PBS, Top Agar, LBM-Agar petri boxes, Agar petri boxes, S9 20%. (See Appendix B.4; page 197)

B.5.4 Equipment

Incubator at 37°C with agitation; incubator at 37°C; centrifuge.

B.5.5 Storage of wastewater samples

All the samples of wastewater were filtered (0.45 μm) with a Millipore Hazardous Waste Filtration System immediately after the sampling and kept frozen until tested, as proposed by several authors [Davidov *et al.*, 2000; Hartmann *et al.*, 1999; Naudin *et al.*, 1995].

B.5.6 Pretreatment

The evening before the test, an overnight culture (see section B.4.14, page 200) of the selected bacterial strain is prepared.

B.5.7 Treatment

Mixture A; add in a 50 ml tube (one tube per each samples and control):

- 2 ml Mini A10x
- 40 μl Growth supplement
- 20 μl $MgSO_4$ 1M
- 20 μl $CaCl_2$ 0.1M
- 80 μl Glucose (50%)
- 100 μl Histidine (20 mg/ml)

- 17.6 ml of water (MQ or wastewater)

Concentration	Sterile MQ water	Wastewaters (+/- filtered 0.22 μm)
Control	17.6 ml	
25%	13.2 ml	4.4 ml
50%	8.8 ml	8.8 ml
100%		17.6 ml

When we have not enough wastewaters, half of these solutions was prepared to obtain a final volume of 10 ml instead of 20 ml.

Without S9

3 ml of this preparation (Mixture A) is put in a tube of 15 ml (in triplicate). And 100 μl of the overnight bacteria culture is added in these tubes and 15-20 μl Ampicillin (if the samples are not filtered and if the bacterial strain is resistant).

2% S9

2.7 ml of this preparation is put in a tube (in triplicate). 100 μl of the overnight bacteria culture and 0.3 ml of S9 solution are added in these tubes.

4% S9

2.4 ml of this preparation is put in a tube (in triplicate). 100 μl of bacteria and 0.6 ml of S9 solution are added in these tubes.

To do with the inoculated Mixture A:

- The inoculated sample are incubated at 37°C for 3 hours with agitation.
- Top agar is heated in a boiling water bath. Petri boxes are identified with the names of samples or control and with the bacterial strain. 5 ml of PBS are added in all tubes for the dilution in the toxicity test.
- After 3 hours of incubation, the samples are centrifuged (4000 rpm for 5 minutes).
- Supernatant are removed.

- Re-suspended the pellet in 3 ml of phosphate buffered saline (PBS) to ensure removal of histidine.
- Second centrifugation: 4000 t/min during 5 minutes.
- Supernatant are well removed, with aspiration.
- Re-suspended in 0.3 ml of PBS (**Mixture B**).

B.5.8 Mutagenicity

Add in a 13 ml tube: (To do 2 times per 3 ml tube)

- 2 ml of Top Agar (at 45°C)
- 0.1 ml of the Mixture B
- 5-10 μl of ampicillin (only if necessary: if the samples is not filtered and if the bacterial strain is resistant)

The contents of test tubes are poured onto the surface of Agar petri plates. When the Top Agar has hardened, the plate are incubated in an inverted position in a 37°C incubator for 48 hours at which time the histidine revertant colonies are counted.

B.5.9 Toxicity assay using induced revertants - First method

Proceed as in the Section B.5.7 and B.5.8, but add 100 μl of the positive control (0.5 mg/ml in DMSO) in 10 ml of the Mixture A to get a final solution of 5 μg/ml.

This is made for sterile bi-distilled water (control+) and for the samples (wastewaters + control+).

B.5.10 Toxicity assay by dilution - Second method

To prepare in advance (during the previous treatment): the adequate number of tube with 5 ml of PBS (3 tubes per each 3 ml tubes, that means 9 tubes per sample)

Make the following dilution in 5 ml of PBS:

- Mix 50 μl of the Mixture B with 5 ml of PBS (1^{st} tube).

- Mix 50 μl (or 100 μl when the bacteria concentrations are low) of the 1^{st} tube with 5 ml of PBS (2^{nd} tube).
- Mix 50 μl (or 100 μl when the bacteria concentrations are low) of the 2^{nd} tube with 5 ml of PBS (3^{th} tube).

Inoculate (in duplicate) a LBM-Agar petri box with 100 μl of the 3^{th} PBS tube. Spread with sterile little balls. The plate are incubated in an inverted position in a 37°C incubator for 18 to 20 hours.

B.6 Mutagenicity ratio

$$MR = \frac{spontaneous + induced\ revertants}{spontaneous\ revertants}$$

$$MR_{adj1} = \frac{MR}{survival\ (induced\ revertants\ method)}$$

$$MR_{adj2} = \frac{MR}{survival\ (dilution\ method)}$$

Appendix C

Complementary results

C.1 Correlation between the removal of Ibuprofen and the residence time

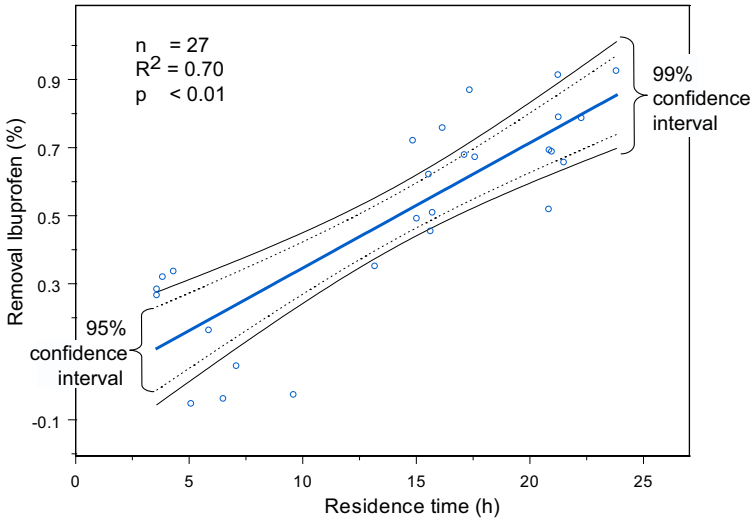

FIGURE C.1: Correlation between the removal of Ibuprofen and the residence time of wastewaters in the STPs (See Section 2.3.2 on page 30)

C.2 Acidic and alkaline hydrolysis of conjugated NSAIDs

C.2.1 Abstract

Besides alkaline and acidic hydrolysis of wastewaters was studied. Alkaline hydrolysis did not increase the concentration of these drugs, indicating that glucuronide metabolites are hydrolysed in parent drugs before the sewage treatment plant.

C.2.2 Introduction

For many acidic NSAIDs, it is well known that urinary excretion of the parent drugs is very low (about 5% of the administered dose, see Table 2.1 on page 21). For the substances studied here, conjugation of parent compounds and their phase I metabolites to their corresponding glucuronides or sulfates is frequent. For instance, after the administration of Ibuprofen, 13% of the dose is excreted in urine as its acyl glucuronide metabolite [Geisslinger *et al.*, 1989], but only 1% of free Ibuprofen. Several methods have been published for the indirect analysis of acyl glucuronide in plasma and urine, by measuring Ibuprofen prior to and after hydrolysis [Castillo & Smith, 1993; El Haj *et al.*, 1999; Bauza *et al.*, 2001]. Hirai *et al.* [1997] describe three kinds of hydrolysis: (1) enzymatic, (2) acidic and (3) alkaline. These hydrolysis are commonly used for the quantification of glucuronide metabolites of NSAIDs in urine. To our knowledge, none of these hydrolysis was tested with wastewaters. Therefore, the aim of this study was to test acidic and alkaline hydrolysis to quantify the glucuronide metabolites of the four substances studied (Ibuprofen, Ketoprofen, Mefenamic acid and Diclofenac).

C.2.3 Experimental section

Hydrolysis was carried out at the beginning of the analytical procedure, just after the filtration. Two types of acidic and alkaline hydrolysis were tested on an influent of Mittleres Emmental STP. Enzymatic hydrolysis was not tested. Indeed, the results of Hirai *et al.* [1997] showed that this hydrolysis can be employed only for one NSAID (Sulindac).

The first acidic hydrolysis, based on El Haj *et al.* [1999], was performed by adding 50 ml of HCl (32%) to obtain a ∼2M HCl solution which was heated at 80°C for 30 minutes. For the second acidic hydrolysis, the pH was adjusted to ∼2 with HCl and heated at 80°C for one hour. In the first alkaline hydrolysis, the sample was adjusted to pH 12 with 5M NaOH, then heated at

80°C for one hour [Castillo & Smith, 1993]. The second alkaline hydrolysis, which was based on Bauza *et al.* [2001], was performed by adding 20 ml of 1M NaOH and heating at 80°C for 30, 60 or 90 minutes. After alkaline hydrolysis, pH was adjusted to 2 with HCl.

TABLE C.1: Various hydrolysis tested

Hydrolysis	Acid or base	Final concentration or adjustment of pH	Temp.	Duration
Acidic	50 ml of HCl	~2M HCl	80°C	30 min.
Acidic	HCl	pH2	80°C	60 min.
Alkaline	5M NaOH	pH12	80°C	60 min.
Alkaline	20 ml of 1M NaOH		80°C	30, 60 and 90 min.

C.2.4 Results and discussion

Both methods of **acidic hydrolysis** showed a decrease of concentrations for the four substances measured in the test sample. When acidic hydrolysis was conducted according to the conditions proposed by El Haj *et al.* [1999], concentrations of Mefenamic acid and Diclofenac were below the limit of detection. Hirai *et al.* [1997] also pointed out that recoveries of Mefenamic acid were decreased and that Diclofenac disappeared with acidic hydrolysis. This shows that these methods of hydrolysis are not suitable to analyze glucuronide metabolites in wastewaters.

The **alkaline hydrolysis** did not seem to degrade the parent substances, and the process of hydrolysis did not increase the concentrations of all of substances in the sample. Unlike urine, where Ibuprofen concentrations were five times higher after hydrolysis [Hirai *et al.*, 1997], influents of Mittleres Emmental STP did not seem to contain glucuronide metabolites. According to Heberer [2002a], the conjugated metabolites can easily be cleaved during sewage treatment and parent compounds will be released into the aquatic environment. According to our results, such metabolites have probably been naturally hydrolyzed before the Mittleres Emmental STP. Indeed, wastewaters are rarely at neutral pH and, for instance, the half-life of Mefenamic acid glucuronide under alkaline condition is lower than at pH 7.4 [McGurk *et al.*, 1996]. This hypothesis is confirmed by the fact that influent concentrations are close to the PECinf calculated with excretion of conjugated and unchanged compounds (see Table 2.4 on page 31).

C.3 Analysis of the NSAIDs and clofibric acid in a surface water sample: Lake Geneva

C.3.1 Experimental section

The analysis of the sample (1l) was performed as described in the Appendix B.1 on page 181.

C.3.2 Results and discussion

TABLE C.2: Concentration [ng/l] of NSAIDs and Clofibric acid in a sample of Lake Geneva and comparison with the literature data and the contamination of STPs

Substances	Lake Geneva [ng/l]	Literature results [ng/l]	Treated waters of studied STPs (min-max) [ng/l]
Clofibric acid	5	<1 - 9 (Lakes)[a]	nd - 270
Ibuprofen	3	nd - 4 (Lakes)[b]	150 - 2500
Ketoprofen	0.4	nd- 200 (Rivers) [c]	100 - 340
Mefenamic acid	5	∼10 (Rivers) [d]	470 - 3000
Diclofenac	1	<1 - 3 (Lakes) [e]	600 - 2500

[a] Buser *et al.* [1998b]
[b] Buser *et al.* [1999]
[c] Stumpf *et al.* [1999]
[d] Ahrer *et al.* [2001]
[e] Buser *et al.* [1998a]

The results presented in Table C.2 show that the concentrations in the sample of Lake Geneva was well correlated with the concentrations in treated wastewaters. Indeed, for the highly concentrated substances in wastewaters (also the highly used: Ibuprofen and Mefenamic acid), the concentrations in the Lake were high. The concentration of Clofibric acid was also relatively important. This contamination was not due to the treated wastewaters where the concentration of clofibric acid was low, but due to the persistent behavior of this substance, which is also detected far away of the population (North Sea up to 7.8 ng/l [Buser *et al.*, 1998b]).

The measured concentrations in this sample was the same order of magnitude than in the literature data.

C.4 Conditions for SPE Extractions of 5-Fluorouracil and Tamoxifen

Table C.3 (page 212) shows the results of several extractions with ENVI-18 (Supelclean; Supelco), Isolute 101 (Separtis), C2 (Isolute; Separtis) and LC-NH2 (Supelclean; Supelco).

Tables C.4 and C.5 (pages 213-214) shows the results of extractions with ENV+ (200mg, 500mg, 1g; ISOLUTE) of 5-Fluorouracil in different volumes of MQwater.

Table C.6 (page 215) shows the results of extractions with MCX and HLB plus (OASIS) of Tamoxifen with different wash steps.

Legends: P1: Phosphate Buffer pH=3; P2: Phosphate Buffer pH=5 (See Appendix B.2, page 189).

TABLE C.3: Conditions for SPE extractions of anticancer drugs (250 ml of bi-distilled water; n=1)

Cartridge Volume	Sample preparation	Column conditioning	Elution (2^{nd} elution)	Recovery (2^{nd} elution)	
				5-FU	TAM
ENVI-18 (Supelco)					
1 g; 6 ml	-	3 ml Acetone, 3 ml MeOH, 3 ml H_2O	3 ml MeOH (3 ml)	0%	10-30% (?)
Isolute 101 (Separtis)					
200 mg; 6 ml	pH 5 with P1	6 ml MeOH, 6 ml P2	3x1 ml MeOH:ethyl acetate (1:1) (3x1 ml)	1% (0%)	
Isolute C2 (Separtis)					
500 mg	1 ml MeOH	3 ml MeOH, 3 ml H_2O	3 ml MeOH:NaCl (9 ml)	-	20% (0%)
500 mg	3 ml MeOH	3 ml MeOH, 3 ml H_2O	3x1 ml MeOH (6 ml)	0% (0%)	7% (0%)
500 mg	5 ml MeOH	3 ml MeOH, 3 ml H_2O	3 ml MeOH:NaCl 4 min (6 ml)	0% (0%)	40-55% (0%)
1 g	7 ml MeOH	6 ml MeOH, 6 ml H_2O	3 ml MeOH:NaCl 4 min (6 ml)	-	77% (5%)
1 g	7 ml MeOH	6 ml MeOH, 6 ml H_2O	10 ml MeOH:NH_4OH (95:5) (9ml)	-	82% (0%)
LC-NH2 (Supelco)					
500 mg; 3 ml	pH 5 with HCl	6 ml MeOH, 6 ml H_2O	4 ml Ethyl acetate (1% acetic acid) (10 ml)	0% (0%)	-

P1: Phosphate Buffer pH=3
P2: Phosphate Buffer pH=5

TABLE C.4: Conditions for extractions of 5-Fluorouracil in bi-distilled water (n=1) with ENV+ (ISOLUTE 6ml)

Bed [mg]	Sample volume	[5-FU] [mg/l]	Sample pretreatment	Column conditioning	Elution (2^{nd} elution)	Recovery (2^{nd} elution)
200	250 ml	0.007	-	3 ml MeOH, 3 ml H_2O	2x1 ml MeOH (16 ml)	∼10% (0%)
200	250 ml	0.007	-	4 ml MeOH, 4 ml H_2O	3x1 ml MeOH (6 ml)	3% (0.4%)
200	250 ml	0.007	pH 4.8 with P1	3 ml MeOH, 3 ml P2	4x1 ml MeOH (12 ml)	2% (0%)
200	250 ml	0.07	pH 4.8 with P1	3 ml MeOH, 3 ml P2	4x1 ml MeOH (6 ml)	45-55% (?%)
500	250 ml	0.007	env. pH 5 with P1; 3 ml MeOH	10 ml MeOH, 10 ml P2	3x2 ml MeOH:ethyl acetate (10 ml)	5-10% (0%)
500	250 ml	0.007	env. pH 5 with P1; 3 ml MeOH	10 ml MeOH, 10 ml P2	3 ml H_2O + 3 x 2 ml MeOH (6 ml MeOH:ethyl acetate)	5-20% (2%)
200	250 ml	0.007	pH 5 with P1	6 ml MeOH, 6 ml P2	4 ml Ethyl acetate (1% acetic acid) (10 ml MeOH:ethyl acetate (1:1) with 5% acetic acid)	∼10% (0%)
200	1 l	0.04	pH 5 with P1	6 ml MeOH, 6 ml P2	3x1 ml MeOH:ethyl acetate (1:1) (8 ml Ethyl acetate with 1% acetic acid)	∼2% (0.5%)
200	10 ml	0.2	pH 5 with P1	6 ml MeOH, 6 ml P2	3x1 ml MeOH:ethyl acetate (1:1) (8 ml Ethyl acetate with 1% acetic acid)	∼110% (1%)
200	250 ml	0.08	pH 5 with P1	6 ml MeOH, 6 ml P2	3x1 ml MeOH:ethyl acetate (1:1)	10-15%
200	100 ml	0.02	pH 5 with P1	6 ml MeOH, 6 ml P2	3x1 ml MeOH:ethyl acetate (1:1)	15-20%

[5-FU]: Final concentration of 5-Fluorouracil

TABLE C.5: Conditions for extractions of 5-Fluorouracil in bi-distilled water (n=1) with ENV+ (ISOLUTE 6ml) (continued)

Bed [mg]	Sample volume	[5-FU] [mg/L]	Sample pretreatment	Column conditioning	Elution (2^{nd} elution)	Recovery (2^{nd} elution)
200	250 ml	0.007	pH 5 with P1; 7.3 g NaCl	6 ml MeOH, 6 ml P2	3x1 ml MeOH:ethyl acetate (1:1)	5%
200	60 ml	0.033	pH 5 with P1	6 ml MeOH, 6 ml P2	3x1 ml MeOH:ethyl acetate (1:1)	30-40%
500	60 ml	0.033	pH 5 with P1	12 ml MeOH, 12 ml P2	3x3 ml MeOH:ethyl acetate (1:1)	90-100%
500	150 ml	0.013	pH 5 with P1	12 ml MeOH, 6 ml P2	2 ml MeOH:ethyl acetate (1:1) (5x2ml)	33% (∼7%)
1000	100 ml	0.013	pH 5 with P1	12 ml MeOH, 12 ml P2	2x3 ml MeOH:ethyl acetate (1:1) (4x2ml)	55-60% (∼7%)
1000	100 ml	0.013	pH 5 with P1	12 ml MeOH, 12 ml P2	2x3 ml MeOH (4x2ml)	72-80% (3%)
1000	200 ml	0.013	pH 5 with P1	12 ml MeOH, 12 ml P2	4x3 ml MeOH:ethyl acetate (1:1)	60-70%
1000	300 ml	0.013	pH 5 with P1	12 ml MeOH, 12 ml P2	4x3 ml MeOH:ethyl acetate (1:1)	40-50%
1000	400 ml	0.013	pH 5 with P1	12 ml MeOH, 12 ml P2	4x3 ml MeOH:ethyl acetate (1:1)	23%
1000	150 ml	0.013	pH 5 with P1	12 ml MeOH, 12 ml P2	4x3 ml MeOH	96%
1000	150 ml	0.0007	pH 5 with P1	12 ml MeOH, 12 ml P2	4x3 ml MeOH	92%

[5-FU]: Final concentration of 5-Fluorouracil

TABLE C.6: Conditions for SPE extraction of Tamoxifen with MCX (30 μm 6 cc 150 mg) and HLB plus (60 μm 225 mg) cartridges (OASIS®)

Cartidge	Sample Vol.	Sample preparation	Column conditioning	Wash steps	Elution (2^{nd} elution)	Recov. (2^{nd} elu.)
MCX HLB plus[1]	0.25 l	pH 2 (HCl)	5 ml MeOH, 1 ml H_2O (pH2)		8 ml MeOH (16 ml) 8 ml MeOH (16ml)	0% (0%) 0% (0%)
HLB plus and MCX	0.25 l	pH 3	5 ml MeOH, 1 ml H_2O		10 ml MeOH:NH_4OH (95:5) (9 ml)	120% (8%)
MCX HLB plus[1]	1 l	pH 3	6 ml MeOH, 1 ml H_2O (pH3)		10 ml MeOH:NH_4OH (95:5) 10 ml MeOH:NH_4OH (95:5)	100% 1-2%
MCX	1 l	pH 3	6 ml MeOH, 1 ml H_2O (pH3)	4 ml 0.1N HCl; 4 ml MeOH; 4 ml MeOH:ACN (30:70)	5 ml MeOH:NH_4OH (95:5) (10 ml)	90% (0%)
MCX	1 l	pH 3	6 ml MeOH, 1 ml H_2O (pH3)	4 ml 0.1N HCl; 4 ml MeOH; 4 ml MeOH:ACN (30:70); **4 ml H_2O:MeOH:NH_4OH** (50:42.5:2.5)	6 ml MeOH:NH_4OH (95:5) (6 ml)	2% (0%)
MCX	1 l	pH 3; **10 ml MeOH**	idem	4 ml 0.1N HCl; 4 ml MeOH; 4 ml MeOH:ACN (30:70)	6 ml MeOH:NH_4OH (95:5)	75% (0%)
MCX	1 l	pH 3; **20 ml MeOH**	idem	idem	idem	77% (0%)
MCX	1 l	pH 3; **50 ml MeOH**	idem	idem	idem	50% (0%)

[1] MCX and HLB were used together (combined), they were conditioned together, but separated before the elution.

C.5 Conditions for 5-Fluorouracil derivatisations

Table C.7 (page 217) shows the results of several tests of derivatisation of 5-Fluorouracil. Various catalysts (triethylamine and K_2CO_3), durations (0h to 3h) and temperatures (20°C to 100°C) were tested.

Table C.8 (page 218) shows the results of several tests of derivatisation of 5-Fluorouracil. Subtle changes in concentrations of Pentafluorobenzyl bromide (PFBBr), in durations (1h-3h) and temperatures (80°C to 150°C) were tested.

Table C.9 (page 219) shows the results of several repeatability tests of derivatisation of 5-Fluorouracil. Most of the changes were in concentrations of Pentafluorobenzyl bromide (PFBBr), in solvents and catalysts (triethylamine, K_2CO_3 and K_2HPO_4) used.

The results show that for a complete and repeatable derivatisation, the quantity of PFBBr have to be relatively important. 400 μl of the solution of PFBBr (2%) was not enough. 600 μl of PFBBr (4%) showed a significant improvement.

The catalysts were in enough quantity, indeed no significant increase of the area were observed with 15 μl of triethylamine (triet.) instead of 7.5 μl.

An increase of temperature was necessary for a complete derivatisation, indeed even with 20% of PFBBr (at 20°C) only 40% of substance was detected. With acetonitrile (ACN), evaporation (and sometimes losses) appeared with a temperature higher than 80°C.

No loses were observed when 5-FU-PFBBr was evaporated till dryness under a gentle stream of nitrogen.

Derivatisation with K_2CO_3 or K_2HPO_4 worked better than with triethylamine, 240% and 315% respectively. K_2HPO_4 seemed very efficient, but an impurity was detected very close to 5-Fluorouracil in GC/MS.

TABLE C.7: Conditions for 5-Fluorouracil derivatisations

Quantity of Pentafluorobenzyl bromide (PFBBr)	Catalyst	Time and Temperature	% Area
15 μl PFBBr in 400 μl TOL	6 μl triet.	1 h - 100°C	100%
10 μl PFBBr in 800 μl TOL	4 μl triet.	1 h - 90°C	90%
10 μl PFBBr in 800 μl TOL	4 μl triet.	1 h - 60°C	0%
10 μl PFBBr in 800 μl TOL	4 μl triet.	1 h - 20°C	0%
15 μl PFBBr in 400 μl TOL	6 μl triet.	1 h - 100°C	100%
15 μl PFBBr in 400 μl TOL	6 μl triet.	1 h - 90°C	95%
15 μl PFBBr in 400 μl TOL	6 μl triet.	1 h - 60°C	20%
15 μl PFBBr in 400 μl TOL	6 μl triet.	1 h - 20°C	3%
600 μl PFBBr/TOL (2%)	6 μl triet.	1 h - 90°C	100%
600 μl PFBBr/TOL (2%)	6 μl triet.	3 h - 60°C	80%
480 μl PFBBr/ACN (15%)	120 μl triet.	0.5 h - 20°C	gelatin
40 μl PFBBr/ACN (15%) + 560 μl ACN	-	direct inj	0%
100 μl PFBBr/ACN (2%) + 1 ml ACN	100 μl K_2CO_3	3 h - 60°C	200%
600 μl PFBBr/TOL (2%)	6 μl triet.	1 h - 90°C	100%
40 μl PFBBr/ACN (15%) + 560 μl ACN	6 μl triet.	1 h - 90°C	gelatin
40 μl PFBBr/ACN (15%) + 560 μl ACN	-	1 h - 90°C	0%
600 μl PFBBr/TOL (2%)	6 μl triet.	1 h - 90°C	100%
600 μl PFBBr/TOL (2%)	6 μl triet.	3 h - 60°C	84%
100 μl PFBBr/ACN (15%) + 1 ml ACN	100 μl K_2CO_3	3 h - 60°C	170%
100 μl PFBBr/ACN (15%) + 1 ml ACN	100 μl K_2CO_3	1 h - 90°C	180%

TOL: Toluene; ACN: Acetonitrile; triet: triethylamine

Table C.8: Duration and Temperature of derivatisations

Quantity of Pentafluorobenzyl bromide (PFBBr)	Catalyst	Time and Temperature	% Area
400 μl PFBBr/TOL (2%)	5 μl triet.	1 h - 100°C	100%
idem	5 μl triet.	1.5 h - 100°C	100-135% (Mean: 117%)
idem	5 μl triet.	2 h - 100°C	117-125% (Mean: 121%)
idem	5 μl triet.	3 h - 100°C	111-121% (Mean: 116%)
100 μl PFBBr/ACN (20%) + 1 ml ACN	100 μl K_2CO_3	1 h - 90°C	100%
200 μl PFBBr/ACN (20%) + 1 ml ACN	100 μl K_2CO_3	1 h - 90°C	100%
100 μl PFBBr/ACN (20%) + 1 ml ACN	100 μl K_2CO_3	2 h - 90°C	100%
idem	100 μl K_2CO_3	2.5 h - 90°C	90%
idem	100 μl K_2CO_3	1 h - 100°C	95%
idem	100 μl K_2CO_3	2 h - 100°C	95%
idem	100 μl K_2CO_3	1 h - 80°C	120%
idem	100 μl K_2CO_3	2 h - 80°C	120%
400 μl PFBBr/TOL (2%)	5 μl triet.	1 h - 100°C	High variation, but a higher area was
idem	5 μl triet.	1 h - 110°C	never observed in
idem	5 μl triet.	1 h - 120°C	the samples with
idem	5 μl triet.	1 h - 150°C	higher temperature than 100°C.

TOL: Toluene; ACN: Acetonitrile; triet: triethylamine

TABLE C.9: Repeatability of various derivatisations followed by a purification step

Quantity of Pentafluorobenzyl bromide (PFBBr)	Catalyst	Temperature	Purification	Solvent	%Area ± SD	n=
600 μl PFBBr/TOL (2%)	7.5 μl triet.	1 h - 100°C	Drying (stream of nitrogen)		100%±7%	3
600 μl PFBBr/TOL (2%) (new solution)	7.5 μl triet.	1 h - 100°C	Drying (stream of nitrogen)		78%±13%	2
600 μl PFBBr/TOL (2%)	7.5 μl triet.	1 h - 100°C	Drying (stream of nitrogen)		100%±3%	6
600 μl PFBBr/TOL (2%)	7.5 μl triet.	1 h - 100°C	SiOH	Hexane / Aceton	65%±23%	3
24 μl PFBBr in 600 μl of TOL (4%)	7.5 μl triet.	1 h - 100°C	SiOH	Hexane / Aceton	170%±9%	2
12 μl PFBBr in 600 μl of TOL (2%)	15 μl triet.	1 h - 100°C	SiOH	Hexane / Aceton	100%±9%	2
40 μl PFBBr in 120 μl of ACN (20%)	20 μl triet.	0.5 h - 20°C	LLE (+centrifugation)	200 μl of Ethyl acetate and 2 ml Hexane	47%±15%	2
100 μl PFBBr/ACN (15%) + 1 ml ACN	100 μl K_2CO_3	1 h - 100°C	SiOH	Hexane / Aceton	240%±7%	3
100 μl PFBBr/ACN (20%) + 1 ml ACN	100 μl K_2CO_3	1 h - 80°C	Drying (stream of nitrogen)		290%±7%	2
20 μl PFBBr + 1 ml ACN	100 μl K_2HPO_4	1 h - 100°C	SiOH	Hexane / Aceton	315%±10%	3
20 μl PFBBr + 1 ml ACN	100 μl K_2HPO_4	1 h - 100°C	Drying (stream of nitrogen)		325%±2%	3

SD: Standard deviation (%); TOL: Toluene; ACN: Acetonitrile; triet: triethylamine

C.6 Conditions for LLE Extractions of Tamoxifen and 5-Fluorouracil

TABLE C.10: Conditions for LLE extractions of Tamoxifen and 5-Fluorouracil in bi-distilled water (n=1)

Solvent	Volume of water	Volume of solvent	Recovery (n=1)	
			5-FU	TAM
Ethyl acetate	0.25 l	2 x 120 ml	0%	15%[a]
Dichloromethane	1 l (+ 100g NaCl)	3 x 60 ml	-	100-110%[b]
Diethyl ether	1 l (+ 100g NaCl)	1 x 120 ml and 2 x 60 ml	-	73-77%[b]

[a]Losses during the rotary evaporation

[b]2 ml of methanol was added to increase the stability of TAM during the rotary evaporation

C.7 Raw data of the Ames test including toxicity assays

All raw data of the results presented in Chapter 4 are shown here. See Section 4.2 on page 60 for a description of the assays and Section 4.2.6 on page 63 for a description of statistical analysis.

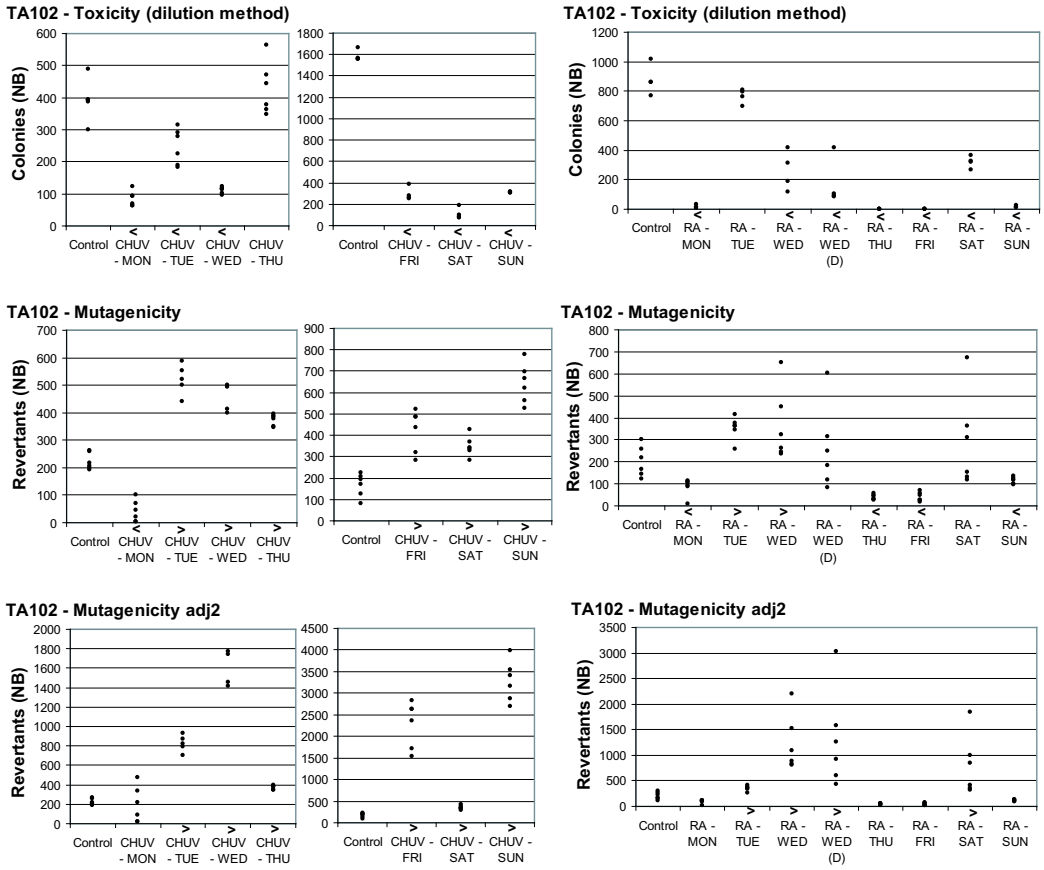

FIGURE C.2: TA102 - Raw data results of the mutagenicity and toxicity assays for the hospital (CHUV) and the Residential area of Lausanne (RA). <: statistically significant lower than in the control. >: statistically significant higher than in the control. D: sampling from 7am to 9pm

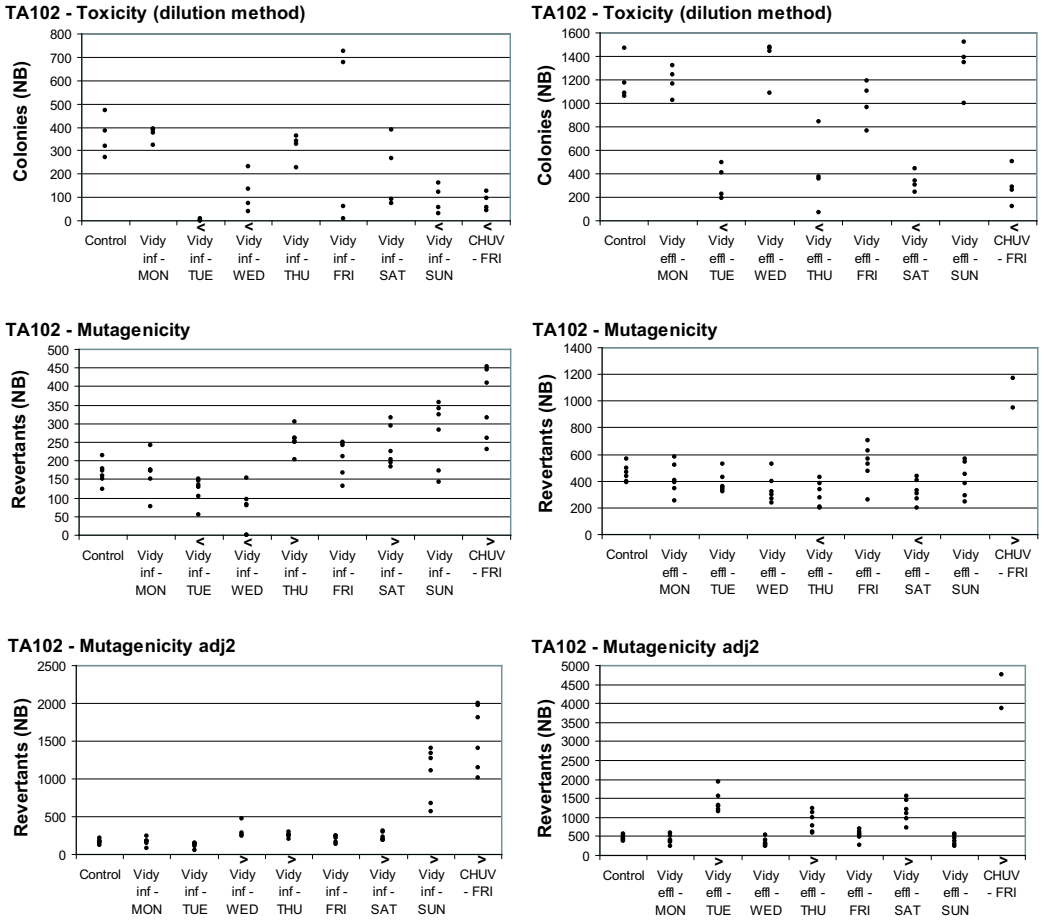

FIGURE C.3: TA102 - Raw data results of the mutagenicity and toxicity assays for Lausanne (Vidy) influents and effluents. <: statistically significant lower than in the control. >: statistically significant higher than in the control

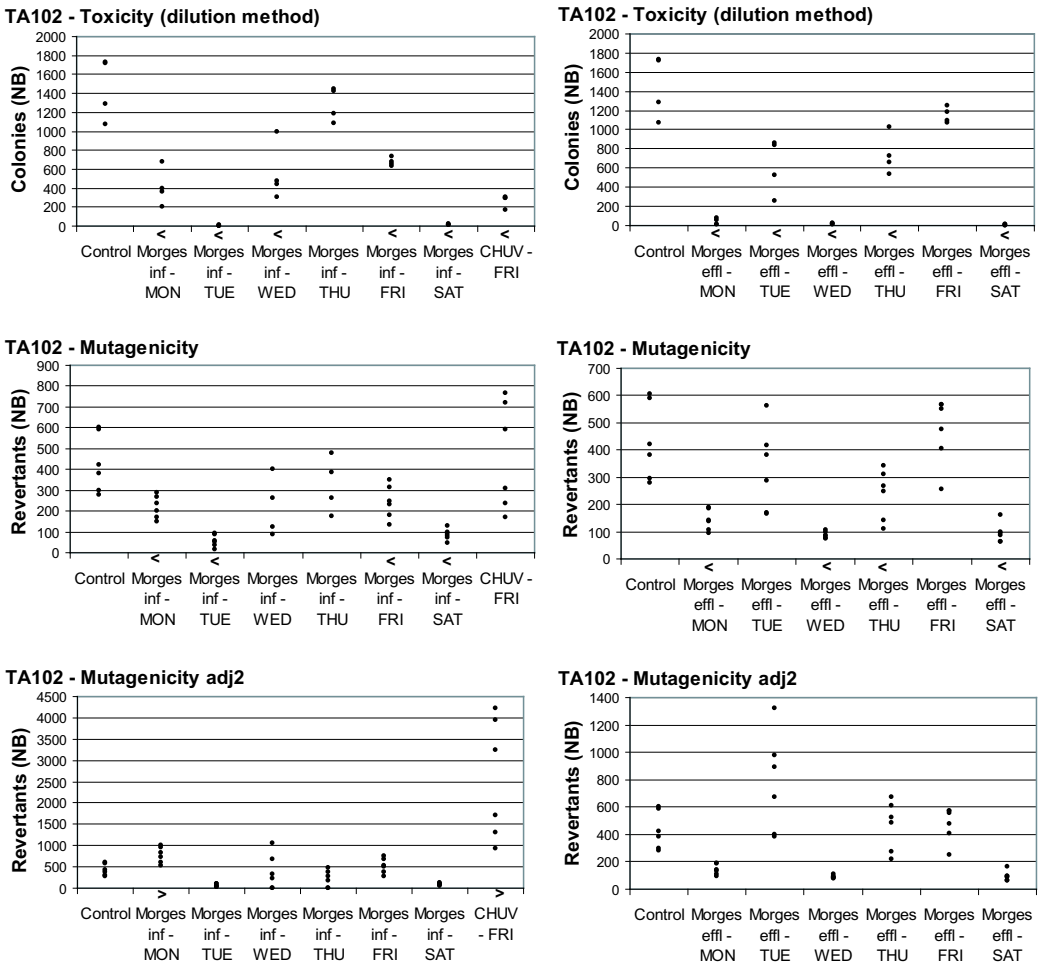

FIGURE C.4: TA102 - Raw data results of the mutagenicity and toxicity assays for Morges influents and effluents. <: statistically significant lower than in the control. >: statistically significant higher than in the control

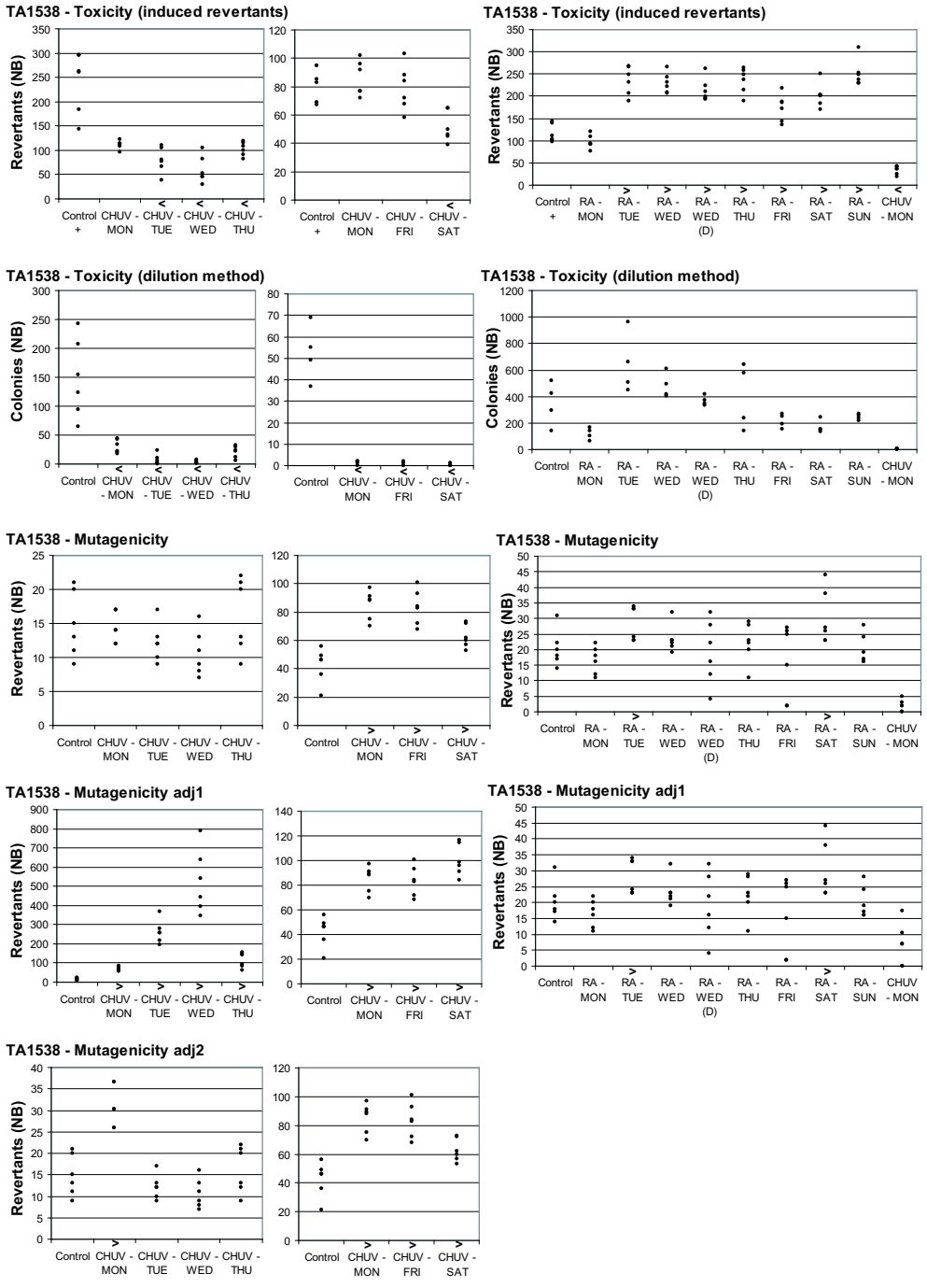

FIGURE C.5: TA1538 - Raw data results of the mutagenicity and toxicity assays for the hospital (CHUV) and the Residential area of Lausanne (RA). <: statistically significant lower than in the control. >: statistically significant higher than in the control. D: sampling from 7am to 9pm

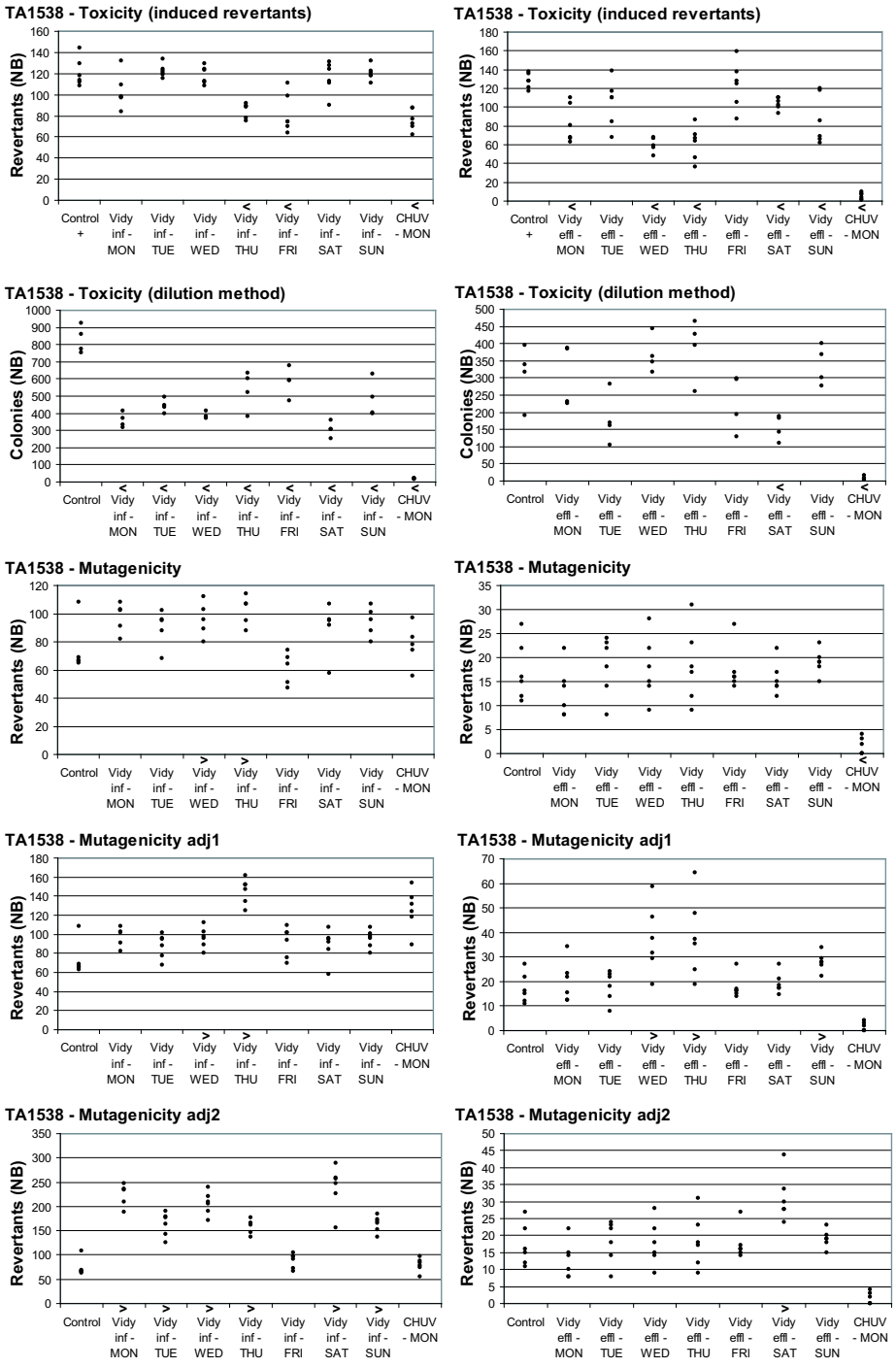

FIGURE C.6: TA1538 - Raw data results of the mutagenicity and toxicity assays for Lausanne (Vidy) influents and effluents. <: statistically significant lower than in the control. >: statistically significant higher than in the control

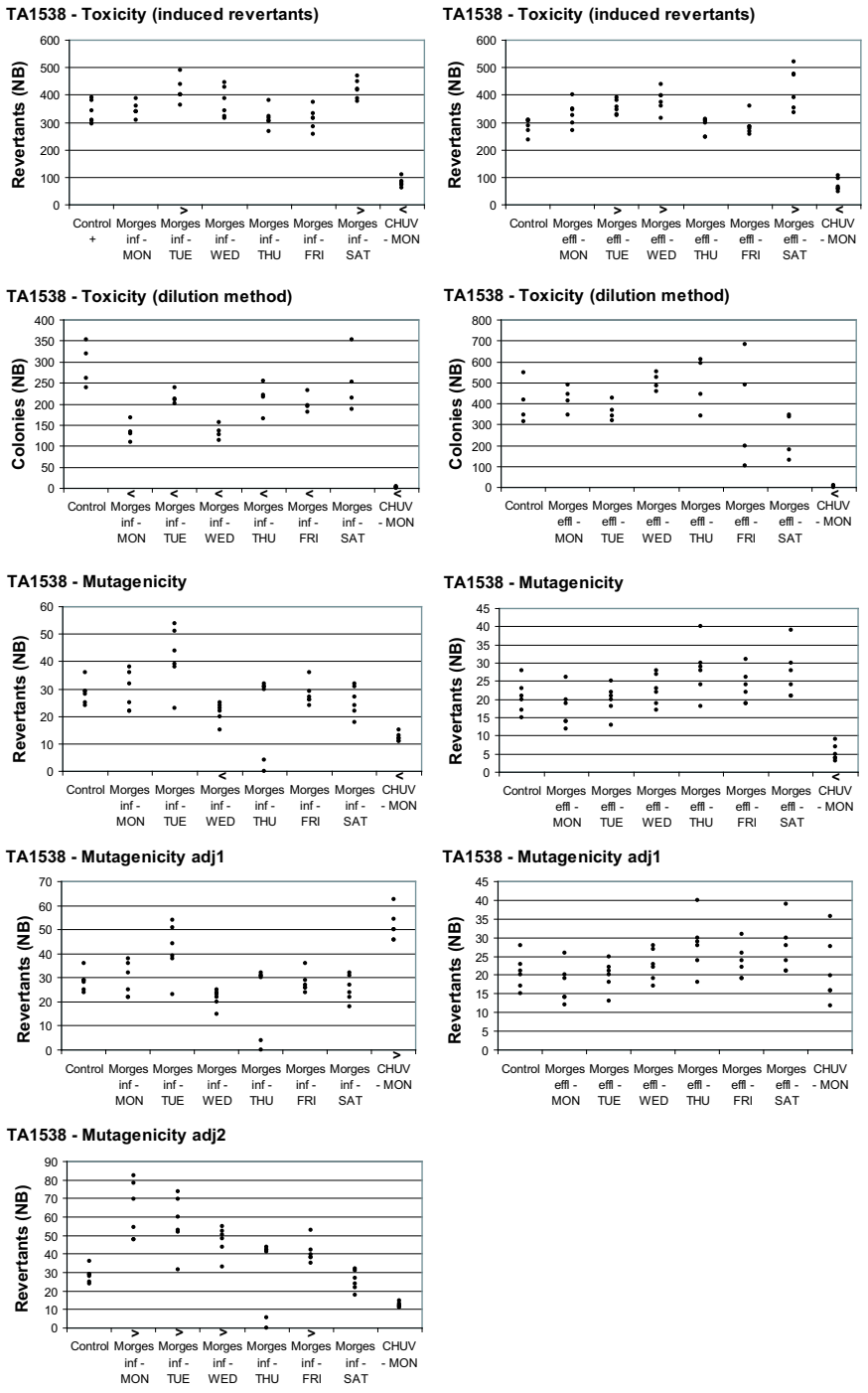

FIGURE C.7: TA1538 - Raw data results of the mutagenicity and toxicity assays for Morges influents and effluents. <: statistically significant lower than in the control. >: statistically significant higher than in the control

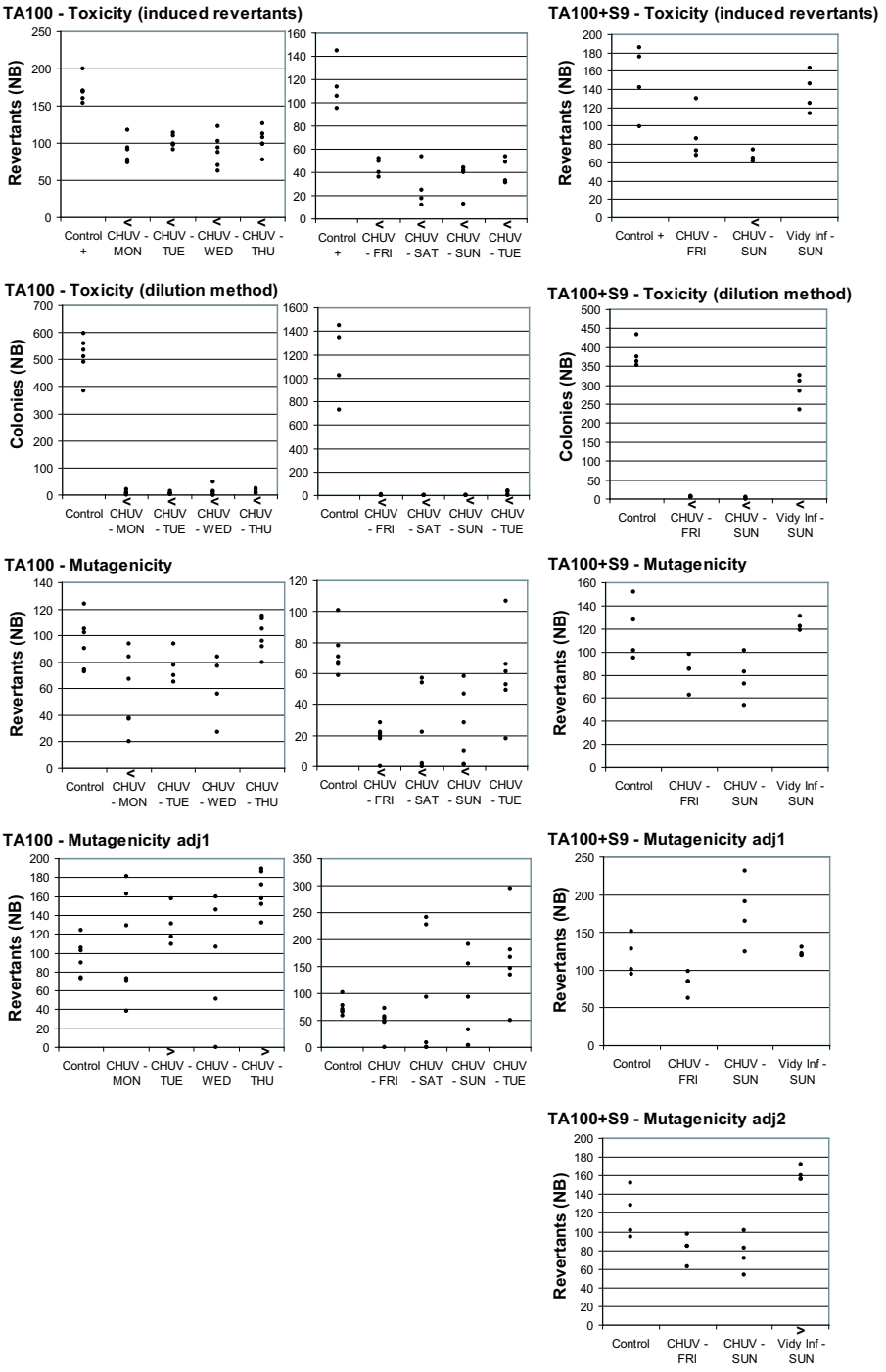

FIGURE C.8: TA100 - Raw data results of the mutagenicity and toxicity assays for the hospital (CHUV) with and without enzymatic activation (S9). <: statistically significant lower than in the control. >: statistically significant higher than in the control

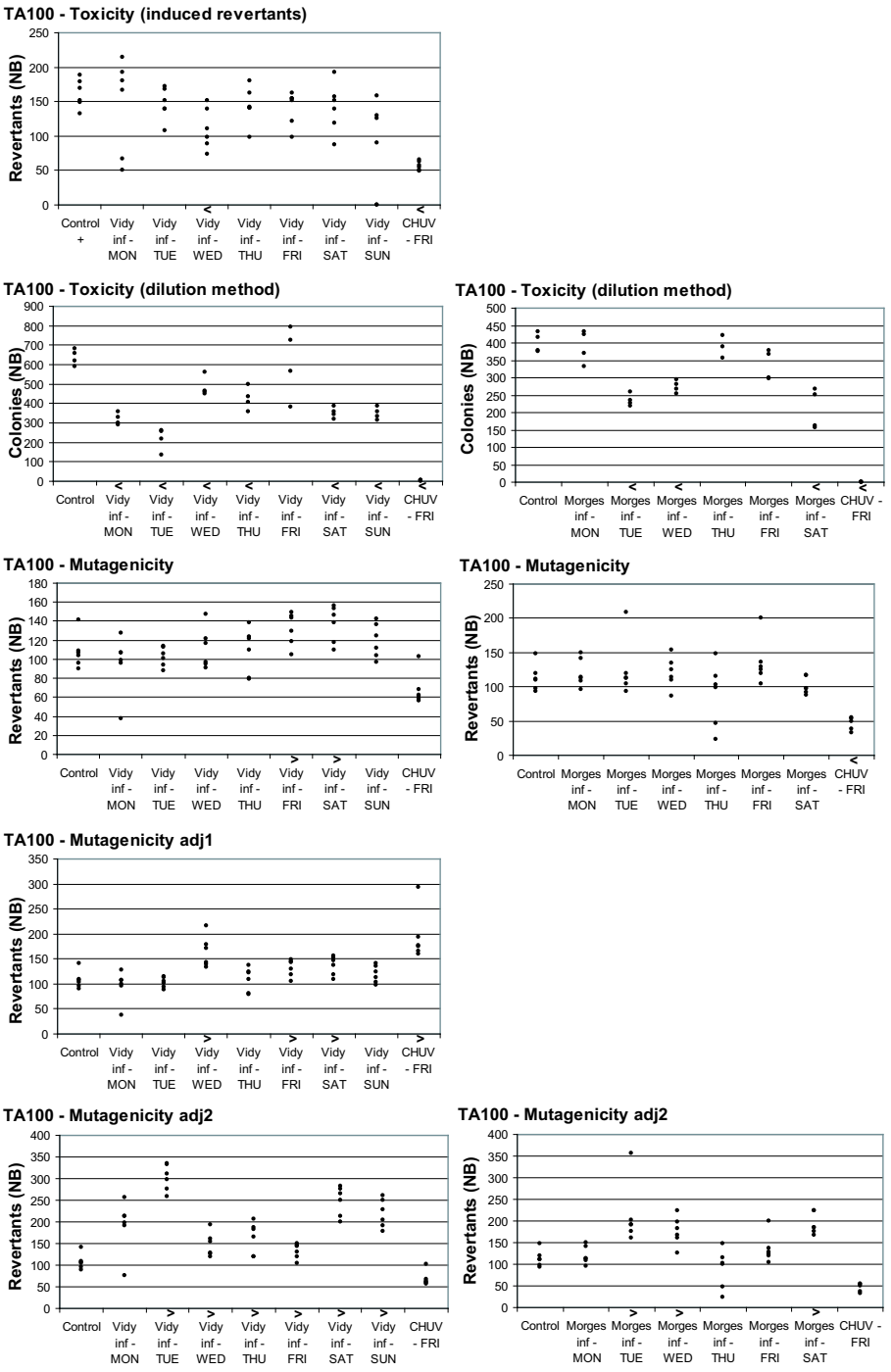

FIGURE C.9: TA100 - Raw data results of the mutagenicity and toxicity assays for Lausanne (Vidy) and Morges influents. <: statistically significant lower than in the control. >: statistically significant higher than in the control

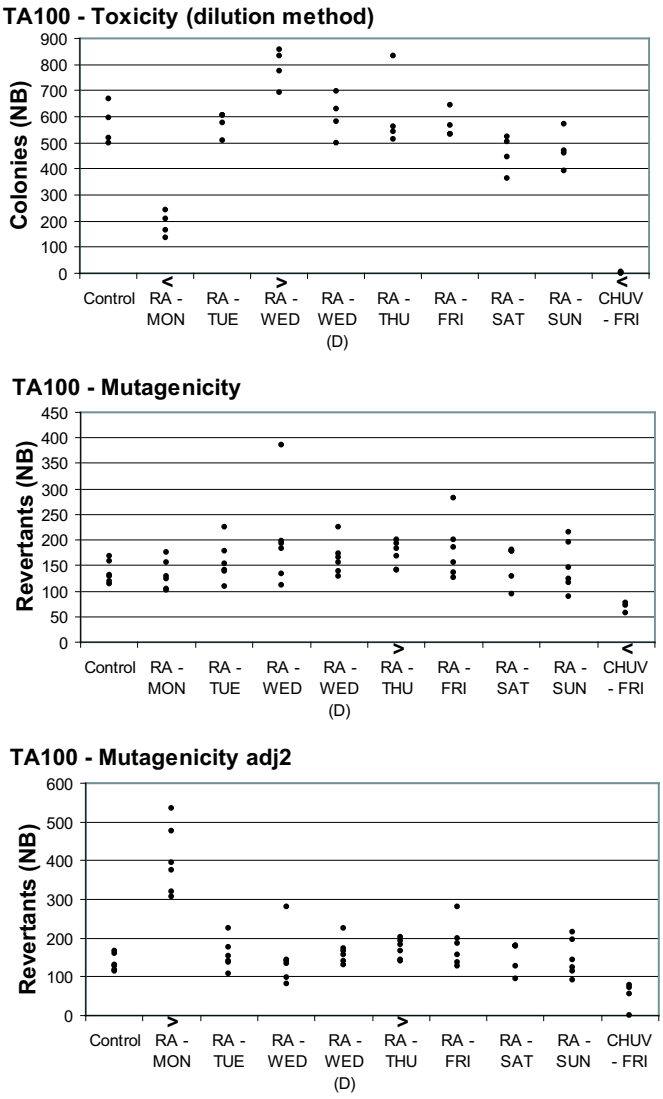

FIGURE C.10: TA100 - Raw data results of the mutagenicity and toxicity assays for the Residential area of Lausanne (RA). <: statistically significant lower than in the control. >: statistically significant higher than in the control. D: sampling from 7am to 9pm

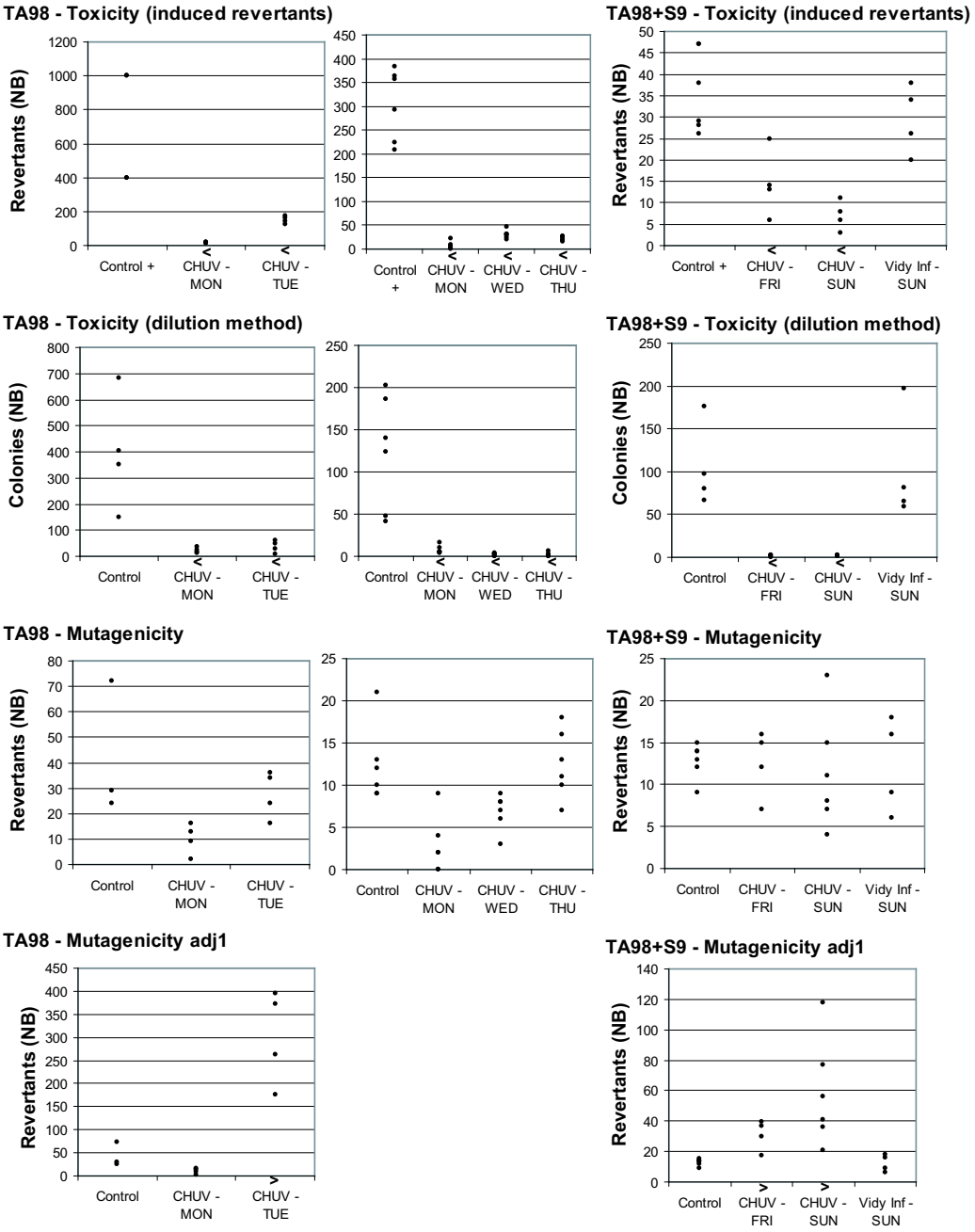

FIGURE C.11: TA98 - Raw data results of the mutagenicity and toxicity assays for the hospital (CHUV) with and without enzymatic activation (S9). <: statistically significant lower than in the control. >: statistically significant higher than in the control

Appendix D

Curriculum Vitae

Annick TAUXE WÜRSCH
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Professional achievements

2002-2005 PhD in the laboratory of environmental chemistry and ecotoxicology (CECOTOX) at EPFL under the supervision of Prof. Tarradellas. Study on the occurrence of pharmaceutical substances in wastewaters and their removal by sewage treatment plants. Determination of the genotoxicity of wastewaters including hospital with high contamination by drugs.

- Laboratory work: analytical methods development (SPE, LLE, GC/MS), and adaptation of genotoxicity test (Ames test)
- Risk assessment
- Student project supervisions

2001-2002 Assistant in the group "Life cycle assessment and durable development" of Prof. Jolliet at EPFL. Evaluation of human health impact of pollutants.

- Study of epidemiological and toxicological data of pollutants (air pollutants, metal, etc.)
- European project on fate and toxicity of pollutants (OMNITOX)
- Databases management

- Life cycle assessment
- Presentation (Sweden, Japan, Basel, etc.) and teaching

2001-2002 **Awareness Campaign** in school for "INFO-Energie", promoting energy saving to children.

1998-1999 Sciences, mathematics and computer **Teaching** for 10 to 16 year old children (La Tour-de-Peilz - Vaud).

1994 NESTEC SA, Vers-chez-les-blanc (Vaud). Two months internship in food research.

Scientific publications and Posters

- Tauxe-Wuersch A., Leong-Morgenthaler P., Tarradellas J., Mutagenicity of wastewaters from hospital and sewage treatment plants, To be submitted.

- Tauxe-Wuersch A., De Alencastro L. F., Grandjean D., Tarradellas J., Trace determination of Tamoxifen and 5-Fluorouracil in hospital and urban wastewaters, Accepted by Intern. J. Environ. Anal. Chem.

- Tauxe-Wuersch A., De Alencastro L. F., Grandjean D., Tarradellas J., 2005. Occurrence in sewage treatment plants and risk assessment of several acidic drugs in Switzerland. Water Research, Vol. 39, pp. 1761-1772.

- Tauxe A., Occurrence of several acidic drugs in sewage. Poster presented at ENVIRPHARMA, European conference on Human & Veterinary Pharmaceuticals in the Environment. 14-16 Avril 2003. Lyon.

- Soulet B., Tauxe A., Tarradellas J., 2002. Analysis of Acidic Drugs in Swiss Wastewaters. Intern. J. Environ. Anal. Chem., Vol. 82, No. 10, pp. 659-667.

- Pennington D., Crettaz P., Tauxe A., et al., 2002. Assessing Human Health Response in Life Cycle Assessment Using ED10s and DALYs: Part 2 - Non-cancer Effects. Risk analysis, Vol. 22, No. 5, pp. 947-963.

- Tauxe A., Health effects of gasoline, diesel and natural gas vehicles. Poster presented at the conference "Energy Technologies for a Sustainable Future" (November 23-24, 2000, PSI, Villigen) and at AGS meeting (January 14-17, 2001, EPFL, Lausanne).

Formations

2001-2003 Complementary Certificate in toxicology (CECT) organised by the "Lemanic Network of Toxicology" (Lausanne and Geneva Universities and EPFL).

- General toxicology - Carcinogenesis - Neurotoxicology
- Genetic toxicology - Analytical toxicology

1999-2001 Postgraduate in environmental sciences (CPSE) at the Swiss Federal Institute of Technology of Lausanne. Nine months research work on "Health Effects of Gasoline, Diesel and Natural Gas Vehicles" awarded with the **"prix ARPEA 2002"** and published in their journal of April 2002.

1994-1998 Licence in biology at the University of Lausanne, including certificates in Zoology, Ecology, Neurosciences & Endocrinology, and Organisms & Populations Biology.

Others

- Organisation of "Nature" excursions and holiday camps for children with Pro-Natura, 1999-2001.
- Training in children education for nature excursion (CEMEA, JV et Pro-Natura, Jeunesse + Sport).
- Horse riding, Theatre, Photography.