

Vers le développement de composés pharmaceutiques durables : Caractérisation de la dégradation et évaluation écotoxicologique d'un analogue d'anticancéreux eco-conçu

Anaïs Espinosa

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par

Anaïs ESPINOSA

Vers le développement de composés pharmaceutiques durables :

Caractérisation de la dégradation et évaluation écotoxicologique d'un analogue d'anticancéreux éco-conçu

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Introduction générale

La présence de résidus de médicaments dans le milieu aquatique est révélée dès la fin des années 70 (Hignite et Azarnoff, 1977) et depuis, l'amélioration des techniques analytiques a permis de détecter des concentrations de plus en plus faibles, révélant alors la présence d'une grande variété de composés. Après excrétion par le patient, les composés pharmaceutiques et leurs métabolites se retrouvent dans les eaux usées et sont souvent partiellement éliminés en station d'épuration (STEP) (Lin et al., 2009). Les transformations que vont subir ces résidus, au cours des traitements des eaux, ou bien dans l'environnement, par des processus de biodégradation et/ou de photodégradation, peuvent conduire à des produits de transformations, appelés TPs, qui peuvent parfois être, (i) plus persistants que les composés parents, (ii) conserver le mode d'action initial et même (iii), être plus toxiques que celui-ci. Ces mélanges de composés et de TPs peuvent atteindre les eaux de surface, les eaux souterraines et contaminent même les eaux de boissons (aus der Beek et al., 2016). Bien que les concentrations rapportées soient de l'ordre du ng/L, ces composés sont spécialement conçus pour exercer des effets biologiques à de faibles concentrations (Sanderson et al., 2003) et ont des impacts sur la santé des écosystèmes comme sur la santé humaine. Citons par exemple l'impact du diclofénac mis en cause dans l'extinction d'une espèce de vautours (Oaks et al., 2004) ou encore les effets observés chez les poissons exposés à des résidus d'hormones synthétiques (Kidd et al., 2007). Les effets perturbateurs endocriniens (Tijani et al., 2013) ou encore sur le phénomène d'antibiorésistance (Martinez, 2009) sont devenus des enjeux de santé globale préoccupants.

La communauté scientifique alerte aussi sur la famille des anticancéreux, en raison de leur utilisation croissante, de leur puissant mode d'action et de leur détection dans les compartiments aquatiques. En effet, les cancers sont la seconde cause de mortalité dans le monde. Avec 19 millions de cas recensés en 2020 et une estimation à 29 millions de cas de nouveau cancer en 2040 (*International Agency for Research on Cancer*)[1] la demande en traitement de chimiothérapie continue de croître à un taux de l'ordre de 10% par an (Johnson et al., 2008). Les anticancéreux représentaient 14,4 % du marché en 2020 contre seulement 4,3% en 2012 (*les entreprises du médicaments)*[2] et face à la demande, de nouvelles molécules ne cessent d'être développées (Tripathi et al., 2020).

Parmi les centaines de composés utilisés pour le traitement des cancers, la classe des antinéoplasiques a pour objectif de détruire ou de ralentir la prolifération des cellules malignes. Ils agissent en causant des dommages à l'ADN ou en empêchant la réplication des chromosomes et entraînent des altérations métaboliques et morphologiques de la cellule, conduisant à la mort cellulaire

[1] International Agency for Research on Cancer 2020, URL https://gco.iarc.fr. (accessed 2.11.22).
[2] Les entreprise du médicament (LEEM), URL https://www.leem.org/marche-mondial. (accessed 2.11.22).

(Johnson et al., 2008; Besse et al., 2012; Booker et al., 2014). Ces composés agissent sur la structure et les fonctions de l'ADN de toutes les cellules et non uniquement sur les cellules cancéreuses, ce mode d'action non spécifique rend ces composés cytotoxiques, mutagènes, tératogènes et cancérigènes (Kümmerer et al., 2000; Johnson et al., 2008; Tripathi et al., 2020). Plusieurs auteurs rapportent leur présence dans l'environnement, jusqu'aux eaux de surface et souterraines et même après potabilisation (Besse et al., 2012; Booker et al., 2014 ; Li et al., 2021). Les très faibles concentrations rapportées pourraient cependant induire des effets sur les organismes aquatiques (Zounková et al., 2007; Kleinert et al., 2021). De plus, leur présence en cocktail peut mener à des effets synergiques (Parrella et al., 2014a). Leurs occurrences et les risques qu'ils représentent sont cependant encore trop peu étudiés (Olalla et al., 2018).

L'étude approfondie de l'écodynamique de ces contaminants organiques doit permettre une meilleure évaluation des risques qu'ils représentent. En parallèle, cette prise de conscience nous pousse à envisager des solutions innovantes pour prévenir cette contamination. Des traitements complémentaires peuvent être ajoutés en amont des STEP, par exemple dans des stations spécifiques au traitement des effluents d'hôpitaux, ou bien en aval, tels que les traitements d'oxydation avancés (AOPs) qui ont fait leurs preuves dans la remédiation de médicaments. Mais ces traitements complémentaires sont cependant coûteux en réactifs et en énergie, certains ne peuvent être appliqués à de gros volumes d'eau à traiter (Nikolaou et al., 2007) et ils peuvent de plus mener eux aussi à des produits de transformation plus persistants ou toxiques que le composé parent (Escher et Fenner, 2011) : ils ne représentent donc pas toujours une solution pérenne.

Nous savons que la structure d'une molécule définit ses propriétés et cette relation trouve son application dans le contexte pharmacologique. Cette réflexion autour de la structure de molécule peut être également appliquée à des fins environnementales. En effet, parmi les différents leviers pour limiter la contamination et les dangers, le concept appelé « benign by design » proposerait alors une molécule éco-conçue qui se dégraderait facilement et mènerait à des produits de transformation inoffensifs une fois excrétés dans l'environnement. Ce travail de thèse se penche sur une approche d'éco-conception appliqué à une molécule modèle, capable d'accueillir une modification de sa structure.

Le méthotrexate (MTX) est l'un des agents antinéoplasiques les plus prescrits dans le monde (Kosjek et Heath, 2011). Analogue de l'acide folique, il fait partie de la famille des antimétabolites, il inhibe la dihydrofolate-réductase (DHFR) et impacte la division cellulaire. Le MTX est utilisé pour traiter des cancers mais aussi pour traiter des cas de psoriasis ou de maladies auto-immunes comme la polyarthrite rhumatoïde (Sabry et al., 2003), son action tératogène est aussi utilisée pour des avortements (Lloyd, 1999). C'est l'un des trois antimétabolites le plus fréquemment détectés dans les eaux usées (Li et al., 2021). Même s'il ne fait pas parti des plus persistants, il est tout de même détecté

en sortie de STEP et même dans des eaux de rivière (Ferrando-Climent et al., 2014; Rabii et al., 2014). De plus, un de ses métabolites, le 7-OH-MTX (Widemann et al., 2000), semble aussi se former par des boues de STEP (Kiffmeyer et al., 1998; Kosjek et al., 2015). Son mode d'action potentiellement toxique, son évolution et sa persistance restent discutés, c'est pourquoi Besse et al. (2012) soulignent que la formation de ce métabolite doit être plus étudié et l'inclut dans la liste des composés pharmaceutiques prioritaires pour leur évaluation.

Dans cette étude, l'approche d'éco-conception repose sur la synthèse d'un bioisostère au MTX dans lequel un atome d'oxygène remplace le groupement N-méthyle. Cet analogue, appelé OMTX, présente une activité anti tumorale *in vivo* comparable au MTX contre la leucémie lymphoïde L-1210 chez la souris (Nair et al., 1981) et aurait donc potentiellement le même mode d'action thérapeutique.

L'hypothèse est alors que cette modification rendrait la molécule plus facilement fragmentable, donc moins persistante et dont la dégradation conduirait à la formation de produits de transformation différents et éventuellement moins toxiques que le MTX. Le travail de cette thèse présente donc une étude comparative entre le devenir et les effets du MTX et ceux du OMTX.

Ce manuscrit est présenté sous un format de thèse sur articles. Un **premier chapitre** introductif regroupe la synthèse bibliographique concernant les problématiques liées à la contamination des eaux par les pharmaceutiques, notamment par les anticancéreux, ainsi que l'évaluation de leurs effets. Ce chapitre présente aussi les leviers existants afin de limiter la contamination de l'environnement et plus particulièrement l'éco-conception de molécules. Ce levier est illustré dans le cas de la molécule d'intérêt, le methotréxate (MTX) et de son analogue éco-conçu l'OMTX étudié dans le cadre du projet ANR EDIFIS coordonné par R. Labruère (Institut de Chimie Moléculaire et des Matériaux d'Orsay, ICCMO). **Le second chapitre** présente ensuite l'état de l'art sur les processus impliqués dans le devenir de résidus de médicaments dans l'environnement, notamment des étapes de transformation menant à la formation de métabolites ou produits de transformation et sur les différentes méthodes analytiques permettant leur suivi et leur identification. Ces deux chapitres bibliographiques sont clos par une présentation des objectifs de la thèse et de la démarche adoptée.

Le **Chapitre 3** présente les résultats de l'étude de photodégradation du MTX et de son analogue éco-conçu avec un premier article paru dans la revue Environmental Science and Pollution Research portant sur le MTX et une deuxième partie sur l'analogue d'intérêt avec un deuxième article en cours de révision pour Chemosphere.

Le **Chapitre 4** présente l'étude de la biodégradation par des boues de station d'épuration des deux molécules. Ce chapitre est découpé en deux sous-parties, la première présente le développement

analytique nécessaire au suivi des produits de transformation dans les matrices d'intérêt et une deuxième sous partie présente les résultats de biodégradation. Cette étude est présentée sous format de manuscrit, la partie biodégradation (**4.2**) pourra être soumise à des revues telle que Environmental Science and Pollution Research, Chemosphere ou Water Research en étant adaptée et complétée avec des éléments présentés dans la partie développement analytique (**4.1**).

Le **Chapitre 5** présente les résultats des tests d'écotoxicité aquatique réalisés à l'INERIS sous la forme d'un manuscrit qui sera soumis prochainement à une revue telle que Ecotoxicology and Environmental Safety.

Et finalement le **Chapitre 6** discute de la portée de ce travail en mettant en lumière les réponses que nous avons apportées aux différentes questions sur l'intérêt de l'éco-conception. Au regard des limitations entrevues et des connaissances actuelles, cette discussion générale propose enfin différentes perspectives de recherche entrouvertes par cette étude.

Chapitre 1. Eco-conception: un levier pour réduire les impacts environnementaux des résidus de médicaments

1.1. Présence de résidus de médicaments dans les eaux et conséquences pour la santé globale

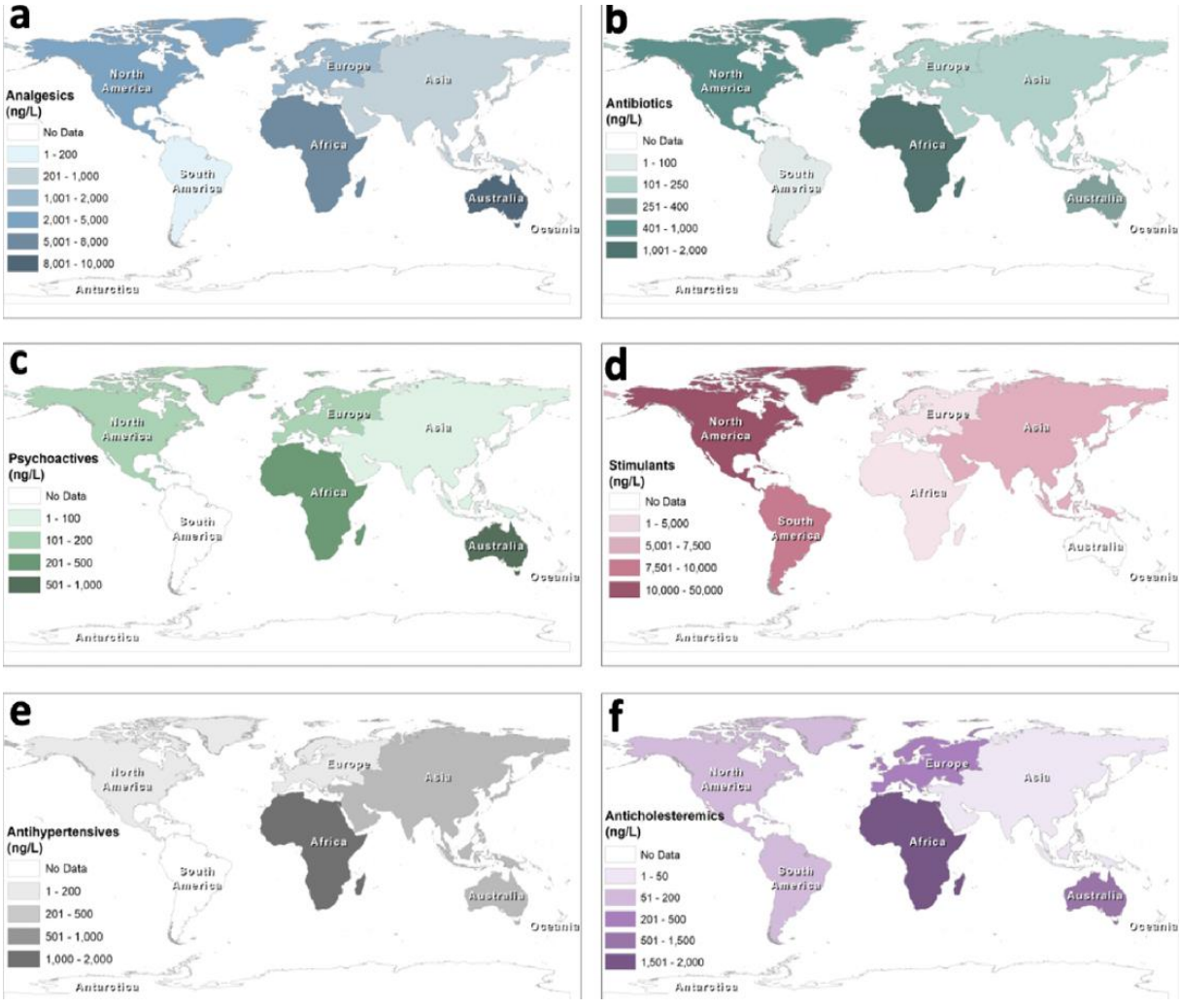
1.1.1. Contamination des milieux aquatiques par les résidus de médicaments

1.1.1.1. De contamination dite émergente à problématique généralisée à l'échelle globale

La découverte de la présence de résidus de médicaments dans les eaux remonte aux années 70. En 1977, Hignite et Azarnoff rapportent la présence d'acide clofibrique et d'acide salicylique dans des effluents de stations d'épuration. Par la suite, les études se multiplient et relatent la présence de composés d'une grande variété de classes de pharmaceutiques dans les eaux usées, mais aussi dans des eaux souterraines, des cours d'eau et de l'eau potable à travers l'Europe (Ternes, 1998; Daughton et Ternes, 1999; Aherne, 1985; Heberer et Stan, 1997). L'évolution des techniques analytiques a permis de descendre à des concentrations de l'ordre du ng/L au pg/L révélant alors la présence de plus en plus de composés. Ils entrent alors dans la liste des « contaminants of emerging concern », une substance chimique est dite « émergente » tant que les informations relatives à cette substance ainsi que les potentiels risques qu'elle représente sont mal documentés dans la littérature scientifique (Sauvé et Desrosiers, 2014).

Les composés pharmaceutiques se retrouvent dans les effluents d'eaux usées en raison de leur métabolisation partielle dans le corps du patient et de leur excrétion via les urines et les fèces (Besse et al., 2012). Cependant ils peuvent aussi provenir des rejets des fabricants menant alors à des concentrations jusqu'à 1 000 fois plus élevées selon une enquête nationale menée aux États-Unis (Phillips et al., 2010). La revue d'Adeleye et al. (2022) fait l'état des lieux de la contamination des eaux usées avant traitement, à travers le monde et en fonction des classes de pharmaceutiques les plus connues. La Figure 1- 1 est extraite de cette revue et présente les concentrations médianes dans des eaux usées pour 6 classes de composés. Il ressort de cette revue que les concentrations les plus hautes concernent les stimulants atteignant une médiane maximale en Amérique du Nord de 43 100 ng/L, le composé le plus souvent détecté est la caféine, qui est une des substances les plus consommées au monde. Ensuite ce sont les analgésiques qui sont présents à des concentrations très élevées dans les eaux usées comme par exemple l'acide acétylsalicylique (aspirine), le diclofénac et le kétoprofène, couramment détectés dans les eaux avec des médianes allant de 105 ng/L en Amérique du Sud à 8970 ng/L en Australie. Vient ensuite la classe des antibiotiques avec des médianes proches en Europe, Asie et Australie (200-300 ng/L), une médiane presque deux fois plus élevée en Amérique du Nord et encore plus élevée en Afrique (1500 ng/L). Les composés les plus courants sont la ciprofloxacine, le sulfaméthoxazole, l'érythromycine, le triméthoprime et la tétracycline. Les antihypertenseurs sont

détectés dans la même fourchette de concentrations, ce sont souvent l'aténolol, le propranolol, le métoprolol, le valsartan et le sotalol. Concernant les anticholestérolémiants, les médianes rapportées en Afrique et en Australie sont bien plus élevées qu'en Asie (*avec seulement 6 et 3 données contre 36 pour l'Asie*). Et pour finir la classe des psychotropes avec la carbamazépine, la fluoxétine, l'amitriptyline et la venlafaxine pour les plus signalés, sont présents dans les influents à des concentrations jusqu'à trois fois inférieures à celles des analgésiques et des antibiotiques. Dans l'ensemble, les concentrations médianes de divers psychoactifs signalées dans les eaux usées vont de 82 ng/L en Asie à 132 ng/L en Amérique du Nord.



Classe Concentration médiane (ng/L) *nombre de données utilisées*	Amérique du Nord	Amérique du Sud	Afrique	Europe	Asie	Australie
a) Analgesiques	2485	105	8000	1707	540	8970
	178	*3*	*91*	*300*	*292*	*17*
b) Antibiotiques	409	62	1530	244	225	300
	193	*7*	*55*	*422*	*516*	*42*
c) Psychoactifs	132	NA	334	129	82	685
	65		*18*	*200*	*73*	*5*
d) Stimulants	43100	8200	4787	4870	5672	NA
	31	*3*	*12*	*37*	*60*	
e) Antihypertenseurs	158	NA	1686	155	304	322
	70		*12*	*182*	*67*	*9*
f) Anticholestérolémiants	146	172	1782	238	31	1470
	50	*4*	*6*	*62*	*36*	*3*

Figure 1- 1 Etat des lieux de la contamination d'eaux usées avant traitement pour différentes classes de médicaments à travers le monde

Selon Adeleye et al. (2022) les différences d'un continent à l'autre dépendent de différents facteurs tels que la densité de population, la démographie desservie par les stations d'épuration (habitant/ WWTP), la présence d'hôpitaux et d'usines de production ainsi qu'à l'accessibilité aux soins de santé et aux habitudes de consommation. Par exemple, la fréquence élevée d'analgésiques dans les eaux usées en Afrique du Sud pourrait être liée à la densité de population par station d'épuration, ou encore à une élimination inappropriée, tandis que des niveaux élevés d'antibiotiques signalés au Kenya, peuvent être dus à l'utilisation généralisée de combinaisons de sulfaméthoxazole et de triméthoprime pour le traitement les infections survenant chez les personnes vivant avec le VIH/SIDA.

Gros et al. (2007) rapportent des efficacités d'élimination très variables, dépendantes du type de traitement appliqué ainsi que des propriétés des composés. C'est pourquoi il est difficile d'établir une tendance générale pour chacune des familles de médicaments. Les différents mécanismes influant sur l'élimination des composés seront détaillés en **Chapitre 2** de ce manuscrit. Finalement, les composés encore présents en sortie de ces STEP vont contaminer les eaux naturelles.

aus der Beek et al. (2016) ont analysé 1016 publications et 150 revues sur cette thématique et recensé pas moins de 631 pharmaceutiques détectés dans des eaux naturelles (eaux de surface, eaux souterraines et eaux potables) sur les 713 analysés. Parmi tous ces composés, 16 sont retrouvés dans toutes les eaux de surface des pays étudiés et leurs concentrations dans les eaux de surface sont présentées en Tableau 1- 1.

Tableau 1- 1 Concentrations moyennes et maximales d'une sélection de pharmaceutiques dans les eaux naturelles à travers le monde extrait de la revue de aus der Beek et al. (2016). Le nombre de données étudiées est donné entre parenthèses

	Global	
Pharmaceutical substance	Average	Maximum
Diclofenac	0.032 (7017)	18.74
Carbamazepine	0.187 (25115)	8.05
Ibuprofen	0.108 (6950)	303.0
Sulfamethoxazole	0.095 (8599)	29.0
Naproxen	0.050 (3229)	32.0
Estrone	0.016 (2228)	5.0
Estradiol	0.003 (297)	0.012
Ethinylestradiol	0.043 (1530)	5.9
Trimethoprim	0.037 (3060)	13.6
Paracetamol	0.161 (937)	230.0
Clofibric acid	0.022 (2947)	7.91
Ciprofloxacin	18.99 (672)	6500
Ofloxacin	0.278 (760)	17.7
Estriol	0.009 (790)	0.48
Norfloxacin	3.457 (628)	520.0
Acetylsalicylic acid	0.922 (254)	20.96

En France, selon le rapport de l'Académie nationale de Pharmacie (2019), les études menées par le Bureau de recherches géologiques et minières (BRGM) et l'Office national des eaux et des milieux aquatiques (ONEMA, aujourd'hui OFB) en métropole et dans les DOM dans le cadre d'une étude sur les micropolluants organiques, rapportent la présence de résidus provenant de différentes classes pharmaceutiques dans les eaux souterraines avec des concentrations de l'ordre du ng/L pour une large variété de composés (Lapworth et al., 2012). Parmi 60 résidus de médicaments détectés, les plus fréquents étaient le paracétamol, la carbamazépine ou encore l'hydrochlorothiazide (Lopez et al., 2015). Les travaux du site pilote SIPIBEL rapportent quant à eux la détection fréquente du sulfaméthoxazole (Lecomte et al., 2018).

Mompelat et al. (2011) quantifient 16 composés pharmaceutiques sur les 20 sélectionnés, dans les eaux de surface et dans les eaux potables, principalement des psychostimulants, anti-inflammatoires non stéroïdiens, des produits de contraste iodés et des anxiolytiques. En effet, les composés les plus persistants peuvent atteindre l'eau potable, la revue de aus der Beek et al. (2016) recense entre 11 et 30 composés au Canada, en Chine, en France, en Suède et aux États-Unis.

Les métabolites et produits de transformation (TPs) sont de plus en plus souvent analysés. Leur présence dans les différents compartiments aquatiques peut poser la même problématique que les composés parents (López-Serna et al., 2012; López-Serna et al., 2013). Une seule molécule peut conduire à une grande variété de TPs. Haddad et al. (2015) recensent 158 TPs formés dans l'environnement pour 15 composés (antibiotiques et anticancéreux) ; certains sont spécifiques à un seul processus, tandis que d'autres peuvent être formés en conditions abiotiques et biotiques. Bonnot et al. (2022) ont recensé

1371 TPs pour 116 composés pharmaceutiques dans une revue bibliographique récente consacrée au devenir environnemental des produits de transformation de produits pharmaceutiques et de soins personnels. Nous aborderons la question des risques posés par ces TPs dans la suite de ce chapitre.

Il est à noter que certaines classes de composés sont moins étudiées. C'est le cas des anticancéreux, qui représentaient encore une minorité des études jusqu'en 2009 (Figure 1- 2). Depuis, de plus en plus d'auteurs se sont intéressés à cette catégorie de médicaments comme illustré dans la partie suivante.

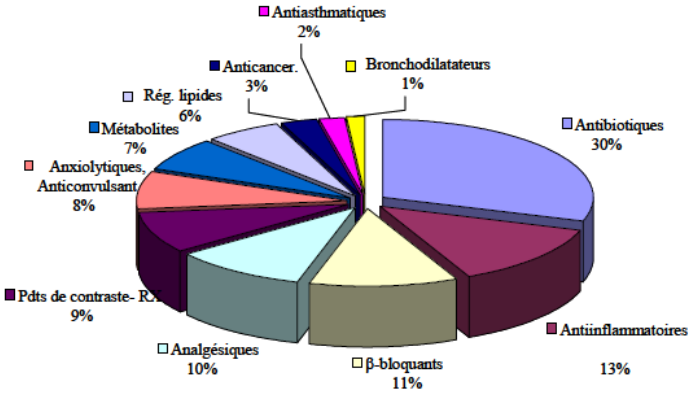


Figure 1- 2 Inventaire des produits pharmaceutiques recherchés par classe thérapeutiques, extrait de Coetsier (2009a)

1.1.2. Le cas des anticancéreux

Comme la majorité des pharmaceutiques, les anticancéreux sont partiellement métabolisés par le corps humain allant d'un taux d'excrétion de la molécule inchangée de 5 à 100% (Turci et al., 2003; Besse et al., 2012). Sur 26 molécules recensées par Besse et al. (2012), plus de la moitié des composés sont excrétés à plus de 50 %. Les principes actifs et leurs métabolites se retrouvent donc dans les effluents d'hôpitaux, mais aussi dans les effluents domestiques. En effet, seulement 13,8 % des anticancéreux utilisés en France sont à attribuer aux hôpitaux, le traitement à domicile est donc majoritaire et par conséquent, les hôpitaux ne sont pas la principale source de leur introduction dans les eaux usées (Johnson et al., 2008; Mompelat et al., 2011; Besse et al., 2012).

Les anticancéreux sont classés comme déchets dangereux selon la Directive n° 2018/C 124/01 de la Commission Européenne, mais leur présence reste moins étudiée que d'autres composés comme vu précédemment (Figure 1- 2). Li et al. (2021) recensent la détection de 33 anticancéreux dans les milieux aquatiques. Les plus couramment étudiés sont le 5-fluorouracile, la cyclophosphamide, le tamoxifène, l'ifosfamide et le méthotrexate (Nassour et al., 2020).

Le Tableau 1- 2 regroupe les anticancéreux pour lesquels nous avons des données des effluents d'hôpitaux jusqu'aux eaux de surface. Dans les effluents d'hôpitaux les plus chargés, après le 5-fluorouracile, qui présente un maximum très élevé car les échantillons proviennent d'un département d'oncologie, l'ifosfamide est détecté entre 4 et 86 µg/L et le méthotrexate avec des concentrations dépassant les 4 µg/L en Slovénie, Chine et Espagne. En France les valeurs sont plus basses avec 900 ng/L pour la cyclophosphamide et 200 ng/L pour le méthotrexate contre environ 4700 ng/L en Espagne.

Pour les influents de STEP provenant de rejets domestiques, les valeurs sont très élevées pour la cyclophosphamide et l'ifosfamide avec 13100 et 2900 ng/L. Le méthotrexate est détecté jusqu'à 300 ng/L en Slovénie, il est aussi retrouvé au Canada de l'ordre de 60 ng/L et à des concentrations maximales au moins deux fois plus basses en Espagne et en France. Dans les effluents de STEP, Catastini et al. (2008) le détectent aux alentours de 30 ng/L en France, en Espagne sa concentration est bien plus basse qu'en amont avec jusqu'à 6 ng/L. Cependant au Canada, en sortie de STEP, Rabii et al. (2014) retrouvent le même niveau de concentration de méthotrexate qu'en entrée, soulevant qu'il n'est pas forcément abattu pendant le traitement avec des concentrations allant jusqu'à la cinquantaine de ng/L.

Dans les eaux de surface le tamoxifène, le 5-fluorouracile et la cyclophosphamide sont retrouvés à de hautes concentrations (100-200 ng/L) en Grande Bretagne, Chine et Australie, alors qu'en France il est détecté de l'ordre de 25 ng/L. Pour le méthotrexate, Ferrando-Climent et al. (2014) le détectent dans une rivière juste après une STEP, au même niveau de concentration que dans l'effluent dont il est issu (jusqu'à 5 ng/L). Finalement, Le méthotrexate a été peu recherché et/ou observé dans des eaux de surface. En effet, même si plusieurs plublications mentionnent une concentration de 6,25 ng/L en citant comme référence Aherne (1985), il s'avère en réalité que Aherne rapporte < 6,25 ng/L, qui correspond à sa limite de quantification

Concernant les données dans les eaux souterraines ou les eaux potables, il n'existe que quelques valeurs pour ces composés : le tamoxifène avec un maximum de 223 ng/L dans des eaux souterraines en Pays (López-Serna et al., 2013) et la cyclophosphamide détecté dans l'eau potable aux environ de 4 ng/L en Chine (Gu et al., 2019).

Tableau 1- 2 Concentrations d'anticancéreux (ng/L) dans différents échantillons aqueux, adapté de la revue de Li et al. (2021)

a) avant entrée en STEP

Composé Conc. ng/L	Pays	Effluents d'hopitaux	Références	Influents de STEP municipales		
Cyclophosphamide	Slovénie	1,080 - 22,100	Isidori et al., 2016	Espagne	< 0.35 - 13,100	Gómez-canela et al., 2012
	Slovénie	22	Česen et al., 2015	Espagne	< 9.3 - 43.8	Negreira et al., 2014
	Espagne	5,73	Gómez-canela et al., 2012	Suisse	< 6 - 143	Steger-Hartmann et al., 1997
	Espagne	< 4 - 4,720	Gómez-canela etVentura, 2014	Slovénie	19 - 27	Isidori et al., 2016
	Suisse	4,486	Steger-Hartmann et al., 1997	Slovénie	< 0.78 - 27	Česen et al., 2015
	Espagne	46 - 3,000	Olalla et al., 2018	Espagne	8 - 26	Ferrando-Climent et al., 2014
	Chine	< 2.0 - 2,000	Yin et al., 2010	Canada	< 4 - 22	Rabii et al., 2014
	Japon	8 - 1,499	Azuma et al., 2016	Chine	12	Lin et al., 2014
	Chine	15 - 1,200	Lin et al., 2014	Suisse	2 - 11	Buerge et al., 2006
	France	30 - 900	Catastini et al., 2008	Espagne	< 4 - 10	Gómez-canela etVentura, 2014
	Espagne	< 3.6 - 200.7	Ferrando-Climent et al., 2013	Espagne	< 2.3 - 6	Isidori et al., 2016
	Allemagne	146	Steger-Hartmann et al., 1996	Canada	9	Garcia et al., 2009
	Espagne	< 1.1 - 43	Ferrando-Climent et al., 2014			
	Espagne	32	Isidori et al., 2016			
	Norvège	< 2 - 21	Thomas et al., 2007			
Ifosfamide	Espagne	<6 - 86,200	Gómez-canela etVentura, 2014	Espagne	< 5.8 - 130.1	Ferrando-Climent et al., 2013
	Chine	< 2 - 10,464	Yin et al., 2010	Espagne	< 0.2 - 43.3	Negreira et al., 2013
	Slovénie	48 - 6,800	Česen et al., 2015	Allemagne	< 6 - 40	Kümmerer et al., 1997
	Espagne	< 0.2 - 4761	Olalla et al., 2018	Espagne	6.49 - 19.1	Martín et al., 2014
	Allemagne	< 6 - 1,914	Kümmerer et al., 1997	Espagne	< 9.3 - 27.9	Negreira et al., 2014
	France	30 - 900	Catastini et al., 2008	Chine	8.3	Lin et al., 2014
	Chine	3.4 - 360	Lin et al., 2014	Suisse	<0.2 - 5	Buerge et al., 2006
	Norvège	< 2.0 - 338	Thomas et al., 2007	Allemagne	< 2900	Ternes 1998
	Espagne	< 5.8 - 227.9	Ferrando-Climent et al., 2013			
	Slovénie	< 4.8 - 48	Isidori et al., 2016			
	Allemagne	24	Steger-Hartmann et al., 1996			
Méthotrexate	Espagne	< 0.1 - 4,756	Olalla et al., 2018	Slovénie	29 - 303	Isidori et al., 2016
	Chine	< 2 - 4,689	Yin et al., 2010	Canada	17 - 60	Rabii et al., 2014
	Slovénie	19 - 3,920	Isidori et al., 2016	Espagne	8.3 - 29	Isidori et al., 2016
	Chine	6.1 - 300	Lin et al., 2014	Espagne	< 1.5 - 23	Ferrando-Climent et al., 2014
	France	200	Catastini et al., 2008	Espagne	2.1 - 20.1	Negreira et al., 2013
	Espagne	< 0.6 - 29	Isidori et al., 2016	Canada	< 59	Garcia et al., 2009

	Espagne	< 1.8 - 19	Ferrando-Climent et al., 2014			
5-Fluorouracile	Autriche	< 8,600 - 124,000	Mahnik et al., 2007 oncologie	Chine	280	Lin et al., 2014
	Autriche	20,000 - 122,000	Mahnik et al., 2004 oncologie	Slovénie	4.7 - 14	Kosjek et al., 2013
	Slovénie	35 - 92	Kosjek et al., 2013			
	Suisse	<5 - 27	Kovalova et al., 2009			
	Slovénie	7	Isidori et al., 2016			
Tamoxifène	Espagne	36 - 170	Ferrando-Climent et al., 2014	Slovénie	11 - 61	Isidori et al., 2016
	Espagne	26.3 - 133.4	Ferrando-Climent et al., 2013	Espagne	30 - 58.3	Ferrando-Climent et al., 2013
	Espagne	7.4	Isidori et al., 2016	Espagne	11 - 58	Ferrando-Climent et al., 2014
	Japon	4 - 44	Azuma et al., 2016	Espagne	< 0.7 - 17.2	Negreira et al., 2013
	Slovénie	< 1 - 10	Isidori et al., 2016	Espagne	6.7 - 15	Isidori et al., 2016
	Suisse	< 1 - 4	Tauxe-wuersch etAlencastro, 2006	Suisse	< 1 - 4	Tauxe-wuersch et Alencastro, 2006

b) après sortie de STEP

Composé Conc. ng/L	**Pays**	**Effluents STEP municipales**	**Référence**	**Pays**	**Eaux de surfaces**	**Référence**
Cyclophosphamide	France	300	Catastini et al., 2008	Australie	< 100	Busetti et al., 2009
	Australie	< 125	Busetti et al., 2009	Chine	0.9 - 96	Lin et al., 2014
	Espagne	< 9.3 - 25	Negreira et al., 2014	Roumanie	< 30 - 64.8	Moldovan, 2006
	Japon	< 0.2 - 22	Azuma et al., 2015	Espagne	< 0.3 - 20	Ferrando-Climent et al., 2014
	Canada	< 4 - 21	Rabii et al., 2014	Japon	< 0.2 - 20	Azuma et al., 2015
	Allemagne	< 10 - 20	Ternes, 1998	Espagne	5.0 - 13.7	Franquet-Griell et al., 2017a
	Suisse	< 6 - 17	Steger-Hartmann et al., 1997	Chine	1.9 - 13	Lin et al., 2014
	Espagne	7-15	Ferrando-Climent et al., 2014	Italie	2.2 - 10.1	Zuccato et al., 2000
	Chine	15	Lin et al., 2014	Espagne	< 1.7	Martín et al., 2011
	Suisse	10-21	Buerge et al., 2006	Suisse	0.05 - 0.17	Buerge et al., 2006
	Espagne	< 4 - 5	Gómez-canela etVentura, 2014			
	UK	0.19 - 3.7	Llewellyn et al., 2011			
	Italie	0.6	Zuccato et al., 2005			
Ifosfamide	Allemagne	< 10 – 2,900	Ternes, 1998	Espagne	< 1 - 41	Valcárcel et al., 2011
	Australie	< 125	Busetti et al., 2009	Espagne	10.1 - 13.9	Franquet-Griell et al., 2017a
	France	100	Catastini et al., 2008	Chine	0.1 - 8.9	Lin et al., 2014
	Norvège	< 2 - 71	Thomas et al., 2007	Suisse	< 0.05 - 0.14	Buerge et al., 2006
	Espagne	<9.3 - 15.9	Negreira et al., 2014	Allemagne	< 20	Ternes 1998
	Chine	10	Lin et al., 2014			
	Suisse	< 0.2 - 6	Buerge et al., 2006			

	Espagne	1.2	Martín et al., 2011			
Méthotrexate	Canada	13 - 53	Rabii et al., 2014	Espagne	< 0.2 - 5	Ferrando-Climent et al., 2014
	France	30	Catastini et al., 2008	UK	< 6.25	Aherne, 1985
	Espagne	< 9.3 - 18.1	Negreira et al., 2014			
	Espagne	< 1.2 - 6	Ferrando-Climent et al., 2014			
	UK	< 6.25	Aherne, 1985			
5-Fluorouracile	Chine	80	Lin et al., 2014	Chine	5.0 - 160	Lin et al., 2014
	Slovénie	4.7 - 14	Kosjek et al., 2013	Slovénie	< LOQ	Kosjek et al., 2013
				Espagne	2.4	Martín et al., 2011
Tamoxifène	UK	143 - 740	Roberts etThomas, 2006	France	< 5.8 - 25	Coetsier et al., 2009b
	France	<5.8 - 102	Coetsier et al., 2009b	UK	27 - 212	Roberts et Thomas, 2006
	Espagne	11 - 42	Ferrando-Climent et al., 2014	Japon	< 0.1 - 76	Azuma et al., 2015
	UK	< 10 - 42	Ashton et al., 2004	Espagne	25 - 38	Ferrando-Climent et al., 2014
	Japon	<0.1 - 9	Azuma et al., 2015	Espagne	0.5 - 25.1	Franquet-Griell et al., 2017a
	Slovénie	7.1	Isidori et al., 2016	Espagne	12.4 - 20.1	López-Serna et al., 2012

Leurs concentrations dans l'environnement restent inférieures à d'autres composés. Cependant, en raison de leur mode d'action, leurs impacts pourraient représenter un risque réel qui doit être évalué (Besse et al., 2012). De plus, des métabolites sont détectés dans des effluents d'hôpitaux, comme par exemple, le métabolite 7-hydroxyméthotrexate (7-OH-MTX), à des concentrations parfois similaires au méthotrexate (MTX). Le même constat est fait pour un métabolite du tamoxifène, le 4-hydroxytamoxifene (Isidori et al., 2016). Les occurrences de tels produits de transformation dans les eaux usées ou naturelles restent encore trop peu étudiées (Besse et al., 2012).

1.1.3. L'évaluation des risques écotoxicologiques est-elle adaptée pour les produits pharmaceutiques ?

De manière générale, la présence des polluants organiques entraine des risques pour les organismes présents dans l'environnement, mais aussi pour la santé humaine, notamment *via* les eaux de boisson. Cette multitude d'expositions intervient dans la notion d'exposome, qui se définit comme « l'ensemble des expositions à des facteurs non génétiques favorisant l'apparition de maladies chroniques, auxquelles un individu est soumis de sa conception *in utero* à sa mort » (INERIS, 2019).

La particularité des composés pharmaceutiques est qu'ils sont conçus pour exercer un effet biologique à très faibles concentrations. Ils sont capables de traverser les membranes et ils sont relativement persistants afin d'éviter d'être inactivés avant d'avoir atteint leur objectif thérapeutique (Sanderson, 2003). La problématique des rejets de composés pharmaceutiques est de plus en plus prise en considération depuis que leurs impacts ont pu être constatés, comme le déclin d'une espèce de Vautour au Pakistan exposée aux résidus de diclofénac (Oaks et al., 2004), ou encore le lien établi entre la présence d'éthinyloestradiol et les effets observés chez des poissons pouvant conduire à son extinction (Kidd et al., 2007). Les effets néfastes ne sont pas exclusivement engendrés par les hormones de synthèse, en effet Tijani et al. (2013) soulignent que plus de 38000 composés ont été identifiés comme étant de potentiels perturbateurs endocriniens. Un autre phénomène important, est l'antibiorésistance, qui impacte directement la santé humaine. Les résidus d'antibiotiques sont suspectés de disséminer de l'antibiorésistance dans l'environnement (Martinez, 2009), c'est pourquoi l'Organisation mondiale de la santé porte ses actions selon une approche « One Health » depuis 2015, incitant à l'articulation de la prévention entre santé humaine, santé animale et environnement.

L'Évaluation du Risque Environnemental (ERE) prend en compte l'exposition potentielle à ces composés et au danger intrinsèque à ceux-ci. Des guides techniques ont été développés, ils décrivent les paramètres à calculer pour mener cette évaluation du risque en deux phases : (I) détermination de l'exposition, se rapportant aux concentrations auxquelles seront exposés les organismes dans les eaux de surface et lorsque la valeur seuil de 10 ng/L est franchie, la phase (II) est enclenchée afin de caractériser les dangers, basée sur la persistance, la bioaccumulation et la toxicité des composés notamment grâce à l'aide d'indicateurs écotoxicologiques, enfin la caractérisation des risques est basée sur le rapport de (I)/(II), qui seront détaillées ci-dessous, Figure 1- 3.

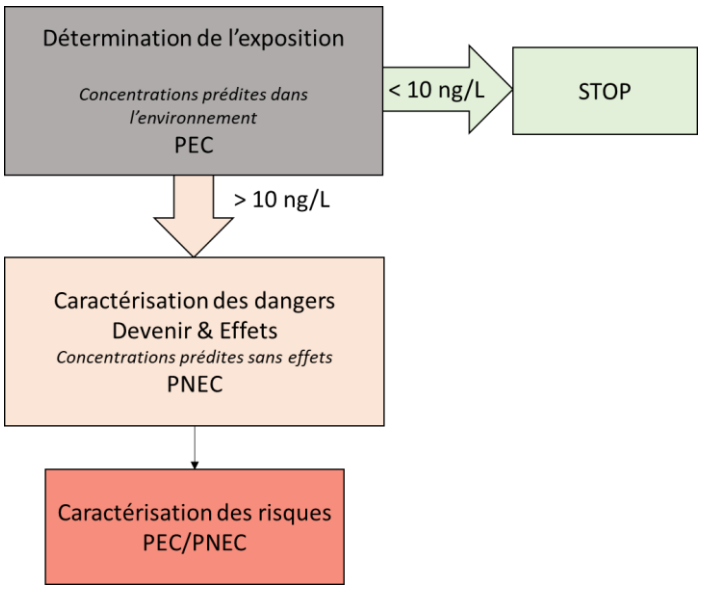


Figure 1- 3 Procédure de l'évaluation du risque environnemental adapté du rapport « Médicaments et environnement » du Conseil général de l'environnement et du développement durable (2010)

La première étape, dite phase I, se réfère au calcul de la PEC (Predicted environnemental concentration ou concentration prédite dans l'environnement) qui peut se passer d'essai et repose souvent sur des calculs théoriques basés sur des données telles que le coefficient de partage octanol/eau (log Kow), la dose journalière maximale, les quantités vendues, le taux d'excrétion ou encore les facteurs de dilution des eaux usées dans les eaux de surface (Schwarz et al., 2021). Lorsque le résultat est inférieur au seuil (10 ng/L dans les eaux superficielles comme milieu récepteur -Figure 1-3, CGEDD 2010), il est conclu une absence de risque environnemental et la deuxième phase d'évaluation des effets n'est pas conduite. Cependant, les anticancéreux sont connus pour être actifs à de très faibles concentrations, pour cette raison, ils se retrouveraient la majeure partie du temps avec une PEC en dessous du seuil et ne passeraient pas en phase II (Kümmerer et al., 2016). De plus, les concentrations estimées peuvent être sous-évaluées si les campagnes de mesures ("monitoring") ne sont pas suffisamment poussées

(Verlicchi et Zambello, 2016 ; Directorat Général de l'environnement de la Commission Européenne, 2019).

La phase II permet de calculer la concentration prédite sans effet toxique (Predicted Non Effect Concentration, PNEC). Cette phase est elle-même divisée en deux étapes (A et B) et concerne le devenir et les effets dans l'environnement. Dans la phase II A, les propriétés de la substance (persistance, bioaccumulation et toxicité, PBT) sont étudiées. Par exemple, le risque de bioaccumulation est basé sur le coefficient de partage Kow (souvent faible pour les anticancéreux, à l'exception du tamoxifène, Olalla et al., 2018). Pour étudier la persistance de la substance dans la STEP, des tests de biodégradabilité peuvent être effectués tels que le « Closed Bottle Test 301D» selon les recommandations de l'OCDE (1992) mais nous verrons plus en détails les informations relatives au devenir des molécules anticancéreuses dans le chapitre suivant.

Olalla et al. (2018) évaluent le risque environnemental de 17 anticancéreux en se basant sur des concentrations mesurées dans deux eaux usées, comme présenté précédemment (cf partie 1.1.2) et caractérisent le devenir et les effets des composés en attribuant des indices pour la persistance, la bioaccumulation et la toxicité, mais pour cette dernière, ils soulignent le manque de données écotoxicologiques nécessaires dans le calcul des concentrations prédites sans effets (PNEC).

Finalement, les résultats indiquant un risque peuvent nécessiter une étude plus approfondie, la Phase II est prolongée (Phase II B), comme par exemple, lorsque les substances ont un log Kow supérieur à 4, ce qui signale un potentiel de bioaccumulation ou lorsque les composés sont considérés non biodégradables (OCDE, 2019).

1.1.3.1. Caractérisation des effets écotoxicologiques

La caractérisation des effets écotoxicologiques se base principalement sur des approches biologiques qui incluent des bioessais, des bioindicateurs, ou encore sur des approches QSAR (Quantitative Structure Activity Relationship), qui sont des modèles d'estimation pour prédire des effets (toxicité) ou des propriétés (bioaccumulation) des substances à partir de leur structure moléculaire, elles même développées à partir de données expérimentales (Marchand et Tissier, 2007).

L'approche expérimentale, pour la caractérisation des effets écotoxicologiques donne des indications de deux types: i) la toxicité aiguë, pour des effets à court terme, comme par exemple des effets de mortalité ou d'inhibition de mobilité, ce sont les tests les plus repandus car les plus faciles à mettre en place (courte durée) ; ii) la toxicité chronique pour des effets à plus long terme, de quelques

jours à plusieurs semaines, avec l'observation d'effets sur la croissance ou la reproduction. Pour l'ensemble de ces tests, aigus ou chroniques, les bioessais sont conduits selon des méthodes standardisés, afin de maîtriser les facteurs influant sur la réponse des organismes et de donc de comparer plus facilement les résultats de différents polluants et entre différents laboratoires (Mater, 2014). Ils permettent d'établir des relations dose-réponse par des méthodes statistiques de régression qui définissent divers indicateurs écotoxicologiques. Comme présenté en Figure 1- 4, l'EC_{50} définit la concentration d'effet affectant 50 % d'une population donnée (lorsqu'il s'agit de mortalité, dans le cas de toxicité aigüe, on parle alors de LC50, ou dose létale). La NOEC (No Observed Effect Concentration) est définie comme étant la plus forte concentration testée pour laquelle aucun effet n'est observé et la LOEC (Lowest Observed Effect Concentration) représente la plus faible concentration pour laquelle un effet est observé.

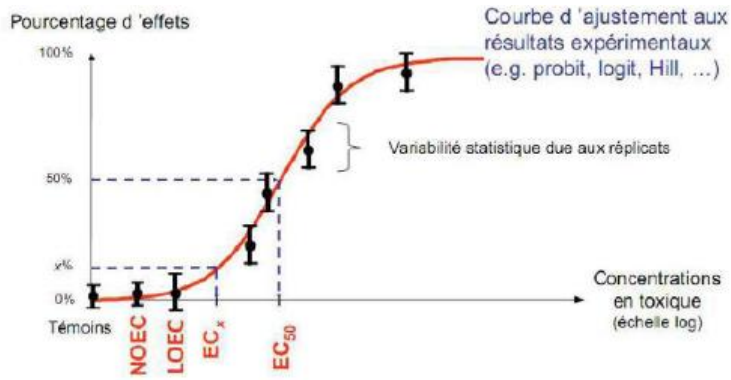


Figure 1- 4 Courbe théorique de la relation dose-réponse extrait de Destrieux (2018)

Les limites de ces tests sont souvent pointées du doigt en raison de leur réalisme écologique limité : en effet, les conditions contrôlées sont éloignées des conditions environnementales et chaque espèce à sa propre sensibilité. Les différents bioessais sont le plus souvent associés pour former des batteries de bioessais en utilisant des représentants de différents niveaux trophiques et permettent alors de réaliser un screening plus complet du potentiel toxique des substances étudiées et surtout de comparer les résultats entre eux, le Tableau 1- 3 compile les bioessais normalisés les plus connus.

Tableau 1- 3 Caractéristiques des bioessais normalisés adapté de Mater (2014)

Organisme	Niveau trophique	Type d'exposition	Durée d'exposition	Critère d'effet	Norme
Daphnia magna **(invertébré)**	Consommateur primaire	Aiguë	24 et 48 h	inhibition de la mobilité	NF EN ISO 6341
Aliivibrio fischeri **(bactérie)**	Décomposeur	Aiguë	15 et 30min	inhibition de la luminescence	NF EN ISO 11348-3
Pseudokirchneriella subcapitata **(algue)**	Producteur primaire	Aiguë /Chronique	72 h	inhibition de la croissance de la population	NR EN ISO 8692
Ceriodaphnia dubia **(invertébré)**	Consommateur primaire	Aiguë	24 et 48 h	inhibition de la mobilité	NF EN ISO 6341 et ISO 20665
Brachionus calyciflorus **(rotifère)**	Consommateur primaire	Chronique	48 h	inhibition de la reproduction	PR NF ISO 20666
Lemna minor **(végétal)**	Producteur primaire	Chronique	7j	inhibition de la croissance de la population	ISO 20079

i. Le cas des anticancéreux

Bien que retrouvés à de faibles concentrations en sortie de stations d'épuration, les anticancéreux peuvent impacter les organismes aquatiques à cause de leurs différents mécanismes d'action (présenté en Tableau 1- 4) cytotoxique, tératogène et/ou mutagène non spécifiques aux cellules cancéreuses et de leurs index thérapeutiques (en lien avec la dose létale) souvent étroits (Wormington et al., 2020).

Tableau 1- 4 Classification ATC (anatomique, thérapeutique et chimique) des anticancéreux, extrait de Besse et al. (2012)

ATC class	Class	Mechanism of action	Detailed mechanism
Antineoplastic agents (ATC L01)	Cytotoxics which interact directly with DNA	Alkylating agents	Attach one or more nucleophile groups to the DNA and inhibit or alter its transcription
		Platinum complexes	Inhibit DNA replication by binding to it
		Intercalating agents	Act by breaking single-stranded DNA
	Cytotoxics which interact indirectly with DNA	Antimetabolites	Structural analogs of purine, pyrimidine or folic acid, act by blocking enzyme activity and disrupting DNA synthesis
		Cytotoxic antibiotics	Intercalate between DNA base pairs and thus disturb the synthesis and/or function of nucleic acids
		Mitotic spindle inhibitors	Halt chromosome segregation by inhibiting mitotic spindle formation
		Topoisomerase inhibitors	Induce or stabilize DNA damage by blocking relegation of double stranded DNA breaks
	Cytostatics	Protein kinase inhibitors	Interact with protein kinases, involved in the regulation of many biological processes (cell growth, migration,survival..), most of the compounds used are Tyrosine kinase inhibitors
		Monoclonal antibodies	Block tumoral cells extracellular receptors
Endocrine therapy (ATC L02)	Hormones and related Anti-hormones and related	GnRH and LhRH analogs	Interfere with gonadotropin release
		Antiestrogens	Direct estrogen receptor antagonism
		Antiaromatase	Inhibit the estrogen biosynthesis
		Antiandrogens	Inhibit the binding of testosterone to its receptor

Kümmerer (2001), Besse et al. (2012) ou encore Toolaram et al. (2014) soulignent l'urgence d'évaluer les occurrences des anticancéreux mais aussi leurs impacts sur la faune et la flore. Au cours des dernières années, de plus en plus d'auteurs ont commencé à s'y intéresser, le Tableau 1- 5 compile les données d'écotoxicité sur une sélection d'organismes et de composés anticancéreux.

Tableau 1- 5 Données d'effets écotoxicologiques sur des organismes aquatiques pour 10 anticancéreux (durée du test)

Composé	Organisme et infos complémentaires	EC_{50} (mg/L)	LOEC (mg/L)	NOEC (mg/L)	Reference
5-Fluorouracile	*B. calyciflorus,* croissance (24 h)*	> 200			Parrella et al., 2014b
	T. platyuiris, mortalité (24 h)*	0.25			Parrella et al., 2014b
	B. calyciflorus, croissance (48 h)**	0.322			Parrella et al., 2014b
	C. dubia, mortalité (24 h)*	501			Parrella et al., 2014b
	C. dubia, reproduction (7 d)**	0.0034			Parrella et al., 2014b
	D. magna, immobilisation (48 h)*	15	5		Zounková et al., 2010
	D. magna, reproduction (21 d)**	0.1	0.05		Zounková et al., 2010
	D. subcapitatus, croissance (72 h)*	48	40		Zounková et al., 2010
	D. magna, immobilisation (48 h)*	>100			Bialk et al., 2017
	D. magna, immobilisation (48 h)*	20.84			Parrella et al., 2014b
	D. magna, immobilisation (48 h)*	36	10	1	Zounková et al., 2007
	D. magna, reproduction (21 d)**	0.026			Parrella et al., 2014b
	L. minor, croissance (7 d)**	0.91			Zaleska-Radziwill et al., 2011
	L. minor, croissance (7 d)**	2.45			Bialk et al., 2017
	P. subcapitata, croissance (96 h)**	0.11	0.01	0.001	Zounková et al., 2007
	P. putida, croissance (16 h)*	0.027	0.01	0.003	Zounková et al., 2007
	P. putida, croissance (16 h)*	0.044	0.03		Zounková et al., 2010
	R. subcapitata, croissance (72 h)**	0.075			Bialk et al., 2017
	R. subcapitata, croissance (72 h)**	0.13			Brezovsek et al., 2014
	R. subcapitata, croissance (72 h)**	0.96			Henschel et al., 1997
	S. subspicatus, croissance**	30.49			Zaleska-Radziwill et al., 2011
	A. fischeri, luminescence inhibition (30 min)*	>100			Bialk et al., 2017
Cyclophosphamide	Crustacé (ECOSAR)	1795			Sanderson et al., 2003
	Algue (ECOSAR)	70			Sanderson et al., 2003
	D. magna, immobilisation (48 h)*	>100			Bialk et al., 2017
	D. magna, immobilisation (48 h)*	>1000	>1000	>1000	Zounková et al., 2007
	D. magna, reproduction (21 d)**	>100			Grung et al., 2008
	L. minor, croissance (7 d)**	>100			Bialk et al., 2017
	P. subcapitata, croissance (96 h)**	930	500	250	Zounková et al., 2007
	P. putida, croissance (16 h)*	>1000	>1000	1000	Zounková et al., 2007
	R. subcapitata, croissance (72 h)**	>100			Bialk et al., 2017
	R. subcapitata, croissance (72 h)**	>320			cecen et al., 2016
	A. fischeri, luminescence inhibition (30 min)*	>100			Bialk et al., 2017

Cytarabine	*D. magna*, immobilisation (48 h)*	200	100		Zounková et al., 2010
	D. magna, reproduction (21 d)**	10	3.7		Zounková et al., 2010
	D. subcapitatus, croissance (72 h)**	53	40		Zounková et al., 2010
	P. putida, croissance (16 h)*	17	10		Zounková et al., 2010
Doxorubicine	*B. calyciflorus*, croissance (24 h)*	12.69			Parrella et al., 2014b
	T. platyuiris, mortalité (24 h)*	0.31			Parrella et al., 2014b
	B. calyciflorus, croissance (48 h)**	7.7			Parrella et al., 2014b
	C. dubia, mortalité (24 h)*	5.18			Parrella et al., 2014b
	D. magna, immobilisation (48 h)*	2.14			Parrella et al., 2014b
	D. magna, immobilisation (48 h)*	2	0.1	0.01	Zounková et al., 2007
	P. subcapitata, croissance (96 h)**	13	10	1	Zounková et al., 2007
	P. putida, croissance (16 h)*	>1000	10	1	Zounková et al., 2007
Etoposide	*B. calyciflorus*, croissance (24 h)*	>120			Parrella et al., 2014b
	T. platyuiris, mortalité (24 h)*	0.31			Parrella et al., 2014b
	B. calyciflorus, croissance (48 h)**	3.7			Parrella et al., 2014b
	C. dubia, reproduction (7 d)**	0.02			Parrella et al., 2014b
	D. magna, immobilisation (48 h)*	30	30	10	Zounková et al., 2007
	D. magna, reproduction (21 d)**	0.24			Parrella et al., 2014b
	P. subcapitata, croissance (96 h)**	250	10	<10	Zounková et al., 2007
	P. putida, croissance (16 h)*	630	250	200	Zounková et al., 2007
Gemcitabine	*D. magna*, immobilisation (48 h)*	110	50		Zounková et al., 2010
	D. magna, reproduction (21 d)**	>1	>1		Zounková et al., 2010
	D. subcapitatus, croissance (72 h)**	45	10		Zounková et al., 2010
	P. putida, croissance (16 h)*	100	50		Zounková et al., 2010
Ifosfamide	Crustacé (ECOSAR)	1795			Sanderson et al., 2003
	Algue (ECOSAR)	11			Sanderson et al., 2003
	D. magna, immobilisation (48 h)*	>100			Bialk et al., 2017
	L. minor, croissance (7 d)**	>100			Bialk et al., 2017
	Poisson (ECOSAR)	140			Sanderson et al., 2003
	R. subcapitata, croissance (72 h)**	>100			Bialk et al., 2017
	A. fischeri, luminescence inhibition (30 min)*	>100			Bialk et al., 2017
Imatinib	*B. calyciflorus*, croissance (24 h)*	3.82			Parrella et al., 2014b
	T. platyuiris, mortalité (24 h)*	43.27			Parrella et al., 2014b
	B. calyciflorus, croissance (48 h)**	0.74			Parrella et al., 2014b
	C. dubia, mortalité (24 h)*	31.92			Parrella et al., 2014b
	C. dubia, reproduction (7 d)**	115			Parrella et al., 2014b
	D. magna, immobilisation (48 h)*	>100			Bialk et al., 2017

	D. magna, immobilisation (48 h)*	72.43	Bialk et al., 2017
	D. magna, immobilisation (48 h)*	11.97	Parrella et al., 2014b
	D. magna, reproduction (21 d)**	0.31	Parrella et al., 2014b
	L. minor, croissance (7 d)**	61.05	Bialk et al., 2017
	R. subcapitata, croissance (72 h)**	5.08	Bialk et al., 2017
	R. subcapitata, croissance (72 h)**	2.29	Brezovsek et al., 2014
	A. fischeri, luminescence inhibition (30 min)*	23.06	Bialk et al., 2017
Methotrexate	Crustacé (ECOSAR)	651	Sanderson et al., 2003
	Algue (ECOSAR)	192	Sanderson et al., 2003
	B. rerio embryos, mortalité (96 h)*	85	Henschel et al., 1997
	C. dubia, reproduction (7 d)**	1.15	Lutterbeck et al., 2014
	D. magna, immobilisation (48 h)*	> 100	Bialk et al., 2017
	D. magna, immobilisation (48 h)*	> 1000	Henschel et al., 1997
	I. galbana, croissance (96 h)**	84	Aguirre-Martínez et al., 2016
	L. sativa, croissance (120 h)**	2.2	Lutterberck et al., 2015b
	L. minor, croissance (7 d)**	0.02	Markovic et al., 2021
	L. minor, croissance (7 d)**	0.16	Bialk et al., 2017
	P. lividus, Fertilization process**	1000	Aguirre-Martínez et al., 2016
	P. lividus, Larval development**	1.5	Aguirre-Martínez et al., 2016
	Poisson (ECOSAR)	383000	Sanderson et al., 2003
	R. subcapitata, croissance (72 h)**	9.51	Bialk et al., 2017
	S. subspicatus, croissance (72 h)**	260	Henschel et al., 1997
	T. pyriformis, croissance (48 h)*	45	Henschel et al., 1997
	A. fischeri, luminescence inhibition (15 min)*	> 2.5	Lai et al., 2017
	A. fischeri, luminescence inhibition (30 min)*	> 100	Bialk et al., 2017
	A. fischeri, luminescence inhibition (30 min)*	900	Aguirre-Martínez et al., 2016
	A. fischeri, luminescence inhibition (30 min)*	> 100	Lutterbeck et al., 2014
	A. fischeri, luminescence inhibiton (30 min)*	< 15	Calza et al., 2014
	A. fischeri, luminescence inhibiton (30 min)*	1220	Henschel et al., 1997
Tamoxifène	*C. dubia*, reproduction (7 d)**	0.00081	DellaGreca et al., 2007
	D. magna, immobilisation (24 h)*	1.53	DellaGreca et al., 2007
	D. magna, immobilisation (48 h)*	>0.2	Bialk et al., 2017
	D. magna, immobilisation (48 h)*	0.21	Orias et al., 2015
	L. minor, croissance (7 d)**	0.18	Bialk et al., 2017
	R. subcapitata, croissance (72 h)**	>0.02	Bialk et al., 2017
	A. fischeri, luminescence inhibition (30 min)*	>0.2	Bialk et al., 2017

Valeurs grisées > 100 mg/L, considéré non toxique

**Acute*

**Chronic*

Certains auteurs rapportent des valeurs très éloignées pour un même composé sur un même organisme, comme par exemple, le test d'immobilisation sur *D. magna* pour le 5-fluorouracile et l'ifosfamide. Enfin, certains organismes sont sensibles à certains composés et non à d'autres, par exemple le 5-fluorouracile est très toxique sur une grande variété d'organismes à l'exception de *A. fischeri*, qui au contraire, est très sensible à l'ifosfamide. Les tests à plus longue exposition comme sur *L. minor* ou sur *R. subcapitata* permettent un temps d'exposition suffisamment long pour que les composés provoquent un effet spécifique à leur mode d'action (inhibition de la réplication de l'ADN et de la division cellulaire) (Białk-Bielińska et al., 2017). Cependant trois types d'algues différentes peuvent donner des résultats différents comme par exemple sur le MTX, *R. subcapitata*, *L. galbana* et *S. subspicatus*, avec des EC_{50} de 9,5, 85 et 260 mg/L respectivement.

Le projet Européen CYTOTHREAT (Commission Européenne, 2015) qui avait pour objectif d'évaluer le devenir et les effets des anticancéreux a révélé que les crustacés étaient les organismes les plus sensibles. Un autre résultat de ce projet a été de proposer également des tests sur des cellules de poisson zèbres comme système modèle fiable pour l'étude d'effets cytotoxiques aigus.

Quant au MTX, sur la croissance de *L. minor* et *R. subcapitata*, *L. savita* et sur la reproduction de *C. dubia* des effets sont visibles, ils semblent alors être des organismes suffisamment sensibles pour obtenir des indications sur la toxicité du MTX. Les données sur *A. fischeri* sont hétérogènes. Calza et al. (2014) et Lai et al. (2017) observent des effets à seulement quelques mg/L mais l'objectif de leurs études n'était pas d'obtenir des EC_{50}, mais plutôt d'observer une évolution de la toxicité au cours de réactions de dégradation du MTX. A l'opposé, d'autres auteurs n'observent pas d'effet majeur en deçà de la centaine de mg/L (Henschel et al. 1997; Lutterbeck et al., 2014; Białk-Bielińska et al., 2017).

Finalement, le choix de l'organisme en fonction de la molécule jouera un grand rôle dans l'évaluation des effets. Certains composés tel que le 5-fluorouracile ont montré des LOEC sur algues et bactéries proches de la concentration trouvée dans les effluents d'eaux usées (Zounková et al., 2007). Cependant lorsque ce n'est pas le cas, il est difficile de conclure car comme nous l'avons vu précédemment, l'utilisation de tests standardisés ne cible pas de mode d'action spécifique. En effet, une exposition chronique à un composé présent à l'état de trace et en particulier à certains stades sensibles du développement est plus pertinente pour expliquer les anomalies observées chez des organismes non-ciblés qu'une exposition aiguë à des doses élevées (Fent et al., 2006). Par exemple, Lutterbeck et al. (2015b) et Muneer et al. (2020) ont révélé une modification cytogénétique en observant des effets sur la longueur des racines, du nombre de racines et de l'indice mitotique de l'oignon *A. cepa* en l'exposant à quelques mg/L d'antinéoplasiques.

Besse et al. (2012) propose des évaluations d'effets plus spécifiques, par exemple l'évaluation des effets génotoxiques sur les spermatozoïdes et les ovocytes des organismes aquatiques exposés. C'est ce que proposent Aguirre-Martinez et al. (2016) avec l'évaluation d'effets sur la fertilisation et le développement des larves d'oursins pour le MTX. Tandis que le processus de fertilisation ne semble pas impacté, l'impact sur le développement est quant à lui visible en dessous du mg/L (Tableau 1- 5).

Kleinert et al. (2021) observent quant à eux des effets sublétaux sur des moules d'eau douce *Elliptio complanata* en présence du 5-fluorouracile et du MTX. Le 5-fluorouracile provoque une augmentation de la dihydrofolate réductase (DHFR), une diminution de la peroxydation des lipides et des cassures de brins d'ADN suggérant que les moules sont métaboliquement endommagées et que l'activité de réparation de l'ADN est réduite. Le MTX quant à lui augmente l'activité de la DHFR dans la gonade. Ces deux composés sont donc biologiquement actifs dans les moules d'eau douce à des concentrations réalistes pour des effluents municipaux, alors que l'évaluation de risque pourrait conclure à une absence de risque environnemental en se basant sur des organismes peu sensibles.

ii. Les effets des produits de transformation (TPs)

La dégradation de tout contaminant organique, qu'elle soit biotique ou abiotique, peut générer des TPs contribuant significativement au risque posé par le composé d'origine. Dans ce manuscrit, les métabolites font référence aux transformations résultant du métabolisme humain ou animal, tandis que les produits de transformation aux molécules résultant de modifications, après l'excrétion des composés parents et des métabolites dans l'environnement, des divers procédés de traitement ou des processus environnementaux abiotiques et biotiques tels que la biotransformation, l'hydrolyse ou la photolyse (Toolaram et al., 2014), c'est ce que nous verrons dans le **Chapitre 2**.

Au niveau Européen, la Directive n° 2006/118 de la Commission Européenne (2006) sur la protection des eaux souterraines mentionne les métabolites et produits de transformation de pesticides, mais pour l'instant côté pharmaceutiques, il n'y a pas encore de réglementation. En effet, comme nous l'avons vu précédemment, les évaluations des risques environnementaux menées pour l'autorisation de produits pharmaceutiques dans l'UE ne prennent pas en compte les métabolites ou autres produits de transformation dans la première phase. Pourtant, Escher et Fenner (2011) rapportent que les processus oxydants conduisent généralement à des TPs plus polaires et par conséquent plus mobiles que les composés d'origine et peuvent aussi parfois être plus persistants. De plus, ils peuvent parfois conserver l'action pharmaceutique/biologique du composé parent, engendrant alors les mêmes problématiques d'impact sur les organismes présents dans l'environnement. Il arrive aussi que certains présentent une toxicité plus élevée que le principe actif lui-même (Schmitt-Jansen et al., 2007; Jiao et al., 2008; Fatta-

Kassinos et al., 2011; Gonçalves et al., 2021). Calza et al. (2014) et Secrétan et al. (2018) ont démontré ainsi que certains des photo-produits du pemetrexed et de la doxorubicine sont plus toxiques que le composé parent grâce à l'utilisation du test d'écotoxicité aigue simple et rapide, sur la bactérie *Aliivibrio fischeri*.

Pour le MTX, le métabolite et TP 2,4-diamino-N10-methyl-pteroic acid (DAMPA) semble inactif (Widemann et al., 2000). Ce n'est pas le cas d'un autre de ses sous-produit, le 7-OH-MTX qui présente un comportement controversé (Fabre et Goldman, 1985). Besse et al. (2012) l'ont proposé alors dans la liste des composés pharmaceutiques prioritaires pour l'évaluation environnementale et recommandent la réalisation d'études d'occurrence. Selon Białk-Bielińska et al. (2017), il semble être moins toxique que le composé parent. Cependant, sa susceptibilité à être photodégradée conduit à une comparaison difficile en raison de l'instabilité des composés lors des essais. Dans cette même étude, aucune donnée n'était exploitable pour une comparaison avec le MTX dans les bioessais avec *A. fischeri* et *D. magna*. Les résultats prédictifs « ecotoxicological Quantitative Structure Activity Relationship, ECOSAR» de Sanderson (2003) sont plus alarmants, ils donnent une EC_{50} de 651 mg/L sur crustacé pour le MTX et 36 mg/L pour le 7-OH-MTX.

Si la toxicité sur *A. fisheri* d'un mélange issu de la photo- et bio-dégradation du MTX semble diminuer au cours du temps (Calza et al., 2014; Lutterbeck et al., 2015a). Calza et al. (2014) mentionnent cependant une légère persistance des effets, mais n'identifient pas le 7-OH-MTX. Lai et al. (2017) observent quant à eux une augmentation de la toxicité après un traitement de catalyse UV en présence d'anions bicarbonate et identifient d'autres TPs comme responsables de la toxicité.

La mise en évidence de la toxicité de TPs peut aussi être faite par des tests spécifiques, ciblant le mode d'action de la molécule. Besse et al. (2012) proposent par exemple d'utiliser des tests *in vivo* tels que le test des comètes (révélant les dommages causés à l'ADN). Il existe aussi des tests bactériens pour évaluer le potentiel écotoxique tel que le SOS Chromotest, ou le UmuC test, ou encore pour évaluer la mutagénicité, le test d'AMES également appelé Mutatest (Zounkova et al., 2007; Zounkova et al., 2010). Muneer et al. (2020) met ainsi en évidence la baisse de mutagénicité d'un mélange issu de l'irradiation UV et Gamma du MTX.

Finalement les données de la littérature sur l'écotoxicité des TPs d'anticancéreux et plus spécifiquement du MTX, sont peu fournies et souvent hétérogènes. La caractérisation des risques liés à ces TPs reste assez peu documentée.

1.1.3.2. Vers une évolution de l'évaluation de médicaments ?

L'obligation d'évaluation pour la délivrance de l'autorisation de mise sur le marché est très récente (European Medicines Agency, 2006) ; elle concerne les nouvelles molécules et non celles déjà sur le marché. L'ensemble de ces substances relèvent d'un système d'autorisation dans le droit communautaire et voient la délivrance de leur autorisation fondée sur un rapport bénéfice-risque qui inclut le risque environnemental. Selon le Conseil général de l'environnement et du développement durable (2010) quand bien même le rapport PEC/PNEC est supérieur à 1 et le risque potentiel pour l'environnement considéré, pour le médicament à usage humain, le bénéfice-risque est défini comme l'évaluation des effets thérapeutiques positifs du médicament au regard du risque pour la santé du patient ou la santé publique lié à la qualité, à la sécurité ou à l'efficacité du médicament. Mais contrairement au médicament vétérinaire qui intègre le risque d'effets indésirables sur l'environnement, celle du médicament à usage humain exclut cette prise en compte car « une molécule utile pour la santé de l'homme ne saurait être interdite pour un simple motif d'atteinte à l'environnement ». Cependant la procédure d'évaluation est amenée à évoluer, notamment pour les perturbateurs endocriniens et les antibiotiques (Schwarz et al., 2021).

Nous savons que la toxicité résultante de mélanges complexes de médicaments à de faibles concentrations pourrait conduire à des interactions dans certains cas, additives ou synergiques, comme vu pour des anticancéreux (Parrella et al., 2014a) et que ces mélanges peuvent tout aussi bien être le composé parent et ses propres TPs (Escher et Fenner, 2011). Pourtant l'évaluation de toxicité considère la molécule isolément alors que les résidus sont issus de multiples familles à effet similaire et auxquelles sont exposés le milieu aquatique et ses écosystèmes (Académie nationale de Pharmacie, 2019). Le guide « Options for a strategic approach to pharmaceuticals in the environment » du Directorat Général de l'environnement de la Commission Européenne (2019) notifie qu'il est indispensable dans un premier temps d'identifier ces composés et enfin d'évaluer les risques qu'ils représentent.

1.1.4. Comment diminuer les risques dus aux résidus de médicaments

1.1.4.1. Les différents leviers

Nous venons de voir que la contamination des eaux par les composés pharmaceutiques et leurs TPs est problématique, même si les concentrations retrouvées dans les eaux sont faibles et que le risque est souvent évalué comme étant négligeable. En effet de manière plus générale, la contamination de l'environnement par les polluants organiques peut poser problème dès lors que la structure chimique et les produits de transformation en étant issus induisent un effet biologique, d'autant plus que leurs effets en cocktail peuvent avoir des impacts additifs ou synergiques.

Pour diminuer les rejets médicamenteux, en France, un plan national sur les résidus de médicaments (PNRM) lancé en 2011 pour l'amélioration de l'évaluation des risques environnementaux et sanitaires, soulevait la nécessité de l'acquisition de connaissances scientifiques et techniques relatives à la présence, au devenir et aux effets de ces médicaments sur l'environnement et sur la santé humaine. Un second axe traitait de la gestion des risques environnementaux et sanitaires par le contrôle et la réduction des émissions de médicaments dans l'environnement. Par exemple, la récupération de médicaments inutilisés par le système CYCLAMED mis en place en France permet de récolter les médicaments non utilisés et de les envoyer à l'incinération pour protéger les consommateurs et l'environnement. Quant à la réduction des usages, de nombreuses initiatives ont été développées pour sensibiliser le public au bon usage des médicaments dans le cadre d'une consommation responsable. Un exemple assez connu est le cas des antibiotiques depuis la découverte du phénomène d'antibiorésistance et qui a donné lieu à plusieurs campagnes de communication, visant à sensibiliser médecins, pharmaciens et patients. D'autres actions à destination des prescripteurs tels que le « Environmentally classified pharmaceuticals » (Médicaments classés pour l'environnement) édité par le Conseil municipal de Stockholm classe les principes actifs en fonction de leur impact sur l'environnement (Académie nationale de Pharmacie, 2019). Dans cette même idée, Daughton et Ruhoy (2013) se penchent sur la classification des médicaments en fonction de leur excrétion, puisque c'est de là que démarre la contamination. Toujours sur l'usage, on peut aussi noter la proposition de la vente de médicament à l'unité afin de limiter les déchets.

D'autres solutions, plus coûteuses, proposent l'amélioration du traitement des eaux usées. La Suisse est le seul pays européen à avoir investi de grosses sommes dans la modernisation de ses STEPs. Le recours aux traitements spécifiques (qui seront détaillés dans le **Chapitre 2**) et le développement de processus innovants notamment sur les effluents d'hôpitaux, bien qu'étant des solutions prometteuses, ne sont pas toujours suffisantes.

Dans son rapport sur les recommandations pour la gestion des produits pharmaceutiques en eau douce, l'OCDE (2019) identifie les différents leviers à prendre en compte pour diminuer ces risques (Tableau 1- 6), intégrant l'ensemble du cycle de vie des produits pharmaceutiques (Figure 1- 5). Dans le cadre de notre étude, nous allons nous intéresser à la première étape du cycle de vie du médicament, dès son design. En effet limiter la contamination peut être pensé en amont de celle-ci et dès la conception de la molécule.



Figure 1- 5 Cycle de vie du médicament extrait des recommandations OCDE (2019)

Tableau 1- 6 Sélection des principales options d'atténuation pour les différentes parties prenantes tout au long du cycle de vie des produits pharmaceutiques, traduit des recommandations OCDE (2019)

Etapes	Partie prenante	Option d'atténuation
Transversale	Gouvernement, industrie, organismes de recherche	Surveillance ciblée et hiérarchisation des principes actifs pharmaceutiques (API) à haut risque Exploiter les nouvelles innovations en matière de surveillance de la qualité de l'eau, de modélisation, d'élaboration de scénarios et d'évaluation des risques Base de données centralisée avec surveillance réglementaire pour partager les ERE et les données de surveillance environnementale des API Normes de qualité environnementale / normes de qualité de l'eau
Design	Industrie	Innovation en pharmacie verte, thérapies biologiques, médecines personnalisées ou de précision
Autorisation	Gouvernement, Industrie	Législation et méthodologie normalisée pour l'évaluation des risques environnementaux et intégration dans l'évaluation des avantages et des risques de l'autorisation de mise sur le marché pharmaceutique Conditions plus strictes pour la mise sur le marché d'un produit pharmaceutique à haut risque pour l'environnement (par exemple, options d'intervention et d'atténuation des risques accrus, étiquetage écologique, prescription uniquement, surveillance post-approbation)
Production	Industrie, gouvernement, organisations intergouvernementales	Marchés publics verts avec critères environnementaux Critères environnementaux pour les bonnes pratiques de fabrication, les limites de rejet d'effluents et la divulgation des rejets d'eaux usées pharmaceutiques des chaînes d'approvisionnement
Consommation (professionnelle)	Agriculture, Santé, Gouvernement	Prévention des émissions par la prévention des maladies et l'utilisation durable des produits pharmaceutiques Amélioration de la santé et du bien-être humain et animal Diagnostics améliorés, prescriptions évitées Amélioration des normes d'hygiène dans les établissements de santé, gestion des étables et manipulation du bétail Médicaments personnalisés, vaccinations, mécanismes de distribution ciblés Prescription d'alternatives pharmaceutiques respectueuses de l'environnement Restrictions ou interdictions des produits pharmaceutiques à haut risque inutiles (par exemple, l'utilisation vétérinaire d'antibiotiques pour des mesures préventives et d'hormones comme stimulateurs de croissance chez le bétail)
Consommation (achats en vente libre, auto-ordonnance)	Secteur de la santé, Industrie, Consommateurs	Éco-étiquetage des produits pharmaceutiques en vente libre à haut risque pour améliorer le choix et la sensibilisation des consommateurs
Collecte et élimination	Services publics de déchets solides, Industrie	Campagnes d'éducation pour éviter l'élimination des produits pharmaceutiques via l'évier ou les toilettes Systèmes publics de collecte pharmaceutique pour les médicaments non utilisés Régimes de responsabilité élargie des producteurs Gestion améliorée du fumier par stockage passif ou fermentation anaérobie dans les usines de biogaz
Traitement des eaux usées	Services d'eaux usées	Modernisation des stations d'épuration
Traitement de l'eau potable	Services d'eau potable	Modernisation des usines de traitement d'eau potable Planification de la sécurité aquatique

1.1.4.2. L'éco-conception

Le terme éco-conception dans son sens large fait appel à toute la chaîne de production, répondant alors au principe de la « green chemistry » dont le but est de réduire ou éliminer l'utilisation et la génération de substances dangereuses. Nous nous intéressons ici à la partie du design de la structure

de la molécule, c'est un domaine de recherche encore peu exploré qui s'inscrit dans une démarche durable pour limiter la contamination de l'environnement.

L'éco-conception d'une molécule vise à ce qu'elle se dégrade facilement et produise alors des TPs inoffensifs une fois excrétés dans l'environnement. Ce concept appelé « benign by design » peut sembler contradictoire, car pour répondre aux exigences pharmaceutiques, d'un point de vue sécurité et stockage, il est nécessaire de proposer des composés stables. Cependant, et comme le souligne Kümmerer (2007), le potentiel de la dégradation des produits chimiques est différent au cours de son cycle de vie. Par conséquent, la réactivité d'une molécule, en terme de cinétique et de thermodynamique, dépend des propriétés de la molécule mais aussi de son environnement : les conditions de stockage (humidité, pureté de la molécule, température, lumière), sont différentes de celle du corps humain (humidité, température plus élevée, enzymes, pH bas, conditions anaérobie…) c'est d'ailleurs le principe du mode d'action des prodrogues, qui sont des molécules activées par le métabolisme dans le corps humain. Enfin, les conditions que subissent les molécules excrétées dans les STEPs et dans l'environnement sont tout aussi différentes et variées.

Un changement dans la structure d'un produit chimique peut avoir des effets considérables sur ses propriétés. Par exemple, lors du développement de pharmaceutiques, des modifications de structure selon le concept de bioisostèrisme permettent de modifier les propriétés d'une molécule dans le but de l'amélioration de la stabilité, de l'absorption orale, ou encore de la distribution, de la métabolisation et de l'excrétion (ADME) de candidats médicaments, tout en conservant leurs propriétés biologiques (Hamada et Kiso, 2012). L'application de modification structurale dans une approche environnementale est un concept plus récent mais a déjà été étudié. Kümmerer (2007) donne l'exemple du benzène qui, après activation métabolique, est cancérogène et n'est que lentement biodégradé. L'insertion d'un atome d'oxygène dans la liaison carbone-hydrogène donne un nouveau composé, le phénol avec des propriétés très différentes : il est facilement biodégradable en aérobie, non cancérigène mais toujours actif contre les bactéries. Kümmerer et al. (2000) démontrent que la réduction de l'impact des produits pharmaceutiques sur l'environnement aquatique est réalisable en modifiant la structure chimique d'un composé tout en exerçant un mode d'action et une activité thérapeutique similaires au travers de l'étude de stéréochimie de la cyclophosphamide et de l'ifosfamide. Rastogi et al. (2015a et b) s'intéressent au re-design de l'atenolol et du métroprolol en étudiant leur dégradation pour identifier quelles parties de la structure de la molécule confère l'activité ou la persistance, afin de proposer un design théorique plus biodégradable et moins toxique tout en gardant l'activité initiale de la molécule. Lee et al. (2000) ont développé une approche où de nouveaux antibiotiques pourraient être désactivés par l'action de la lumière, limitant donc le phénomène d'antibiorésistance dans l'environnement.

Les propriétés des molécules donnent des indications sur leur comportement, par exemple le Kow, indicateur d'hydrophobicité, est directement corrélé à la potentielle bioaccumulation (BCF) du composé et donne des indications sur sa toxicité. Ou encore par exemple, pour l'impact de la dégradation photochimique, les sous-structures appelées chromophores sont déjà connues, comme pour l'hydrolyse (par exemple les groupes ester) et la biodégradation (Kümmerer, 2007). Cette connexion entre la structure d'une molécule et ses propriétés, appuyée par des outils *in silico* tels que l'analyse QSAR, permettrait de mieux anticiper les comportements de molécules et peut être mis à profit dans une démarche d'éco-conception.

Comme vu précédemment, l'autorisation de mise sur le marché des nouveaux médicaments à usage humain, doit être accompagnée d'évaluation du risque environnemental, le document guide de l'European medicines agency (EMEA) fixe une PEC au seuil de 0,01 µg L^{-1} mais notifie que cette valeur est amenée à être modifiée en fonction des données de toxicité chronique disponible. On peut imaginer que dans un futur proche, l'éco-conception permettra de devancer ces problématiques. Comme vu en Tableau 1- 6, cette démarche innovante est encouragée par l'OCDE (2019) pour i) le développement de nouvelles substances plus efficacement biodégradées mais conservant leurs propriétés et efficacité pharmaceutiques, ou ii) la reconception de produits pharmaceutiques existants avec une meilleure biodégradabilité environnementale.

1.1.4.3. Analogues du méthotrexate : objets d'étude et questions posées

Cette thèse propose d'étudier la validité d'une démarche d'éco-conception suite à la modification de la structure chimique d'une molécule pharmaceutique problématique. Pour étudier la validité d'une telle démarche, nous avons choisi le méthotrexate (MTX), un cytotoxique de la classe des antimétabolites, analogue de l'acide folique. Il est un inhibiteur compétitif de la dihydrofolate-réductase (DHFR) et impacte donc la division cellulaire. Ce choix, déjà argumenté quant à la problématique de la contamination par les anticancéreux et appuyé par la formation d'un TP potentiellement actif et persistant, le 7-OH-MTX, comme énoncé en partie 1.1.3.1,ii. Le MTX a aussi été sélectionné pour sa capacité à accueillir une modification structurale particulière. Cette thèse s'intègre dans le projet EDIFIS qui étudie donc l'impact du MTX en comparaison avec différents analogues éco-conçus ou « éco-drogues » synthétisés par l'ICMMO. Les analogues éco-conçus synthétisés et évalués pharmacologiquement par l'ICMMO seront présentés en **Chapitre 3**. Cependant, dans le cadre de la thèse, seul un analogue a été sélectionné comme étant prometteur. Cet analogue (Figure 1- 6, à droite) présente un atome d'oxygène qui remplace la fraction N-méthyle du MTX (Figure 1- 6, à gauche), nous l'appelons donc l'analogue oxygéné ou OMTX.

La stratégie de ce projet repose sur l'inactivation programmée de l'analogue qu'on pourrait alors qualifier « d'écodrogue ». Cette molécule présente une modification bioisostérique (modification structurale garantissant des propriétés physico-chimiques similaires) lui conférant le même mode d'action thérapeutique que la molécule initiale. La synthèse de cet analogue est déjà décrite dans la littérature et il présente une activité antitumorale *in vivo* comparable au MTX contre la leucémie lymphoïde L-1210 chez la souris (Nair et al., 1981). **L'hypothèse est alors qu'il présenterait une fraction sensible dite auto-immolable ou « self-immolative scaffold » le rendant plus facilement fragmentable, donc moins persistant et donnerait lieu à des produits de transformations (TPs) différents et éventuellement moins toxiques que le MTX.**

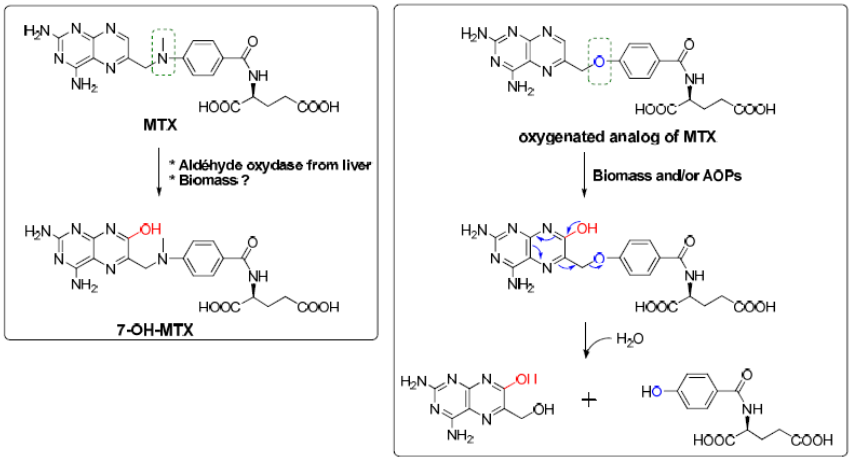


Figure 1- 6 Hypothèses sur le devenir environnemental du MTX (à gauche) en comparaison avec son analogue éco-conçu, le OMTX (à droite)

Le travail de cette thèse a essentiellement consisté en une étude comparative entre le devenir et les effets du MTX et celui du OMTX. Cette comparaison a porté sur (i) la caractérisation de la dégradation des deux molécules durant différents processus susceptibles d'intervenir dans l'environnement, la photodégradation dans les eaux et la biodégradation par des boues activées ; (ii) l'identification et le suivi des produits formés au cours de ces processus de transformation et finalement (iii) la caractérisation des effets des molécules et de leur produits de transformation par des tests ciblés sur leur mode d'action et par le biais de tests d'écotoxicité aquatique normalisés (*A. fischeri, R. subcapitata*) mais également au regard d'impacts potentiellement induits sur les communautés microbiennes de boues de station d'épuration**.**

Afin de préciser les objectifs scientifiques et méthodologiques de la thèse, le chapitre suivant propose une analyse critique des connaissances disponibles : d'une part, sur les processus de transformation étudiés dans le cas spécifique des médicaments anticancéreux et du MTX en particulier et d'autre part, sur les questions méthodologiques concernant à l'analyse de ces composés et l'identification de leurs produits de transformation dans des eaux naturelles ou usées. Ces questions sont centrales pour améliorer les procédures à mettre en œuvre dans l'éco-conception.

Chapitre 2. Processus impliqués dans le devenir environnemental des anticancéreux et méthodes analytiques pour suivre les produits de transformation

2.1. Devenir des résidus de médicaments dans les eaux

Afin d'envisager la limitation de la contamination de l'environnement et d'étudier la démarche d'éco-conception de composés pharmaceutiques, il est important de comprendre les processus mis en jeu tout au long du parcours de ces résidus schématisés en Figure 2.1-1. Le principe actif pharmaceutique, une fois consommé par le patient, devient un contaminant dès son excrétion dans les eaux usées. Lorsque l'on parle de résidu, il est question d'une partie du principe actif, mais aussi de ses métabolites potentiels, ce sont donc plusieurs composés qui se retrouvent dans les eaux usées. Comme vu précédemment, on peut aussi noter des rejets ponctuels mais élevés sur les sites de production. Ces eaux usées arrivent dans les stations d'épuration dont la fonction première n'est pas de dégrader spécifiquement les contaminants mais de réduire la charge en matières organiques (Bound et Voulvoulis, 2005). Les procédés conventionnels les plus répandus font appel à des processus biologiques par l'utilisation de boues activées (Ahmed et al., 2017). Différents paramètres entrent alors en compte, conduisant à l'élimination totale ou partielle des contaminants, notamment par des processus d'adsorption aux boues (Figure 2.1-1,i) et de biodégradation par ces boues (Figure 2.1-1,ii). Des processus de photodégradation par l'action du soleil peuvent mener à la dégradation des composés sortant des STEP (Figure 2.1-1,iii), l'action d'UV est aussi un processus utilisé lors de traitements spécifiques en amont des STEP, par exemple pour traiter des effluents d'hôpitaux, ou bien en aval, notamment lors de la potabilisation des eaux (Figure 2.1-1,iv). La photodégradation et les traitements spécifiques, appelés AOPs (Advanced Oxidation Processes - Procédés d'Oxydation Avancée) seront détaillés dans les parties suivantes**.** L'ensemble de ces processus conduisant à l'élimination des composés mène à la formation de produits de transformation (TPs).

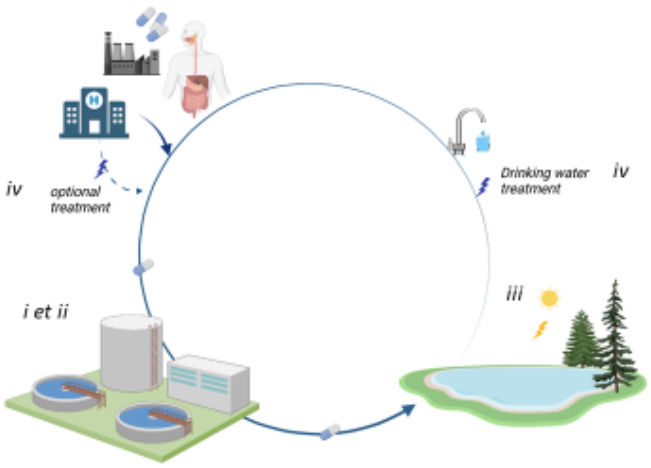


Figure 2.1-1 Parcours schématisé des résidus de médicaments après excrétion du patient

2.1.1. Processus responsables de l'élimination en station d'épuration

En STEP, les deux voies majeures d'élimination des composés pharmaceutiques sont l'adsorption aux boues et la biodégradation. Quant aux processus de volatilisation, de photodégradation et d'hydrolyse ou d'oxydation, ils restent négligeables pour la plupart des composés (Kosjek et Heath, 2011; Verlicchi et al., 2012).

L'efficacité de leur élimination varie selon le type de technologie utilisée : boues activées (activated sludge en anglais, noté « AS »), bioréacteurs à membrane, réacteurs à biofilms. D'autres méthodes physiques et physico-chimiques telles que la coagulation-floculation, l'adsorption sur charbon actif et la filtration sur membrane peuvent être utilisées pour éliminer les produits pharmaceutiques de l'eau (Luo et al., 2014). Nous nous intéresserons principalement aux boues activées, technologie la plus largement répandue en France (Ahmed et al., 2017). L'efficacité du traitement biologique dépend aussi des propriétés physico chimiques des composés, du temps de rétention hydraulique ou temps de séjour, le temps de rétention solide ou âge des boues, mais aussi d'autres facteurs tel que les conditions climatiques (pluie entraînant une dilution, température, ensoleillement etc…) (Gros et al., 2007).

2.1.1.1. Sorption par les boues

Les paramètres physico-chimiques des composés influent sur leur voie d'élimination. La constante de dissociation (pKa), le coefficient de partage eau/octanol (Kow), le coefficient de distribution (Kd) aussi appelé coefficient d'adsorption, nous donnent des indications quant à leur répartition entre la phase aqueuse et la phase particulaire/solide des boues (Pomiès et al., 2013).

Le coefficient Koc (Kd normalisé par rapport à la teneur en carbone organique de la phase solide), quant à lui, traduit l'aptitude de la molécule à être adsorbée sur la matière organique, les boues activées en étant constituées de 70 à 80 % (Çeçen et Gül, 2021). Plus le Koc est élevé, plus la molécule se liera préférentiellement à la phase solide par rapport à la phase aqueuse. Une molécule est considérée mobile si le Koc est inférieur à 100.

Le Tableau 2.1- 1 regroupe les propriétés de 6 anticancéreux et comme la plupart des pharmaceutiques, leur Kow <1 indiquent une forte polarité et une affinité avec la phase aqueuse (Rowney et al., 2009). Le 5-fluorouracile et l'ifosfamide présentent des Koc très élevés indiquant une potentielle adsorption à la matière organique, tandis que le méthotrexate dont le Koc est égal à 1, est faiblement sorbé sur les boues d'épuration (Toolaram et al., 2014; Kosjek et al., 2015). Les valeurs des pKa, relativement faibles du méthotrexate impliquent que le composé sera dissocié à des valeurs de pH autour de 7, ce qui augmente sa mobilité aqueuse. Ce comportement rend ce composé plus susceptible d'être biodégradé (Kiffmeyer et al., 1998; Toolaram et al., 2014 ; Kosjek et al., 2015).

Tableau 2.1- 1 Paramètres physico chimiques d'anticancéreux adapté de Toolaram et al. (2014)

Propriétés	Cyclophosphamide	Ifosfamide	5-Fluorouracile	Methotrexate	Gemcitabine	Imatinib

pK_a	2.84	1.45	pK_{a1} = 8.0
			pK_{a2} = 13
				pK_{a1} 4.7
				*pK_{a2} 3.36
				*pK_{a3} 5.71
					3.6	pK_{a1} = 8.07;
						pK_{a2} = 3.73
						pK_{a3} = 2.5;
						pK_{a4} = 1.52

log K_{ow}	0.6	0.86	−1	−1.85	−1.22	3
Solubilité (mg/L)	4.00×10^4	4×10^3	1.11×10^4	0.26×10^4	5.14×10^4	1.0×10^3
Constante d'Henry (atm-cu m/mole)	1.4×10^{-11}	1.4×10^{-11}	1.66×10^{-10}	1.54×10^{-31}	1.70×10^{-17}	N.D.
Pression de vapeur (mmHg)	4.45×10^{-5}	3.0×10^{-5}	2.7×10^{-6}	2.1×10^{-9}	1.7×10^{-9}	N.D.
K_{oc}	52	70	8	1	1.4	7.9
BCF	3	3	3	3.2	1	1

*(Rubino, 2001)

2.1.1.2. Biodégradation par des boues activées

Les produits pharmaceutiques polaires sont peu adsorbés et sont principalement éliminés par dégradation microbienne (Quintana et al., 2005).

Cette dégradation est réalisée par les microorganismes présents dans les boues de station d'épuration, regroupées en agrégats et appelés flocs de boues activées. Ces flocs sont présents en suspension et mis en contact avec l'eau à épurer, on parle alors de « cultures libres ». Ces boues activées sont essentiellement composées de microorganismes hétérotrophes : plusieurs centaines d'espèces bactériennes différentes, champignons, protozoaires et métazoaires, qui utilisent les éléments de pollution carbonée (matière organique), azotée et phosphorée comme source d'énergie pour leur croissance, nécessitant alors de l'oxygène. De l'air est alors injecté dans ces bassins, appelés bassins d'aération mais aussi bassins de nitrification puisque l'ammonium y est oxydé en nitrites (Bassompiere, 2007).

Ensuite, l'élimination totale de la pollution azotée est poursuivie en convertissant les nitrates (NO_3) et les nitrites (NO_2^-) en azote (N_2) par le passagage en conditions anaérobie effectué en coupant l'arrivée d'air. Cette étape appelée dénitrification est faite par des bactéries spécifiques autotrophes (qui utilisent le CO_2). Les phases de nitrification et dénitrification sont alternées en injectant ou non de l'air dans le bassin. La dégradation des polluants à majoritairement lieu lors de la phase aérobie.

Comme le détaillent Luo et al. (2014), la biodégradation des micropolluants peut se produire via différents mécanismes et conduire à une dégradation totale (minéralisation) ou partielle (accumulation de TPs) :

1) croissance d'un petit sous-ensemble d'organismes oligotrophes spécialisés, qui est moins courante dans les STEP et plus susceptible de se produire dans les eaux réceptrices ou les sédiments (Daughton et Ternes, 1999).

2) le co-métabolisme, dans lequel les micropolluants sont décomposés par des enzymes générées pour d'autres dégradations de la sous-station primaire (par exemple, l'ammoniac monooxygénase, et ne sont pas utilisés comme source de carbone et d'énergie pour la croissance microbienne notamment dans le cas de composés lentement biodégradés (Çeçen et Gül, 2021).

De plus, ces contaminants pourraient perturber les procédés de traitement des eaux usées en raison de leur toxicité sur les communautés microbiennes des boues et conduire à un mauvais abattement de la demande biologique en oxygène (DBO), perturber les processus de nitrification et de dénitrification ou encore entrainer la défloculation des boues (Love et Bott, 2002; Alvarino et al., 2014; Amarei et al., 2017). Ces perturbations peuvent parfois mener à une défaillance du fonctionnement de la STEP (Ren, 2004; Yao et al., 2012).

Les études menées sur la biodégradation d'anticancéreux en condition de laboratoire indiquent que certains composés se dégradent facilement comme par exemple la cytarabine (Kümmerer et Al-Ahmad, 1997) ou la gemcitabine (Franquet-Griell et al., 2017b) tandis que d'autres semblent être récalcitrants comme l'ifosfamide, la cyclophosphamide (Kümmerer et al., 2000; Buerge et al., 2006 ; Franquet-Griell et al., 2017b) ou encore le 5-fluorouracile (Kümmerer et Al-Ahmad, 1997). Pour le méthotrexate, Henschel et al. (1997) et Lutterbeck et al. (2015a) rapportent une faible biodégradabilité selon le test « Biodegradation Closed Bottle», tandis que Wang et al. (2018) le classent comme composé facilement biodégradable lors d'une expérimentation en bioréacteur à membrane osmotique en condition anaérobie. Kosjek et al. (2015) observent la biodégradation du composé en quelques jours en présence et en absence de milieu nutritif et à différentes concentrations de boue activée. Onesios et al. (2009) soulignent que les différences dans la conception expérimentale (aérobie, anaérobie, dimensionnement, temps de séjour, âge des boues, apport de milieu nutritif etc…) peuvent impacter la façon dont les micro-organismes transforment les composés. Le rôle de la concentration initiale est déterminant : en effet, apporté à haute concentration, le composé peut être utilisé comme source de carbone et d'énergie par les microorganismes, alors qu'un composé apporté à de faibles concentrations sera plutôt dégradé par co métabolisme.

2.1.1.3. Dégradation abiotique

i. Photodégradation : quelques généralités

La lumière peut interagir avec un composé et engendrer des réactions menant à sa dégradation. La photodégradation causée par l'irradiation solaire est considérée comme une voie majeure dans l'élimination des résidus de médicaments dans l'environnement (Boreen et al., 2003; Dąbrowska et al., 2004; Fatta-Kassinos et al., 2011). La première loi de la photochimie mentionne que « seule une radiation lumineuse absorbée par la molécule peut initier une réaction », ce qui implique une interaction entre le composé et une onde électromagnétique (loi de Grotthus-Draper). En effet, beaucoup de ces composés pharmaceutiques possèdent des cycles aromatiques, des hétéroatomes et d'autres groupes chromophores fonctionnels qui peuvent absorber le rayonnement solaire ou réagir avec des espèces transitoires photogénérées dans l'eau naturelle. Boreen et al. (2003) mentionnent aussi que certains composés contiennent également des groupements structuraux, tels que phénol, nitro et les groupes naphoxyle, qui sont similaires à ceux que l'on trouve dans les pesticides et qui subissent de la photodégradation. La deuxième loi mentionne que pour chaque photon de lumière absorbé, une molécule au plus est activée pour faire une réaction chimique (Stark-Einstein) : le nombre de molécules absorbant la lumière est donc proportionnel à la quantité de lumière absorbée.

L'absorption d'énergie lumineuse conduit la molécule à un état excité. Le diagramme de Jablonski (Figure 2.1- 2a) rend compte de l'interaction entre la lumière et la matière et des différents transferts susceptibles de survenir entre les niveaux énergétiques, puis le retour à l'état fondamental peut suivre divers mécanismes concurrents (Figure 2.1- 2b) (Wayne, 2005):

- La désactivation de l'espèce excitée par différents processus (lorsqu'il s'agit d'une désactivation par émission d'une radiation, on parle dans ce cas de luminescence (Figure 2.1- 2b,voie vi). Si cette émission se produit entre deux états de même multiplicité, elle est nommée fluorescence. Si elle se produit entre deux états de multiplicité différente, elle est appelée phosphorescence. Ou par un processus de quenching, une molécule M peut soulager AB∗ de son surplus d'énergie (voie vii).

-La transformation de l'espèce excitée suivant divers mécanismes. L'espèce peut se dissocier en fragments réactifs (voie i), un cas particulier de dissociation est celui de l'ionisation (voie viii). L'espèce peut aussi réagir directement (voie ii) ou s'isomériser (voie iii). Elle peut aussi transférer ou perdre son excitation par des processus intermoléculaires ou se réorganiser par des processus intramoléculaires. Le transfert d'énergie représentée par les voies iv et v sur la figure, conduit à des espèces excitées, qui peuvent alors participer à n'importe lequel des processus généraux.

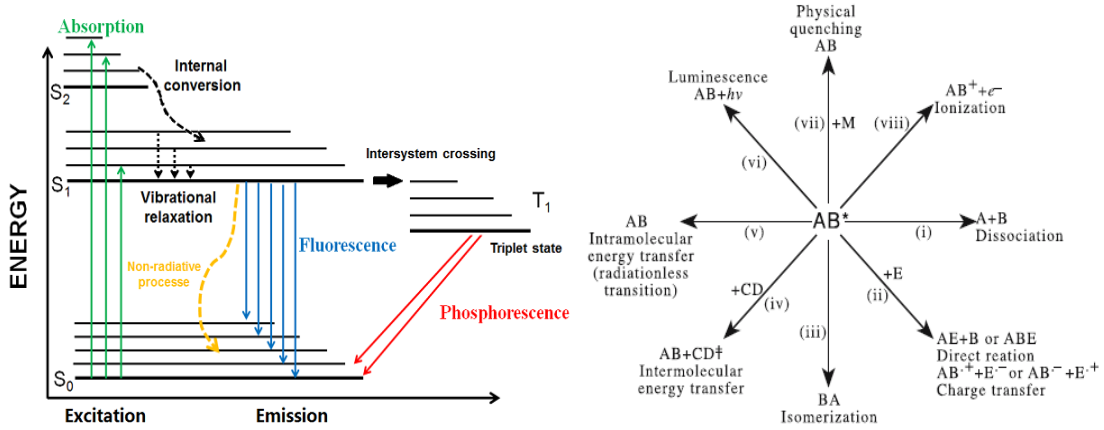


Figure 2.1- 2a) Diagramme de Jablonski [3]

b) Mécanismes de retour à l'état fondamental extrait de Wayne (2005)

Ces deux types de réactions sont en concurrence et la prépondérance de l'une sur l'autre est évaluée par le rendement quantique Φ :

$$\Phi = \frac{nombre\ de\ molécules\ ayant\ réagi}{nombre\ de\ photons\ absorbés}$$

[3] Institut de biologie structurale, n.d. Photophysique des Protéines fluorescentes. URL : https://www.ibs.fr/recherche/groupes-de-recherche/groupe-imagerie-integree-de-la-reponse-au-stress/equipe-pixel/projets/article/photophysique-des-proteines-fluorescentes (accessed 2.11.22).

$$\Phi = \frac{N\ x\ \frac{d[M]}{dt} x\ l}{10^3\ x\ I_0\ x\ (1-10^{-A})}$$

Avec d[M]/dt = variation initiale de la concentration de la molécule M en mol/L pendant la dt en s, *N* = nombre d'Avogadro = 6,022 x 10^{23}, I_0 = flux de photons incident en photons en cm^{-2} s^{-1}, A = absorbance à la longueur d'onde d'excitation.

ii. Photodégradation des anticancéreux dans les eaux

Il existe deux types de processus photolytiques : la photolyse directe et la photodégradation indirecte (Figure 2.1- 3) :

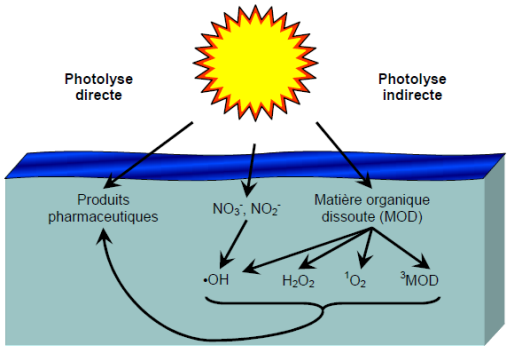


Figure 2.1- 3 Photolyse directe et indirecte dans le milieu aquatique naturel extrait de Coetsier (2009a)

Lors de la photolyse directe les rayons UV agissent directement sur les composés, tandis que lors de la photolyse indirecte, ce sont des intermédiaires chimiques très oxydants comme par exemple, l'oxygène singulet ($^1O_2$), des radicaux hydroxyles (HO°) ou des radicaux alkyle peroxyles (°OOR) créés sous l'action de rayons UV qui agissent sur les molécules organiques. La matière organique dissoute (DOM) et les ions nitrates sont connus comme photoinducteurs ou photosensibilisateurs et jouent un rôle clé dans la dégradation des contaminants organiques dans le système aquatique (Andreozzi et al., 2003). Cependant, la nature et/ou la concentration des DOM, dont les substances humiques ou fulviques, peuvent les entrainer à avoir des impacts variables sur les photoréactions. A forte concentration, elles jouent essentiellement un rôle de filtre solaire. La nature des DOM module leurs capacités chromophores, de production d'entités réactives et/ou de transfert (de proton, d'électron ou d'énergie) et donc à favoriser ou non des photodégradations induites (Andreazzi et al., 2003).

Concernant les anticancéreux, certains se dégradent facilement en photolyse directe, en accord avec leur spectre d'absorption, tels que le méthotrexate, la doxorubicine ou le 5-fluorouracile (Calza et al., 2014 ; Lin et al., 2014). D'autres composés comme l'ifosfamide, la cyclophosphamide ou encore la

mitomycine ne sont pas susceptibles de subir une photolyse directe, mais peuvent se dégrader *via* une photolyse indirecte comme la plupart des composés organiques (Lin et al., 2014).

Les principes de la photochimie peuvent être utilisés dans le cadre de traitements avancés, comme vu sur la Figure 2.1-1, iv. Ces procédés font intervenir des processus physiques, chimiques, ou hybrides peuvent être employés comme pré-traitement ou en post-traitement, pour assainir les eaux avant leur rejet dans l'environnement.

iii. Procédés d'oxydation avancée (AOPs)

Lorsque les composés sont récalcitrants à la biodégradation, mais aussi à la photolyse directe telle que les traitement UV-C seuls (compris entre 200 et 280 nm), des traitements plus poussés tels que les procédés d'oxydation avancée (AOPs) ont fait leurs preuves, comme par exemple sur les anticancéreux cytarabine, ifosfamide et la cyclophosphamide (Franquet-Griell et al., 2017b).

Les AOPs ont d'abord été introduits pour le traitement des eaux potables dans les années 1980, puis leur utilisation a été étendue au traitement de différents types d'effluents d'eaux usées, notamment pour éliminer les odeurs et les composés organiques volatiles, pour décolorer des eaux, ou pour désinfecter des eaux potables. Ces procédés permettent la dégradation de produits phytosanitaires et pharmaceutiques et souvent employé dans le traitement des effluents industriels (Zaviska et al., 2009).

Le concept initialement établi par Glaze et al. (1987) définit les AOPs comme des processus impliquant la génération de radicaux tels que OH°, HO_2° ou encore O_2^{-}°, espèces hautement actives capables de réagir rapidement et de manière non sélective sur la plupart des composés organiques. Il existe une multitude de procédés mais les plus efficaces sont ceux qui conduisent à la formation des radicaux hydroxyles OH° car ils ont un pouvoir oxydant supérieur à celui des oxydants traditionnels (Andreozzi, 1999). De manière générale les réactions d'oxydation de composés pharmaceutiques par des mécanismes radicalaires OH° peuvent être décrit selon Litter (2005) :

Initiation:	RH + OH°	→	R° + H_2O
Propagation :	R° +R'H	→	RH + R'°
	R° + O2	→	ROO°
Terminaison :	R° + R°	→	R-R
	R° + OH°	→	R-OH
	OH° + OH°	→	H_2O_2

Ces radicaux peuvent être formés à partir d'ozone, de peroxyde d'hydrogène, d'oxygène ou d'autres composés en utilisant des couplages de procédés chimiques, photolytiques, catalytiques. Quelques exemples sont donnés en Figure 2.1- 4. Il existe aussi des AOPS basés sur des radicaux

persulfates, ou bien en utilisant des procédés basés sur les ultrasons ou encore d'électrochimie. Ces couplages permettent une optimisation de la génération de radicaux et une augmentation de la cinétique de dégradation.

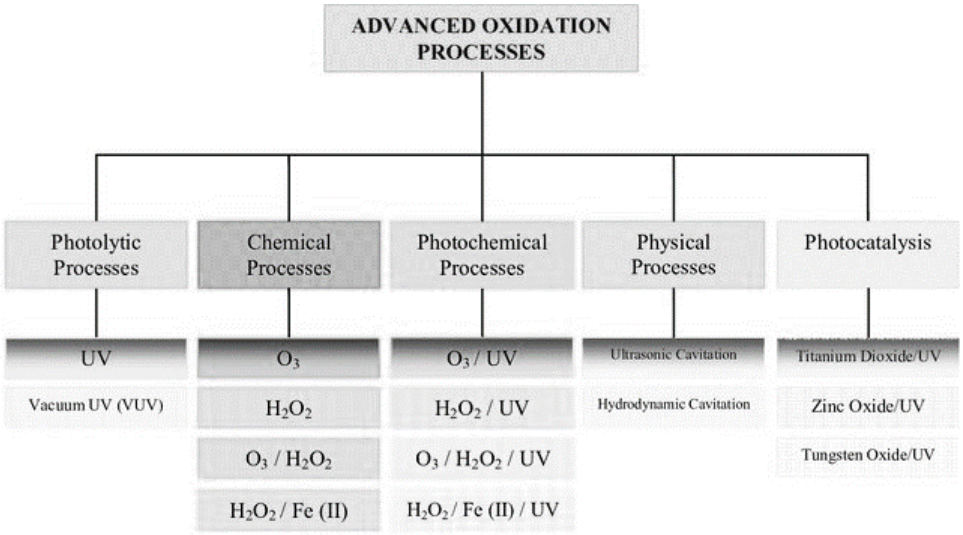


Figure 2.1- 4 Exemples de procédés d'oxydation avancée, extrait de Chirwa et Bamuza-Pemu (2010)

En traitement des eaux, l'action du rayonnement en UV-C induit un effet bactériostatique et ou bactéricide en perturbant la structure de l'ADN des cellules des micro-organismes et est donc utilisé pour la désinfection (ANSES, 2010). De plus, comme nous l'avons dans la partie précédente, la photo-irradiation énère des radicaux et permet la dégradation de composés. En effet, à 254 nm, l'énergie des photons est capable de provoquer une rupture homolytique ou hétérolytique dans une molécule (Wayne, 2005). Cependant l'efficacité de cette irradiation seule n'est pas toujours optimale pour dégrader tous les micropolluants (Legrini et al., 1993). Le couplage des radiations UV avec des oxydants puissants (comme O_3 ou encore H_2O_2) permet de dégrader les polluants par photodégradation en utilisant les rayons UV pour exciter les molécules polluantes et les dégrader, par oxydation *via* une action directe des oxydants mais aussi par oxydation *via* une photolyse de l'oxydant induisant la formation de radicaux libres hydroxyles (Zaviska et al., 2009). Parmi les AOPs présentés en Figure 2.1- 4, le couplage UV/ H_2O_2, largement utilisé pour le traitement des eaux (Legrini et al., 1993), permet d'accélérer la réaction en produisant plus de radicaux hydroxyles par mole de réactif. L'irradiation UV provoque l'excitation de la molécule du peroxyde d'hydrogène qui subit une scission homolytique entre la liaison O-O d'où il résulte la formation de deux radicaux HO° qui initie la réaction (Zaviska et al., 2009)

Initiation :	$H_2O_2 + hv$	→	$2OH°$
Propagation :	$OH° + H_2O_2$	→	$H_2O + HO_2°$
	$HO_2° + H_2O_2$	→	$OH° + H_2O + O_2$
	$OH° + HO_2^-$	→	$HO_2^- + OH^-$
Terminaison :	$2HO_2°$	→	$H_2O_2 + O_2$
	$OH° + HO_2°$	→	$H_2O + O_2$
	$2OH°$	→	H_2O_2

Les méthodes d'oxydation chimique telles que l'ozonation/H_2O_2, la photolyse UV/H_2O_2 et les procédés photo-Fenton se sont avérées être les meilleurs procédés pour l'élimination de pesticides et de produits pharmaceutiques (Ahmed et al., 2017). Pieczyńska et al. (2017) rapportent que le procédé photo-Fenton est une méthode efficace pour l'élimination des cytostatiques, entraînant un degré élevé de minéralisation. Ces auteurs soulignent aussi que les nombreuses études en condition de laboratoire dans l'eau distillée peuvent donner des résultats éloignés de la réalité, car dans les eaux de surface ou eaux usées les matrices sont plus complexes et contiennent des mélanges de médicaments.

Concernant le méthotrexate, comme présenté dans le Tableau 2.1- 2, les différentes techniques de traitement rapportent toutes une très bonne dégradation de ce composé, certaines techniques telles que les AOPs mènent cependant à une meilleure minéralisation.

Tableau 2.1- 2 Efficacité de différents types de traitements ou AOPs sur le méthotrexate, adapté de Zhang et al. (2021)

AOPs	Conditions	échantillon	[MTX] (mg/L)	% de dégradation	Minéralisation	Ref.
Chloration	[HOCl] = 2 mg/L, pH 7, durée = 10 min	eau pure	1	98.2%	NA	Yin et al., 2017
Chloration	[HOCl]:[MTX] = 100, pH 8.6, durée = 120 min	eau pure	1	100%	NA	Roig et al., 2014
$UV_{(200–440nm)}$ H_2O_2	[H_2O_2] = 9.8 mM, pH 6.4, durée = 256 min	eau pure	20	100%	~ 60%	Lutterbeck et al., 2015a
$UV_{(200–440\ nm)}$ Fenton	[Fe^{2+}] = 1.56 mM, [H_2O_2] = 9.8 mM, pH 5, durée = 256 min	eau pure	20	100%	78%	Lutterbeck et al., 2015a
$UV_{(254\ nm)}$ PMS	[PMS] = 0.3 mM, pH 3.3, durée = 30 min	eau pure	5	~ 95%	~ 30%	Kanjal et al., 2020
Photocatalysis $UV_{(200–440\ nm)}$	[TiO_2] = 500 mg/L, pH 6.4,durée = 256 min	eau pure	20	100%	72%	Lutterbeck et al., 2015a
Photocatalysis $UV_{(360nm)}$	[TiO_2] = 200 mg/L, pH 6.4, durée = 60 min	eau pure	15	100%	NA	Calza et al., 2014
Photocatalysis $UV_{(352nm)}$	UV-A lamp (3.0 mW/cm^2, 352 nm), [TiO_2] = 1 mg/L, pH 8.0, durée = 90 min	eau pure	15	100%	NA	Lai et al., 2017
Photocatalysis UV $_{(352nm)}$	UV-A lamp (3.0 mW/cm^2, 352 nm), [TiO_2] = 1 mg/L, [HCO_3^-] = 400 mg/L, pH 8.0, durée = 10 min	eau pure en présence d'ions bicarbonates	15	100%	NA	Lai et al., 2017
Oxydation Electrochimique	j = 30 mA/cm^2, [Na_2SO_4] = 200 mg/L, pH 7, durée = 5 min	eau pure	0,5	100%	NA	Barışçı et al., 2018
Oxydation Electrochimiqu	j = 16 mA/cm^2, pH 6, durée = 360 min	eau pure	50	100%	71%	Siedlecka et al., 2018
Ozonation	0.6 mg O_3/mg DOC	eau usée	0,005	~ 100%	NA	Li et al., 2016
Ozonation	1mg O_3/mg DOC	eau provenant d'une station municipale avant traitement de l'eau	10	97%	NA	Garcia-Ac et al., 2010

Bien que souvent efficaces, ces procédés avancés ne sont pas adaptés à de gros volume ou débits et représentent un cout élevé, ils ne peuvent donc pas être utilisés à grande échelle (Nikolaou et al., 2007). De plus ils ne mènent pas toujours à une minéralisation totale et peuvent conduire à une grande variété de TPs, surtout si des radicaux sont impliqués (Fatta-Kassinos et al., 2011) et ces TPs peuvent parfois être plus toxiques que la molécule mère, comme nous l'avons vu au **Chapitre 1** en partie 1.1.3.1,ii ce qui est notamment le cas pour les TPs du MTX produits par UV/TiO_2 en présence de HCO_3^- dans l'étude de Lai et al. (2017).

On retiendra de cette analyse de la littérature que peu de travaux ont porté spécifiquement sur la biodégradation et la photodégradation des anticancéreux et du MTX en particulier. Ces études ont été menées en laboratoire dans des conditions parfois peu représentatives de la réalité. Par ailleurs, nous avons également vu au **Chapitre 1** que les connaissances sur la toxicité des produits de transformation sont encore relativement fragmentaires.

2.1.1.4. Produits de transformation des anticancéreux

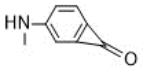
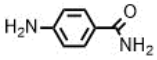
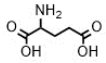
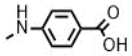
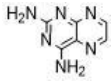
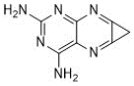
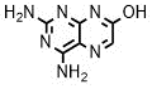
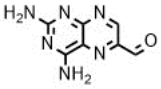
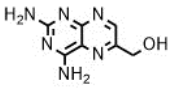
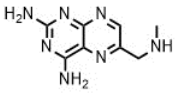
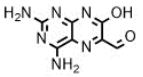
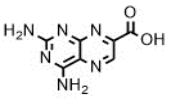
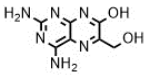
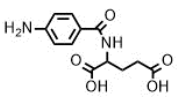
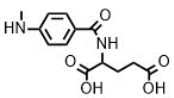
Comme nous l'avons vu au **Chapitre 1**, certains TPs peuvent être plus toxiques et parfois plus persistants que les molécules mères mais ces effets restent mal évalués. Il est donc nécessaire dans un premier temps de pouvoir identifier ces molécules.

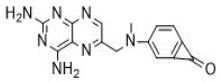
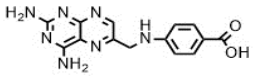
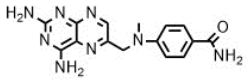
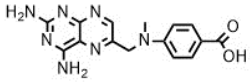
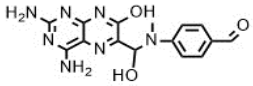
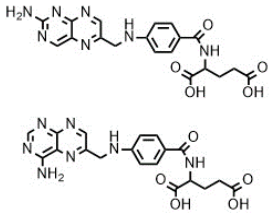
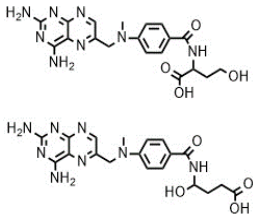
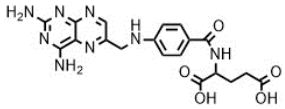
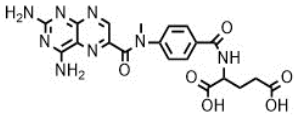
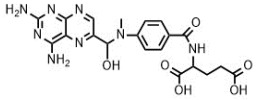
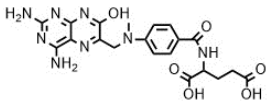
Onesios et al. (2009) ou encore Xu et al. (2016) soulignent que la composition de la boue de station d'épuration et que les conditions peuvent influencer les voies de dégradation et mener à des TPs différents, ce qui complique aussi les comparaisons entre études. Les transformations abiotiques, quant à elles, peuvent mener à une plus grande variété de TPs selon Fatta Kassinos (2011). Mais, ici aussi les conditions mais aussi la concentration initiale peut impacter les voies de dégradation, Fatta Kassinos (2011) recommande l'emploi de tests standardisés, très peu utilisés par la communauté scientifique. Cependant les TPs suivis sont souvent des TPs déjà connus. Pour réaliser l'identification de nouvelles molécules, il est souvent nécessaire de travailler à haute concentration.

Différents travaux comme ceux de Calza et al. (2014), Kosjek et al. (2015), Lutterbeck et al. (2015a), Lai et al. (2017) et Kanjal et al. (2020) font appel à des spectromètres de masse comportant des analyseurs à haute resolution dans l'identification de photo-TPs et de TPs issus de la biodégradation par des boues de STEP du MTX. Kiffmeyer et al. (1998) ont travaillé uniquement sur le TP majoritaire qu'ils observent au cours de l'étude de biodégradation du MTX. Ces auteurs ont proposé le 7-OH-MTX en se basant sur ses spectres UV et IR.

Au total 28 TPs ont été proposés par différents auteurs et sont présentés dans le Tableau 2.1- 3.

Tableau 2.1- 3 Produits des transformation du MTX : structures et conditions d'obtention

Masse (Da)	structure	Lai et al., 2017	Calza et al., 2014	Lutterbeck et al., 2015a	Kosjek et al., 2015	Kanjal et al., 2020	Kiffmeyer et al.,1998
		Eau MilliQ	Eau MilliQ	UV/H2O2		Eau MilliQ	
		Avec ou sans HCO3-	+	+	Aerated activated sludge	UV/	Aerated activated sludge
		UV/TiO2	UV	Biodegradation par boue activées		Peroxy-monosulfate	
			Ou UV/TiO2				
133		non	non	non	non	oui	X
136		oui*	non	non	non	non	X
147		oui*	non	non	non	non	X
151		non	non	non	oui	oui	X
162		oui	oui	non	non	non	X
174		non	non	non	non	oui	X
178		oui	oui	oui	non	non	X
190		oui	non	oui	oui	oui	X
192		oui*	non	non	non	non	X
205		non	non	non	oui	non	X
206a		oui	non	oui	non	non	X
206b		non	non	non	oui	oui	X
208		oui	oui	oui**	non	non	X
266		oui*	oui**	oui**	non	non	X
280		oui	oui**	oui**	non	non	X

307		non	non	non	non	oui	X
311		non	non	non	oui	non	X
324		oui	oui	oui	oui	non	X
325 (DAMPA)		oui	non	non	oui	oui	X
341		non	non	non	oui	non	X
425		oui	non	non	non	non	X
426		non	oui	non	non	non	X
440		oui	non	non	non	non	X
468		oui	non	oui	non	non	X
470		non	non	oui	non	non	X
470 (7-OH-MTX)		oui*	non	non	oui	non	oui

**Vérifié avec étalon analytique*

*** Déduction selon la fragmentation*

X Pas d'identification recherchée

Certains TPs ne sont identifiés que par un seul auteur, tandis que d'autres sont communs à plusieurs conditions. TP 324 et 190 sont observés par 4 auteurs, TP 324 correspondrait à l'élimination de

l'acide succinique $C_4H_6O_4$ par suite du clivage de la liaison C–N dans le segment acide glutamique (Calza et al., 2014 ; Kosjek et al. 2015). Le TP 190 quant à lui, observé majoritairement par Kosjek et al. (2015), proviendrait de la perte d'une molécule d'eau du TP 208 tandis que Lutterbeck et al. (2015a) proposent la N-Dealkylation du TP 468, donc pour des TPs identiques, différentes voies de formation peuvent être envisagées.

Le TP470, correspondant au 7-OH-MTX semble se former en condition biotiques et abiotiques. C'est ce TP qui était observé par Kiffmeyer et al. (1998) comme majoritaire et persistant (identification basée sur son spectre UV et IR). Cependant pour Kosjek et al. (2015), ce composé est minoritaire et non persistant. Lai et al. (2017) arrivent à la même conclusion et montrent sa formation uniquement en présence de TiO_2. Lutterbeck et al. (2015a) rapportent aussi un hydroxylation de la molécule en position N-alpha mais soulignent que le schéma de fragmentation obtenu ne permet pas d'indiquer la place du –OH sur la structure. Le TP 325 correspond au DAMPA, il résulte de l'hydrolyse de la partie acide glutamique : ce composé est un métabolite connu (Widemann et al., 2000), mais se forme aussi dans les boues de STEP et en conditions abiotiques. Les TPs 206a et b correspondent à un clivage C-N de la molécule. Lutterbeck et al. (2015a) et Lai et al. (2017) proposent la structure 206a qui serait issue directement du MTX tandis que Kosjek et al. (2015) et Kanjal et al. (2020) proposent un sous-produit du DAMPA en fonction des évolutions observées.

Pour résumer, le MTX est susceptible de subir de nombreuses réactions : l'hydrolyse qui semble majoritaire lors de la biodégradation par les boues, l'hydroxylation, le clivage de la liaison C – N, mais d'autres types de réactions ont été observées telle que, la déméthylation ou encore l'oxydation de l'acide glutamique. Les questions non résolues portent notamment sur l'influence des conditions environnementales qui vont moduler les processus de photodégradation (pH, intensité du rayonnement) ou de biodégradation intervenant dans une boue activée (concentration, origine et âge de la boue), leur intensité et leur cinétiques et les schémas réactionnels impliqués dans la transformation du MTX. La partie suivante est consacrée aux méthodes analytiques relatives à la quantification des composés dans des échantillons aqueux, ainsi que les méthodes permettant l'identification des TPs en faisant à nouveau un focus sur les connaissances disponibles pour le cas du MTX.

2.1.2. L'analyse des résidus et l'identification des TPs

Nous avons vu que pour assurer une évaluation globale de l'impact écotoxicologique d'un composé, des méthodes analytiques sont nécessaires pour étudier leurs occurrences dans les eaux. En plus de sa présence, son devenir doit être pris en compte et donc des méthodes permettant l'identification de TPs sont nécessaires.

Les étapes clés nécessaires à l'analyse de pharmaceutiques à l'état de traces dans les eaux seront présentées ci-après : préparation d'échantillon, séparation et détection en fonction de l'objectif : suivi d'un processus ou d'une cinétique et/ou identification des produits de transformation.

2.1.2.1. Extraction, purification, concentration

L'extraction sur phase solide (SPE) permet l'extraction, la concentration et la purification de composés présents dans des matrices liquides par l'adsorption de ceux-ci sur une phase solide. Le choix de cette phase solide, appelée sorbant, est important car la rétention des analytes dépend de leurs interactions avec les groupements fonctionnels présents à sa surface, mais dépend également du type de matrice analysé et de ses interactions avec à la fois le sorbant et avec les analytes (Pavlović et al., 2007). Les interactions peuvent être de type hydrophobes (apolaire-apolaire), hydrophiles (polaire-polaire, liaison H, dipôle-dipôle…) ou encore électrostatiques. Les sorbants, souvent conditionnés sous forme de cartouche, sont constitués de silice ou de polymères, fonctionnalisés ou non. Les cartouches les plus utilisées pour l'analyse de composés pharmaceutiques en phase aqueuse sont de type HLB® (Richardson et Kimura, 2016). Elles sont de nature polymérique (mélange de polystyrène-divinylbenzène et N-vinylpyrrolidone) et présentent à la fois un caractère hydrophile et lipophile. Weigel et al. (2004) ont démontré leur efficacité sur une grande variété de pharmaceutiques dans des eaux de surfaces. Elles ont aussi fait leurs preuves dans de nombreuses études sur les cytostatiques dont le MTX, dans des eaux de surfaces mais aussi eaux usées (Yin et al., 2010; Martín et al., 2011; Ferrando-Climent et al., 2013; Negreira et al., 2013; Galaon et al., 2017).

Cette étape d'extraction peut être intégrée à la procédure analytique de deux manières, de manière classique et indépendante de l'analyse, dite « offline » ou bien directement dans le système d'injection d'échantillon, dite « online » :

i. SPE offline

Son principe de base est analogue à celui de la chromatographie de partage et se déroule en quatre étapes : le conditionnement de la phase stationnaire, le chargement de l'échantillon, le(s) lavage(s) de la cartouche et (après un séchage éventuel) l'élution (Figure 2.1- 5)

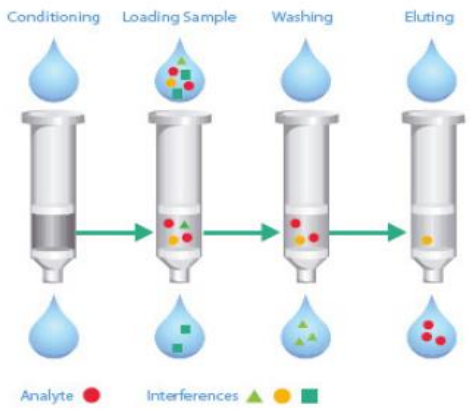


Figure 2.1- 5 Etapes de la SPE [4]

Chaque étape peut être optimisée, par exemple l'acidification peut influencer les récupérations des analytes, en particulier dans le cas de composés acides relatifs, tels que la cyclophosfamide ou le méthotrexate (Ferrando-Climent et al., 2013). Par contre un pH neutre ou basique est préconisé pour les composés basiques, alors que certains composés amphotères seront quant à eux, aussi bien retenus à pH acide que basique. Par ailleurs, l'ajout de Na_2EDTA permet d'éviter la chélation de l'analyte aux ions métalliques ou aux protéines présents dans la matrice (Castiglioni et al., 2005; Ferrando-Climent et al., 2013). Le choix de l'éluant est aussi un facteur important et doit être optimisé afin d'abaisser les limites de quantification. La plupart des méthodes publiées sur la détermination des concentrations en pharmaceutiques en solution aqueuse sont basées sur une extraction en phase solide (SPE) hors ligne suivie d'une LC-MS/MS qui permettent d'abaisser les limites de quantifications (LOQ) (Castiglioni et al., 2005; Roberts et Thomas, 2006; Langford et Thomas, 2009; Yin et al., 2010; Martín et al., 2011; Verlicchi et al., 2012; Ferrando-Climent et al., 2013 ; Galaon et al., 2017; Santos et al., 2018).

ii. SPE online

La méthode online, régie par les mêmes principes que la méthode offline, est une méthode automatisée couplée à l'analyse. La cartouche SPE, qui contrairement aux cartouches offlines, est réutilisable, est placée en amont de la colonne analytique. L'échantillon est ainsi chargé dans la cartouche, tandis qu'une pompe maintient la colonne UHPLC équilibrée (Figure 2.1- 6a). A la fin de cette étape, la vanne programmable commute entre les modes de chargement et d'élution (Figure 2.1- 6b), afin de désorber les analytes de la cartouche SPE par la phase mobile de la LC et de les transférer dans la colonne analytique, en mode « back flush » et selon le gradient chromatographique choisi.

[4] Gilson. Guide to SPE Automation. URL https://fr.gilson.com. (accessed 2.11.22).

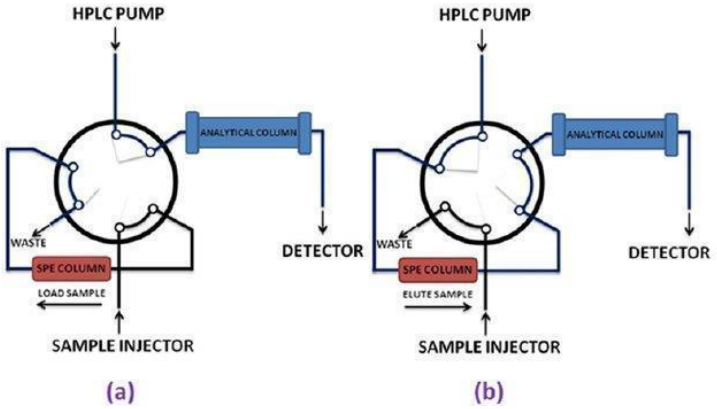


Figure 2.1- 6 Configuration d'un système SPE online avec une vanne de commutation à 2 positions et 6 voies ; (a) Position de charge et (b) position d'injection selon Lucci et al. (2012)

Des méthodes dites online-SPE-HPLC-MS/MS permettent de réduire le volume d'échantillon, minimisent leurs manipulations et donc réduisent la possibilité de contamination, elles réduisent aussi la consommation de solvants et représentent un gain de temps significatif en laboratoire. Cette technique présente donc beaucoup d'avantages par rapport à la méthode conventionnelle, de nombreuses études prouvent sa robustesse et sa sensibilité (Oliferova et al., 2006). Elle est utilisée pour l'analyse des résidus cytostatiques (Garcia-Ac et al., 2009b; Kovalova et al., 2009; Rabii et al., 2014). Une des limitations est cependant à noter : la nécessité d'utiliser des solvants compatibles avec la colonne analytique et le détecteur limite souvent le choix des mélanges utilisables (Togola et al., 2014).

2.1.2.2. Séparation et détection

Les premières méthodes de détection des pharmaceutiques dans l'environnement faisaient appel à la chromatographie en phase gazeuse couplée à la spectrométrie de masse (GC-MS) (Hignite et Azarnoff, 1977) ou encore aux immunoessais dans le cas de stéroïdes et du méthotrexate (Aherne, 1985); mais très vite le choix va se porter sur des techniques de chromatographie liquide couplée à un spectromètre de masse (LC-MS) plus adaptées aux composés polaires (Figure 2.1-7). Finalement, l'évolution des techniques et l'utilisation de la LC-MS en tandem, a permis d'atteindre des limites de détection inférieures au ng/L, tout à fait adaptées pour l'analyse de traces dans différents compartiments aquatiques tel que les eaux de surface, les eaux souterraines ou encore les eaux potables (Petrović et al., 2005).

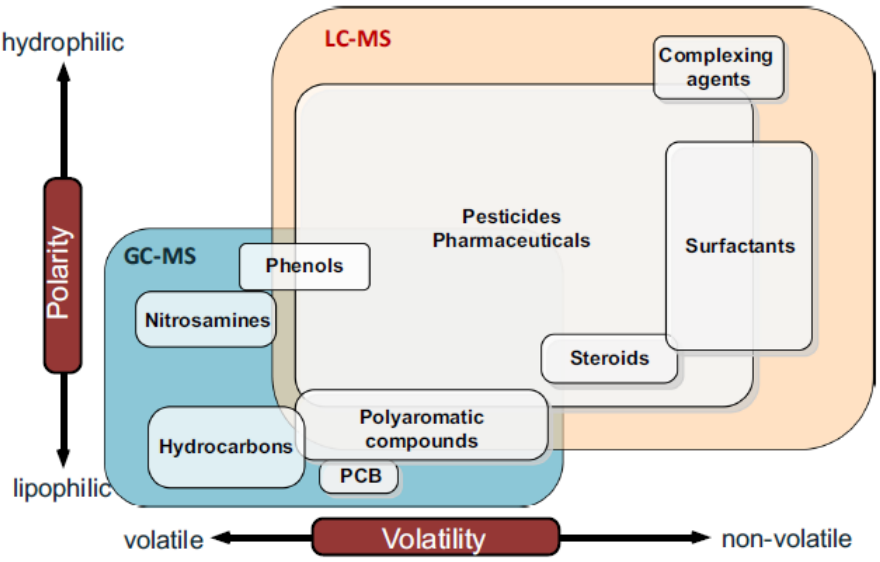


Figure 2.1-7 Domaine d'application de la GC-MS et de la LC-MS en fonction des composés analysés (Altenburger et al., 2019)

La LC-MS/MS fait appel au couplage d'une technique séparative, la chromatographie Liquide Haute performance (HPLC, souvent abrégée en LC) ou LC Ultra Haute Performance (UHPLC) qui permet la répartition des analytes entre une phase mobile et une phase stationnaire suivant leur affinité avec celles-ci. Ainsi l'échantillon est « décortiqué » avant d'atteindre le détecteur (séparé de sa matrice, d'interférents ou d'autres molécules). Cette technique séparative est couplée à un détecteur, le spectromètre de masse (MS) qui fonctionne en plusieurs étapes : l'Introduction, l'ionisation, la séparation et la détection. L'effluent issu de la colonne chromatographique est introduit dans une interface (ESI, APPI, APCI), permettant à la fois la volatilisation du milieu (à pression atmosphérique) et la production d'ions qui sont ensuite focalisés vers le spectromètre de masse. Dans le cas de l'electrospray (ESI), la vaporisation est obtenue par l'action conjuguée de gaz de nébulisation (N_2) et de gaz séchant à contre-courant (N_2 également) dans un fort champ électrique (obtenu par différence de potentiel entre le capillaire métallique d'introduction et une contre-électrode). Ce champ induit une accumulation de charges à la surface des gouttelettes nébulisées, d'où des explosions coulombiennes et la formation de microgouttelettes chargées. L'obtention des ions en phase gazeuse est ensuite obtenue par fission successive des microgouttelettes (théorie de Dole et Röllgen) ou par désorption (théorie d'Iribarne et Thomson). Ce mode d'ionisation, qui peut être utilisé en mode positif ou négatif, est une ionisation douce qui fournit une faible énergie aux ions. Ainsi, les ions formés sont essentiellement de type pseudo-moléculaires, $[M+nH]^{n+}$ en mode positif ou $[M-nH]^{n-}$ en mode négatif (avec n=1 la plupart du temps). Les ions seront ensuite séparés selon leurs rapports masses sur nombre de charges noté m/z (où m est la masse du composé et z représente sa charge associée) dans un analyseur avant d'être détectés.

De nombreux types d'analyseurs existent, basés sur des champs magnétiques et/ou électriques : magnétiques, à temps de vol, quadripolaires, pièges d'ions, à résonance cyclotronique, etc... Chacun d'eux est caractérisé par son pouvoir de résolution entre deux ions de *m/z* voisins, sa gamme de masse, sa précision et sa capacité de transmission des ions entre la source où ils sont générés et le détecteur.

Le courant d'ions ayant traversé l'analyseur est converti en un courant électrique utilisable par un détecteur. Ce courant électrique est proportionnel à l'abondance des ions incidents. Les données obtenues sont exploitables *via* un traitement informatique. L'utilisation de spectromètre de masse de type triple quadripôle (QqQ) est largement utilisé pour la quantification de pharmaceutiques dans les eaux (Petrović et al., 2005) et notamment pour les cytostatiques. Le triple quadripôle résulte de l'association de trois quadripôles en série et permet d'effectuer des analyses de spectrométrie de masse en tandem (MS-MS). Un quadripôle constitue la cellule de collision et est placé entre deux analyseurs.

i. Approche quantitative

L'approche quantitative relève d'une méthode d'analyse dite « ciblée » puisque l'objectif est de détecter et de quantifier des composés connus et prédéfinis en amont de l'analyse. Notons tout de même qu'il existe des approches non ciblées, qui permettent l'exploitation des données *a posteriori*, ces méthodes sont dites semi-quantitatives et nécessitent l'emploi d'analyseurs haute résolution (Togola et al., 2015).

Quand des étalons analytiques sont disponibles, ils permettent le développement l'optimisation de la méthode adaptée aux composés choisis. Comme vu précédemment**,** la spectrométrie de masse en tandem est la technique de choix dans l'analyse des résidus de médicaments dans les eaux et l'utilisation du triple quadripôle est largement répandue. Les mode SRM (Single Reaction Monitoring) et MRM (multiple reaction monitoring) permettent le suivi d'une molécule identifiée, pour laquelle on connait donc la masse et les fragments associés à une énergie de collision donnée suite à l'optimisation des différents paramètres. Comme montré en Figure 2.1- 8, le spectromètre sélectionne un ion précurseur m/z=a avec le premier quadripôle. Ce dernier va ensuite être fragmenté en appliquant une énergie de collision optimale en cellule de collision (CID) constituée par le quadripôle 2, puis l'ion fragment obtenu le plus intense et le plus spécifique sera sélectionné par le dernier quadripôle (SRM). Le couple ion précurseur/ ion fragment est appelé transition. Lorsque l'on choisit au moins 2 ions fragments, il s'agit alors de MRM, cette méthode apporte une double spécificité.

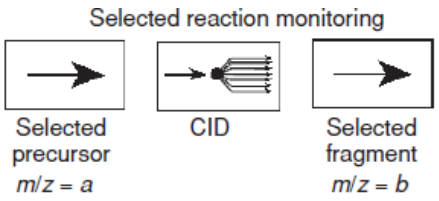


Figure 2.1- 8 MRM Mode extrait de Hoffmann et Stroobant (2007)

Finalement, les étapes de pré-concentration en SPE online ou offline et l'analyse par HPLC-MS/MS sur triple quadripôle permettent de quantifier les faibles concentrations d'anticancéreux présentes dans les matrices aqueuses environnementales. Le Tableau 2.1- 4 rapporte les estimations des limites de quantification (LOQ) de l'ordre du ng/L pour plusieurs auteurs, les variations de sensibilité proviennent notamment de perturbations de l'étape d'ionisation par des interférents, un phénomène appelé « effet matrice ».

Tableau 2.1- 4 Limite de détection et de quantification pour des anticancéreux dans différents types d'échantillons aqueux, déterminé par SPE offline ou online- SPE*-HPLC-MS/MS sur triple quadripôle

Composé (ng/L)	Riviere		Effluent d'eaux usées		Inffluent d'eaux usées		Référence
	LODs	**LOQs**	**LODs**	**LOQs**	**LODs**	**LOQs**	
Cyclophosphamide	1.7	5.5	2.3	7.7	2.1	7.1	Martin et al., 2011
	5	-	-	-	-	-	Santos et al., 2018
	-	-	-	1.9	-	-	Castiglioni et al., 2005
	-	-	-	-	-	2.4	Galaon et al., 2017
	-	-	9	26	5	4	*Garcia et al., 2009a
	-	-	-	-	4	13	*Rabii et al., 2014
	-	-	2	-	-	-	Yin et al., 2010
Cytarabine	1.4	4.8	1.5	4.9	1.5	4.9	Martin et al., 2011
Doxorubicine	5.3	18	4.3	14	4.2	14	Martin et al., 2011
Etoposide	2.2	7.2	3.0	9.8	3.4	11	Martin et al., 2011
5-Fluorouracile	46	-	46	-	46	-	Santos et al., 2018
Gemcitabine	1.0	3.3	2.6	8.7	1.4	4.8	Martin et al., 2011
Ifosfamide	1.3	4.4	1.1	3.8	1.7	5.8	Martin et al., 2011
	-	-	-	-	4	14	*Rabii et al., 2014
	-	-	2	-	-	-	Yin et al., 2010
	-	-	-	-	-	1.8	Galaon et al., 2017
Methotrexate	0.1	0.3	0.1	0.3	0.1	0.3	Martin et al., 2011
	-	-	16	47	11	32	*Garcia et al., 2009a
	-	-	-	0.83	-	-	Castiglioni et al., 2005
	-	-	-	-	-	1.3	Galaon et al., 2017
	-	-	-	-	12	36	*Rabii et al., 2014
	-	-	5	-	-	-	Yin et al., 2010
Tamoxifène	-	-	-	-	-	1.1	Galaon et al., 2017

ii. Identification

La spectrométrie de masse permet aussi l'identification de produits de transformation. Afin de mener l'élucidation structurale des composés inconnus générés au cours de dégradations, plusieurs modes d'acquisition peuvent être utilisés :

Le mode full scan en LC-MS: Le premier quadripôle de l'instrument balaye une large gamme de masses et le gaz de collision n'est pas appliqué dans la cellule de collision, ainsi elle joue juste le rôle de filtre de masse. Quant au troisième quadripôle, il reste transparent afin que tous les ions parviennent au détecteur. Cette première étape permet de détecter les ions pseudo-moléculaires $[M + H]^+$ et $[M - H]^-$ des divers composés, donnant donc une indication sur leur composition élémentaire. Cependant, dans une matrice complexe, il n'est pas toujours possible d'observer le courant d'ion total (TIC) (Kosjek et al., 2015).

Ensuite l'association MS/MS permet plusieurs types d'acquisitions présentés ci-dessous en Figure 2.1-9, afin d'obtenir des informations sur la structure des molécules, ou bien de chercher une molécule en fonction des informations dont on dispose sur sa structure.

- Le mode descendant aussi appelé product, fragment ou encore daughter ion Scan : Le premier quadripôle (MS1) sert à sélectionner l'ion précurseur spécifique, encore appelé ion précurseur en se focalisant sur son rapport *m/z*. Il est alors fragmenté dans la cellule de collision CID (collision induced dissociation) puis les ions fragments, sont ensuite séparés au niveau de MS2. Le spectre obtenu peut présenter à la fois l'ion précurseur et ses ions fragments et permet d'acquérir des informations structurelles de l'ion m/z sélectionné en amont par le biais d'ions fragments spécifiques à la molécule.

- Le mode ascendant aussi appelé parent, ou precursor ion scan: MS1 balaie une gamme de masse tandis que MS2 est focalisé sur un seul rapport *m/z*. Tous les ions générés en source et capables de donner un fragment de même rapport *m/z* seront détectés. Ce mode est utilisé pour repérer les composés capables de présenter un fragment commun dans un mélange complexe.

- Le mode perte de neutre ou neutral loss scan: MS1 et MS2 balaient une gamme de masse simultanément et avec un décalage de masse constant. Le spectre établi présentera alors tous les ions précurseurs capables de se fragmenter en générant un neutre de masse égal au décalage imposé. Comme le mode ascendant, ce mode est utilisé lors du screening de mélanges complexes

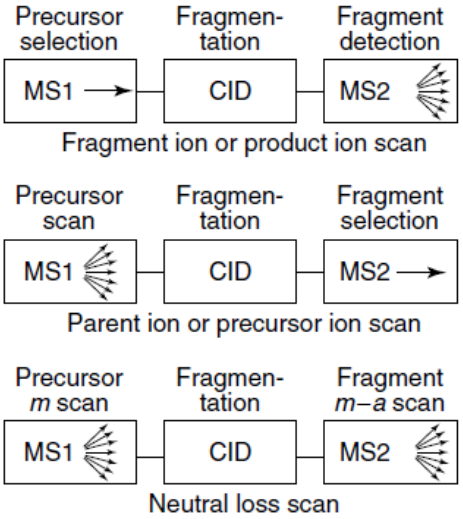


Figure 2.1-9 Modes d'acquisition en MS/MS extrait de Hoffmann et Stroobant (2007)

Petrovic et al. (2007) rapportent l'utilisation du triple quadripôle dans l'identification de photoproduits de β-bloquants, d'agents régulateurs des lipides, de médicaments psychotropes ou encore de produits de contraste et d'antibiotiques. Cependant la basse résolution ne permet pas de confirmer une structure puisque le nombre de formules brutes possibles est fonction de la masse mesurée et de l'exactitude relative (Bart Emary et Zhang, 2013). La haute résolution reste la référence dans l'identification de TPs. Schymanski et al. (2014) proposent différents niveaux de confiance pour l'identification des composés inconnus, basé sur des données analytiques telles que la formule moléculaire exacte ou encore la masse de fragments tel que présenté ci-dessous, en Figure 2.1-10.

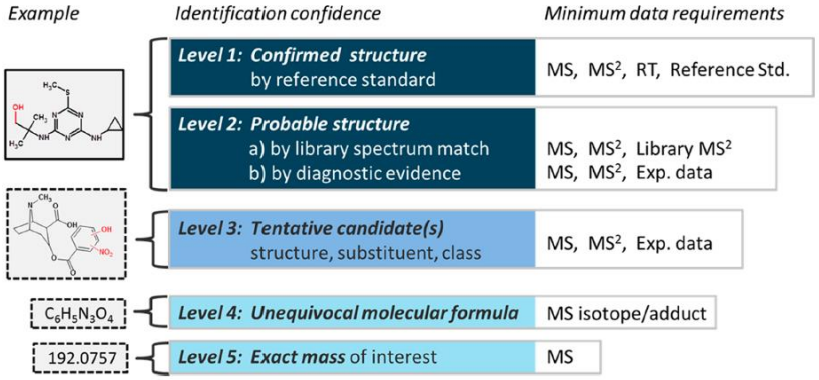


Figure 2.1-10 Niveau de confiance dans l'identification de TPs selon Schymanski et al. (2014)

Il reste à souligner la non-exhaustivité des résultats d'identification de composés, les TPs identifiés ne représentent pas l'intégralité de l'échantillon initial. En effet, l'identification reste dépendante des différentes étapes de préparation et d'analyses vu précédemment : les composés identifiables sont des

composés qui ont pu être extraits lors de la préparation d'échantillons, qui étaient analysables et détectable selon les conditions d'analyses.

Il est important de souligner que parmi dans les 28 TPs du MTX signalés dans la littérature Tableau 2.1- 3, seulement 6 TPs ont été confirmés par l'utilisation d'un standard, portant l'identification du 7-OH-MTX et des TPs 266,147,192,136 à un niveau 1 (Lai et al., 2017). Les TPs 280,266 et 208 (Calza et al., 2014 ; Lutterbeck et al., 2015a) sont renseignés par des informations sur leur fragmentation portant leur identification à un niveau 2b. Pour les autre TPs, il s'agit d'un niveau inférieur ou non précisé.

Cette analyse fait ressortir que les méthodes analytiques mises en œuvre pour quantifier le MTX et d'autres anticancéreux et pour identifier les produits de transformation demandent d'être continuellement adaptées à la nature des matrices environnementales et aux questions posées par chaque étude expérimentale. On constate également que le niveau de confiance obtenu sur l'identification de TPs et la proposition de voies réactionnelles dans des schémas de transformation sont très dépendants du niveau de performance analytique des chaines analytiques et de la disponibilité de standards pour l'identification moléculaire. Le matériel dont on dispose pour chaque étude est donc un facteur limitant que l'on peut en partie lever *via* des collaborations. C'est ce que nous avons recherché en travaillant en collaboration avec l'unité UMR SayFood, (AgroParisTech, Université Paris-Saclay, INRAE) afin de confirmer les hypothèses de structures issues d'analyses sur triple quadripole, par des analyses en haute résolution.

2.2. Objectifs de la thèse et démarche générale

Ce travail de thèse questionne par différentes approches expérimentales la validité d'une démarche d'éco-conception en comparant la molécule MTX à un analogue éco-conçu, le OMTX. Si l'on reformule, la question centrale auquel il nous faut répondre est la suivante :

La modification de la structure du méthotrexate est-elle en faveur d'une diminution des risques environnementaux ?

Pour répondre à cette question, nous avons deux objectifs principaux : caractériser la dégradation des deux molécules **(I)** et caractériser les effets de ces molécules et de leurs TPs **(II).** Pour répondre au premier objectif, nous proposons de :

a) étudier les cinétiques de dégradation des deux molécules durant des processus abiotiques (photodégradation) et biotique (biodégradation par des boues de station d'épuration)

b) identifier et suivre les produits formés

Le second objectif vise à :

c) suivre l'évolution de la cytotoxicité résultante de la photo et de la biodégradation

d) évaluer l'impact des mélanges produits parents/TPs sur la boue de station d'épuration exposée au cours de la biodégradation

e) caractériser la toxicité de ces composés sur des organismes aquatiques modèles

Cependant, pour répondre à ces objectifs nous avons été confrontés à certaines limitations : l'accès à un analyseur haute résolution limité, la nécessité de développer une méthode analytique permettant le suivi dans une matrice complexe lors de la biodégradation par les boues de station d'épuration en l'absence de standards pour la majorité des TPs, ainsi qu'une quantité restreinte de OMTX synthétisé dans le cadre du projet EDIFIS.

La démarche adoptée présentée sur le schéma Figure 2.2- 1 est construite comme suit :

-L'étude de la photodégradation en eau pure permettant l'identification des TPs ainsi que le suivi de l'évolution de la cytotoxicité (encadré bleu sur le schéma) – **Chapitre 3** :

En effet, une matrice simple permet le suivi des TPs sans préparation d'échantillons qui peut s'avérer sélective (cf partie 2.1.2). Pour l'identification des TPs des techniques de pré-concentration sont cependant nécessaires et ont été utilisées. Différentes conditions d'irradiation en photolyse directe ont été étudiées afin de générer une large variété de TPs, car le MTX y est sensible (cf 2.1.1.3). Une première étape d'identification a été faite en basse résolution, mais a dû être ensuite confirmée en haute résolution. De plus, le suivi de l'évolution de la cytotoxicité, spécifique du mode d'action des composés permet de s'assurer de la perte du mode d'action initial.

-Le développement d'une méthode analytique adaptée au suivi des TPs dans les boues de station d'épuration, (encadré rouge) – **Chapitre 4.1** :

Comme nous l'avons vu partie 2.1.1.4, certains TPs peuvent être spécifiques à la biodégradation. En dehors des photoproduits déterminés lors de l'étape précédente, la méthode a été adaptée et

enrichie en TPs suspectés en s'appuyant sur la littérature**.** Le MTX est plus susceptible d'être biodégradé car plutôt présent en phase aqueuse et peu sorbé en phase solide. En conséquence, la méthode a été donc développée sur le surnageant de ces boues. Le développement analytique spécifique à cette matrice a été nécessaire pour permettre le suivi des composés identifiés**.**

-L'étude de la biodégradation par des boues de STEP (encadré marron) - **Chapitre 4.2** :

Les composés formés ont été suivis au cours d'une incubation en conditions contrôlées selon la méthode développée et l'évolution de la cytotoxicité sera aussi évaluée. Nous avons cherché à évaluer l'impact en retour sur les communautés microbiennes de la boue activée exposées aux composés par le biais de différents indicateurs microbiologiques et enzymatiques (cf. partie 2.1.1.2).

-L'évaluation de l'impact écotoxicologique des molécules étudiée (encadré vert) - **Chapitre 5** :

Cette évaluation a été réalisée par des tests simples, rapides et consommant peu de produit. Il s'agit de tests d'écotoxicité aquatique normalisés permettant la comparaison avec la littérature présentée dans la partie 1.1.3.1i, tels que des tests sur algues, ou des tests plus rapides tels que sur *A. fischeri* permettant le suivi de l'évolution de la toxicité au cours de la génération de TPs.

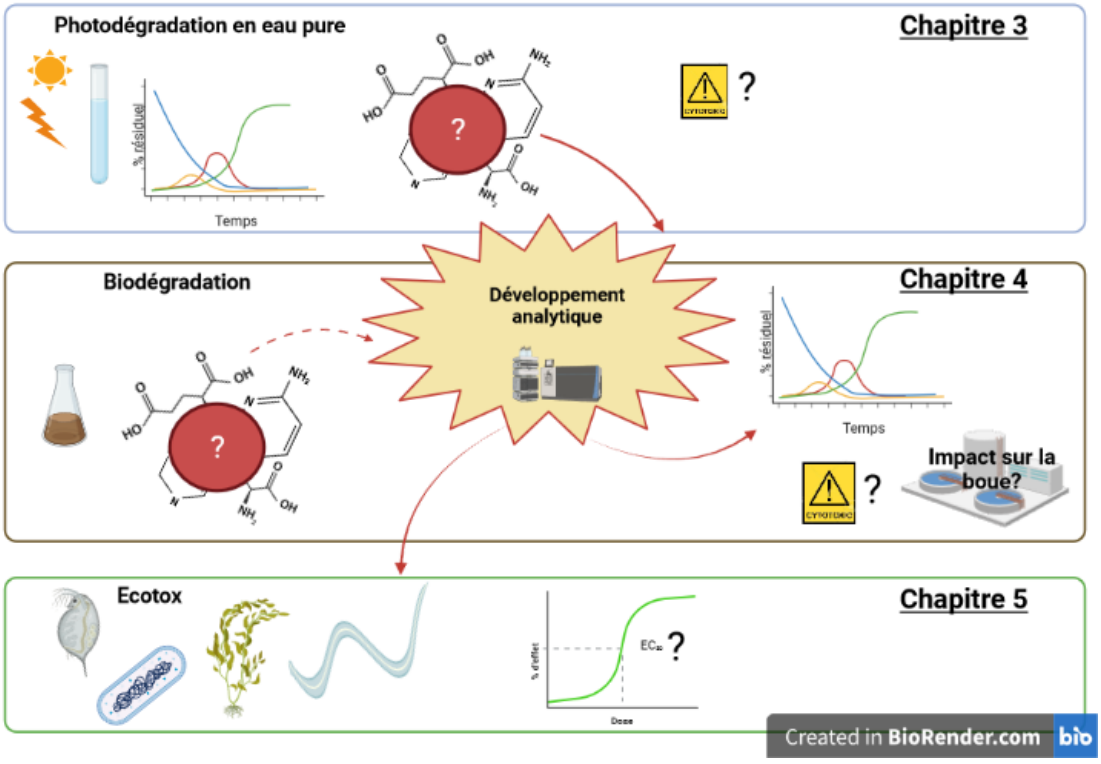


Figure 2.2- 1 Schéma de la démarche générale du travail de thèse

2.3. Références

Académie nationale de Pharmacie, 2019. Médicaments et environnement. https://www.acadpharm.org/dos_public/Rapport_Medicaments_Environnement_2019.04.24_VF.pdf

Adeleye, A.S., Xue, J., Zhao, Y., Taylor, A.A., Zenobio, J.E., Sun, Y., Han, Z., Salawu, O.A., Zhu, Y., 2022. Abundance, fate, and effects of pharmaceuticals and personal care products in aquatic environments. Journal of Hazardous Materials 424, 127284. https://doi.org/10.1016/j.jhazmat.2021.127284

Aguirre-Martínez, G.V., Okello, C., Salamanca, M.J., Garrido, C., Del Valls, T.A., Martín-Díaz, M.L., 2016. Is the step-wise tiered approach for ERA of pharmaceuticals useful for the assessment of cancer therapeutic drugs present in marine environment? Environmental Research 144, 43–59. https://doi.org/10.1016/j.envres.2015.10.028

Aherne, G.W., 1985. Immunoassays in the Analysis of Water. International Journal of Environmental Analytical Chemistry 21, 79–88. https://doi.org/10.1080/03067318508078372

Ahmed, M.B., Zhou, J.L., Ngo, H.H., Guo, W., Thomaidis, N.S., Xu, J., 2017. Progress in the biological and chemical treatment technologies for emerging contaminant removal from wastewater: A critical review. Journal of Hazardous Materials 323, 274–298. https://doi.org/10.1016/j.jhazmat.2016.04.045

Altenburger, R., Brack, W., Burgess, R.M., Busch, W., Escher, B.I., Focks, A., Mark Hewitt, L., Jacobsen, B.N., de Alda, M.L., Ait-Aissa, S., Backhaus, T., Ginebreda, A., Hilscherová, K., Hollender, J., Hollert, H., Neale, P.A., Schulze, T., Schymanski, E.L., Teodorovic, I., Tindall, A.J., de Aragão Umbuzeiro, G., Vrana, B., Zonja, B., Krauss, M., 2019. Future water quality monitoring: improving the balance between exposure and toxicity assessments of real-world pollutant mixtures. Environmental Sciences Europe 31, 12. https://doi.org/10.1186/s12302-019-0193-1

Alvarino, T., Katsou, E., Malamis, S., Suarez, S., Omil, F., Fatone, F., 2014. Inhibition of biomass activity in the via nitrite nitrogen removal processes by veterinary pharmaceuticals. Bioresource Technology 152, 477–483. https://doi.org/10.1016/j.biortech.2013.10.107

Amariei, G., Boltes, K., Rosal, R., León, P., 2017. Toxicological interactions of ibuprofen and triclosan on biological activity of activated sludge. Journal of Hazardous Materials 334, 193–200. https://doi.org/10.1016/j.jhazmat.2017.04.018

Andreozzi, R., 1999. Advanced oxidation processes (AOP) for water purification and recovery. Catalysis Today 53, 51–59. https://doi.org/10.1016/S0920-5861(99)00102-9

ANSES, 2010. Évaluation de l'innocuité des réacteurs équipés de lampes à rayonnements ultraviolets et de l'efficacité de ces procédés pour la désinfection des eaux destinées à la consommation humaine. https://www.anses.fr/fr/content/evaluation-de-l%E2%80%99innocuit%C3%A9-des-r%C3%A9acteurs-%C3%A9quip%C3%A9s-de-lampes-%C3%A0-rayonnements-ultraviolets-et-d-0 (accessed 2.11.22).

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, 167–184. https://doi.org/10.1016/j.scitotenv.2004.04.062

aus der Beek, T., Weber, F.-A., Bergmann, A., Hickmann, S., Ebert, I., Hein, A., Küster, A., 2016. Pharmaceuticals in the environment-Global occurrences and perspectives: Pharmaceuticals in the global environment. Environmental Toxicology and Chemistry 35, 823–835. https://doi.org/10.1002/etc.3339

Azuma, T., Arima, N., Tsukada, A., Hirami, S., Matsuoka, R., Moriwake, R., Ishiuchi, H., Inoyama, T., Teranishi, Y., Yamaoka, M., Mino, Y., Hayashi, T., Fujita, Y., Masada, M., 2016. Detection of pharmaceuticals and phytochemicals together with their metabolites in hospital effluents in Japan, and their contribution to sewage treatment plant influents. Science of The Total Environment 548–549, 189–197. https://doi.org/10.1016/j.scitotenv.2015.12.157

Azuma, T., Ishiuchi, H., Inoyama, T., Teranishi, Y., Yamaoka, M., Sato, T., Mino, Y., 2015. Occurrence and fate of selected anticancer, antimicrobial, and psychotropic pharmaceuticals in an urban river in a subcatchment of the Yodo River basin, Japan. Environmental Science and Pollution Research 22, 18676–18686. https://doi.org/10.1007/s11356-015-5013-6

Barışçı, S., Turkay, O., Ulusoy, E., Şeker, M.G., Yüksel, E., Dimoglo, A., 2018. Electro-oxidation of cytostatic drugs: Experimental and theoretical identification of by-products and evaluation of ecotoxicological effects. Chemical Engineering Journal 334, 1820–1827. https://doi.org/10.1016/j.cej.2017.11.105

Bart Emary, W., Zhang, N.R., 2013. Utility of High-Resolution Mass Spectrometry for New Drug Discovery Applications, in: Korfmacher, W.A. (Ed.), Mass Spectrometry for Drug Discovery and Drug Development. John Wiley & Sons, Inc., Hoboken, NJ, USA, pp. 37–54. https://doi.org/10.1002/9781118516157.ch2

Bassompiere, C., 2007. Procédé à boues activées pour le traitement d'effluents papetiers : de la conception d'un pilote à la validation de modèles. Institut National Polytechnique de Grenoble. Thèse. 232 pp.

Besse, J.-P., Latour, J.-F., Garric, J., 2012. Anticancer drugs in surface waters. Environment International 39, 73–86. https://doi.org/10.1016/j.envint.2011.10.002

Białk-Bielińska, A., Mulkiewicz, E., Stokowski, M., Stolte, S., Stepnowski, P., 2017. Acute aquatic toxicity assessment of six anti-cancer drugs and one metabolite using biotest battery – Biological effects and stability under test conditions. Chemosphere 189, 689–698. https://doi.org/10.1016/j.chemosphere.2017.08.174

Bonnot K., Benoit P., Mamy L., Patureau P., 2022. Transformation products of PPCPs in the environment: review of existing knowledge and classification of formation pathways according to parent molecule chemical structures. Critical Reviews in Environmental Science and Technology, in press.

Booker, V., Halsall, C., Llewellyn, N., Johnson, A., Williams, R., 2014. Prioritising anticancer drugs for environmental monitoring and risk assessment purposes. Science of The Total Environment 473–474, 159–170. https://doi.org/10.1016/j.scitotenv.2013.11.145

Boreen, A.L., Arnold, W.A., McNeill, K., 2003. Photodegradation of pharmaceuticals in the aquatic environment: A review. Aquatic Sciences - Research Across Boundaries 65, 320–341. https://doi.org/10.1007/s00027-003-0672-7

Bound, J.P., Voulvoulis, N., 2005. Household Disposal of Pharmaceuticals as a Pathway for Aquatic Contamination in the United Kingdom. Environmental Health Perspectives 113, 1705–1711. https://doi.org/10.1289/ehp.8315

Brezovšek, P., Eleršek, T., Filipič, M., 2014. Toxicities of four anti-neoplastic drugs and their binary mixtures tested on the green alga Pseudokirchneriella subcapitata and the cyanobacterium Synechococcus leopoliensis. Water Research 52, 168–177. https://doi.org/10.1016/j.watres.2014.01.007

Buerge, I.J., Buser, H.-R., Poiger, T., Müller, M.D., 2006. Occurrence and Fate of the Cytostatic Drugs Cyclophosphamide and Ifosfamide in Wastewater and Surface Waters. Environmental Science & Technology 40, 7242–7250. https://doi.org/10.1021/es0609405

Busetti, F., Linge, K.L., Heitz, A., 2009. Analysis of pharmaceuticals in indirect potable reuse systems using solid-phase extraction and liquid chromatography–tandem mass spectrometry. Journal of Chromatography A 1216, 5807–5818. https://doi.org/10.1016/j.chroma.2009.06.001

Calza, P., Medana, C., Sarro, M., Rosato, V., Aigotti, R., Baiocchi, C., Minero, C., 2014. Photocatalytic degradation of selected anticancer drugs and identification of their transformation products in water by liquid chromatography–high resolution mass spectrometry. Journal of Chromatography A 1362, 135–144. https://doi.org/10.1016/j.chroma.2014.08.035

Castiglioni, S., Bagnati, R., Calamari, D., Fanelli, R., Zuccato, E., 2005. A multiresidue analytical method using solid-phase extraction and high-pressure liquid chromatography tandem mass spectrometry to measure pharmaceuticals of different therapeutic classes in urban wastewaters. Journal of Chromatography A 1092, 206–215. https://doi.org/10.1016/j.chroma.2005.07.012

Catastini, C., Mullot, J.-U., Boukari, S., Mazellier, P., Levi, Y., Cervantes, P., Ormsby, J.-N., 2008. Identification de molécules anticancéreuses dans les effluents hospitaliers. European journal of water quality 39, 171–180. https://doi.org/10.1051/water/2008004

Çeçen, F., Gül, G., 2021. Biodegradation of five pharmaceuticals: estimation by predictive models and comparison with activated sludge data. International Journal of Environmental Science and Technology 18, 327–340. https://doi.org/10.1007/s13762-020-02820-y

Česen, M., Eleršek, T., Novak, M., Žegura, B., Kosjek, T., Filipič, M., Heath, E., 2016. Ecotoxicity and genotoxicity of cyclophosphamide, ifosfamide, their metabolites/transformation products and their mixtures. Environmental Pollution 210, 192–201. https://doi.org/10.1016/j.envpol.2015.12.017

Česen, M., Kosjek, T., Laimou-Geraniou, M., Kompare, B., Širok, B., Lambropolou, D., Heath, E., 2015. Occurrence of cyclophosphamide and ifosfamide in aqueous environment and their removal by biological and abiotic wastewater treatment processes. Science of The Total Environment 527–528, 465–473. https://doi.org/10.1016/j.scitotenv.2015.04.109

Chirwa, E.M.N, Bamuza-Pemu, E.E, 2010. Investigation of photocatalysis as an alternative to other advanced oxidation processes for the treatment of filter backwash water. https://doi.org/10.13140/RG.2.1.1368.3042

Coetsier, C., 2009a. Approche intégrée de la gestion environnementale Des produits pharmaceutiques dans des rejets de Stations d'épuration urbaines et leur milieu récepteur : occurrence, impact et traitements tertiaires d'élimination (Génie des procédés). Université Montpellier II - Sciences et Techniques du Languedoc. Thèse. 294 pp.

Coetsier, C.M., Spinelli, S., Lin, L., Roig, B., Touraud, E., 2009b. Discharge of pharmaceutical products (PPs) through a conventional biological sewage treatment plant: MECs vs PECs? Environment International 35, 787–792. https://doi.org/10.1016/j.envint.2009.01.008

Commission Européenne, 2019. Directorate-General for Environment, Kümmerer, K., *Options for a strategic approach to pharmaceuticals in the environment : final report*, Publications Office, https://data.europa.eu/doi/10.2779/87838 (accessed 2.11.22).

Commission Européenne, 2018. Directive n° 2018/C 124/01 notice on technical guidance on the classification of waste. https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=uriserv:OJ.C_.2018.124.01.0001.01.ENG (accessed 2.11.22).

Commission Européenne, 2015. Projet Cytothreat : Fate and effects of cytostatic pharmaceuticals in the environment and the identification of biomarkers for and improved risk assessment on environmental exposure. https://cordis.europa.eu/project/id/265264/fr (accessed 2.11.22).

Commission Européenne, 2006. Directive n° 2006/118/CE sur la protection des eaux souterraines contre la pollution et la détérioration. https://aida.ineris.fr/consultation_document/923 (accessed 2.11.22).

Conseil général de l'environnement et du développement durable, 2010. Médicament et environnement : La régulation du médicament vis-à-vis du risque environnemental. Rapport N° 007058-01 - M.,Bouvier, F. Durand, R. Guillet. 118 pp. https://cgedd.documentation.developpement-durable.gouv.fr/documents/Affaires-0006157/007058-01_rapport.pdf

Dąbrowska, D., Kot-Wasik, A., Namieśnik, J., 2004. The Importance of Degradation in the Fate of Selected Organic Compounds in the Environment. Part II. Photodegradation and Biodegradation. Polish Journal of Environmental Studies 13, 617–626.

Daughton, C.G., Ruhoy, I.S., 2013. Lower-dose prescribing: Minimizing "side effects" of pharmaceuticals on society and the environment. Science of The Total Environment 443, 324–337. https://doi.org/10.1016/j.scitotenv.2012.10.092

Daughton, C.G., Ternes, T.A., 1999. Pharmaceuticals and personal care products in the environment: agents of subtle change? Environmental Health Perspectives 107 Suppl 6, 907–938. https://doi.org/10.1289/ehp.99107s6907

DellaGreca, M., Iesce, M.R., Isidori, M., Nardelli, A., Previtera, L., Rubino, M., 2007. Phototransformation products of tamoxifen by sunlight in water. Toxicity of the drug and its derivatives on aquatic organisms. Chemosphere 67, 1933–1939. https://doi.org/10.1016/j.chemosphere.2006.12.001

Destrieux, D., 2018. Résidus de médicaments d'un cours d'eau urbain : constitution d'une base de données pour la gestion des risques écotoxicologiques (Ecologie, Environnement). Université Paul Sabatier, Toulouse III. Thèse 431 pp.

Domingues, E., Gomes, J., Quina, M., Quinta-Ferreira, R., Martins, R., 2018. Detoxification of Olive Mill Wastewaters by Fenton's Process. Catalysts 8, 662. https://doi.org/10.3390/catal8120662

Escher, B.I., Fenner, K., 2011. Recent Advances in Environmental Risk Assessment of Transformation Products. Environmental Science & Technology 45, 3835–3847. https://doi.org/10.1021/es1030799

European Medicines Agency, 2006. Guideline on the Environmental risk assessment of medicinal products for human use , EMEA/CHMP/SWP/4447/00 corr 21*. https://www.ema.europa.eu/en/environmental-risk-assessment-medicinal-products-human-use (accessed 2.11.22).

Fabre, G., Goldman, I.D., 1985. Formation of 7-hydroxymethotrexate polyglutamyl derivatives and their cytotoxicity in human chronic myelogenous leukemia cells, in vitro. Cancer Research 45, 80–85.

Fatta-Kassinos, D., Vasquez, M.I., Kümmerer, K., 2011. Transformation products of pharmaceuticals in surface waters and wastewater formed during photolysis and advanced oxidation processes – Degradation, elucidation of byproducts and assessment of their biological potency. Chemosphere 85, 693–709. https://doi.org/10.1016/j.chemosphere.2011.06.082

Fent, K., Weston, A., Caminada, D., 2006. Ecotoxicology of human pharmaceuticals. Aquatic Toxicology 76, 122–159. https://doi.org/10.1016/j.aquatox.2005.09.009

Ferrando-Climent, L., Rodriguez-Mozaz, S., Barceló, D., 2014. Incidence of anticancer drugs in an aquatic urban system: From hospital effluents through urban wastewater to natural environment. Environmental Pollution 193, 216–223. https://doi.org/10.1016/j.envpol.2014.07.002

Ferrando-Climent, L., Rodriguez-Mozaz, S., Barceló, D., 2013. Development of a UPLC-MS/MS method for the determination of ten anticancer drugs in hospital and urban wastewaters, and its application for the screening of human metabolites assisted by information-dependent acquisition tool (IDA) in sewage samples. Analytical and Bioanalytical Chemistry 405, 5937–5952. https://doi.org/10.1007/s00216-013-6794-4

Franquet-Griell, H., Cornadó, D., Caixach, J., Ventura, F., Lacorte, S., 2017a. Determination of cytostatic drugs in Besòs River (NE Spain) and comparison with predicted environmental concentrations. Environmental Science and Pollution Research 24, 6492–6503. https://doi.org/10.1007/s11356-016-8337-y

Franquet-Griell, H., Medina, A., Sans, C., Lacorte, S., 2017b. Biological and photochemical degradation of cytostatic drugs under laboratory conditions. Journal of Hazardous Materials 323, 319–328. https://doi.org/10.1016/j.jhazmat.2016.06.057

Galaon, T., Petre, J., Iancu, V.I., Cruceru, L., Niculescu, M., Chiriac, F.L., Puiu, D.M., Mihalache, M., 2017. Detection of cytostatic drugs in municipal waste water and their transfer to surface water, in: SIMI 2017. Presented at the International Symposium "The Environment and the Industry," National Research and Development Institute for Industrial Ecology, pp. 229–236. https://doi.org/10.21698/simi.2017.0029

Garcia-Ac, A., Broséus, R., Vincent, S., Barbeau, B., Prévost, M., Sauvé, S., 2010. Oxidation kinetics of cyclophosphamide and methotrexate by ozone in drinking water. Chemosphere 79, 1056–1063. https://doi.org/10.1016/j.chemosphere.2010.03.032

Garcia-Ac, A., Segura, P.A., Gagnon, C., Sauvé, S., 2009a. Determination of bezafibrate, methotrexate, cyclophosphamide, orlistat and enalapril in waste and surface waters using on-line solid-phase extraction liquid chromatography coupled to polarity-switching electrospray tandem mass spectrometry. Journal of Environmental Monitoring 11, 830. https://doi.org/10.1039/b817570e

Garcia-Ac, A., Segura, P.A., Viglino, L., Fürtös, A., Gagnon, C., Prévost, M., Sauvé, S., 2009b. On-line solid-phase extraction of large-volume injections coupled to liquid chromatography-tandem mass spectrometry for the quantitation and confirmation of 14 selected trace organic contaminants in drinking and surface water. Journal of Chromatography A 1216, 8518–8527. https://doi.org/10.1016/j.chroma.2009.10.015

Glaze, W.H., Kang, J.-W., Chapin, D.H., 1987. The Chemistry of Water Treatment Processes Involving Ozone, Hydrogen Peroxide and Ultraviolet Radiation. Ozone: Science & Engineering 9, 335–352. https://doi.org/10.1080/01919518708552148

Gómez-Canela, C., Ventura, F., Caixach, J., Lacorte, S., 2014. Occurrence of cytostatic compounds in hospital effluents and wastewaters, determined by liquid chromatography coupled to high-resolution mass spectrometry. Analytical and Bioanalytical Chemistry 406, 3801–3814. https://doi.org/10.1007/s00216-014-7805-9

Gómez-Canela, C., Cortés-Francisco, N., Oliva, X., Pujol, C., Ventura, F., Lacorte, S., Caixach, J., 2012. Occurrence of cyclophosphamide and epirubicin in wastewaters by direct injection analysis–liquid chromatography–high-resolution mass spectrometry. Environmental Science and Pollution Research 19, 3210–3218. https://doi.org/10.1007/s11356-012-0826-z

Gonçalves, N.P.F., Iezzi, L., Belay, M.H., Dulio, V., Alygizakis, N., Dal Bello, F., Medana, C., Calza, P., 2021. Elucidation of the photoinduced transformations of Aliskiren in river water using liquid chromatography high-resolution mass spectrometry. Science of The Total Environment 800, 149547. https://doi.org/10.1016/j.scitotenv.2021.149547

Gros, M., Petrović, M., Barceló, D., 2007. Wastewater treatment plants as a pathway for aquatic contamination by pharmaceuticals in the ebro river basin (Northeast Spain). Environmental Toxicology and Chemistry 26, 1553. https://doi.org/10.1897/06-495R.1

Grung, M., Källqvist, T., Sakshaug, S., Skurtveit, S., Thomas, K.V., 2008. Environmental assessment of Norwegian priority pharmaceuticals based on the EMEA guideline. Ecotoxicology and Environmental Safety 71, 328–340. https://doi.org/10.1016/j.ecoenv.2007.10.015

Gu, G., Yin, H., Zhu, Q., Shen, L., Zhang, K., Liu, M., Wu, Q., 2019. Recognition of the prioritized types and individual of pharmaceuticals and personal care products (PPCPs) in the drinking water of Shanghai and a health risk assessment. Human and Ecological Risk Assessment: An International Journal 25, 1207–1221. https://doi.org/10.1080/10807039.2018.1461009

Haddad, T., Baginska, E., Kümmerer, K., 2015. Transformation products of antibiotic and cytostatic drugs in the aquatic cycle that result from effluent treatment and abiotic/biotic reactions in the environment: An increasing challenge calling for higher emphasis on measures at the beginning of the pipe. Water Research 72, 75–126. https://doi.org/10.1016/j.watres.2014.12.042

Hamada, Y., Kiso, Y., 2012. The application of bioisosteres in drug design for novel drug discovery: focusing on acid protease inhibitors. Expert Opinion on Drug Discovery 7, 903–922. https://doi.org/10.1517/17460441.2012.712513

Heberer, Th., Stan, H.-J., 1997. Determination of Clofibric Acid and N-(Phenylsulfonyl)-Sarcosine in Sewage, River and Drinking Water. International Journal of Environmental Analytical Chemistry 67, 113–124. https://doi.org/10.1080/03067319708031398

Henschel, K.-P., Wenzel, A., Diedrich, M., Fliedner, A., 1997. Environmental Hazard Assessment of Pharmaceuticals. Regulatory Toxicology and Pharmacology 25, 220–225. https://doi.org/10.1006/rtph.1997.1102

Hignite, C., Azarnoff, D.L., 1977. Drugs and drug metabolites as environmental contaminants: Chlorophenoxyisobutyrate and salicylic acid in sewage water effluent. Life Sciences 20, 337–341. https://doi.org/10.1016/0024-3205(77)90329-0

Hoffmann, E. de, Stroobant, V., 2007. Mass spectrometry: principles and applications, 3rd ed. ed. J. Wiley, Chichester, West Sussex, England ; Hoboken, NJ. 489pp

INERIS, 2019. Exposome et évaluation de risques : décryptage .. Fiche exposome 2 pp. DCO-19-174131-06880A. https://www.ineris.fr/sites/ineris.fr/files/2019-09/ONG_fiche_exposome_2019_vDEF.pdf

Isidori, M., Lavorgna, M., Russo, C., Kundi, M., Žegura, B., Novak, M., Filipič, M., Mišík, M., Knasmueller, S., de Alda, M.L., Barceló, D., Žonja, B., Česen, M., Ščančar, J., Kosjek, T., Heath, E., 2016. Chemical and toxicological characterisation of anticancer drugs in hospital and municipal wastewaters from Slovenia and Spain. Environmental Pollution 219, 275–287. https://doi.org/10.1016/j.envpol.2016.10.039

Jiao, S., Zheng, S., Yin, D., Wang, L., Chen, L., 2008. Aqueous photolysis of tetracycline and toxicity of photolytic products to luminescent bacteria. Chemosphere 73, 377–382. https://doi.org/10.1016/j.chemosphere.2008.05.042

Johnson, A.C., Jürgens, M.D., Williams, R.J., Kümmerer, K., Kortenkamp, A., Sumpter, J.P., 2008. Do cytotoxic chemotherapy drugs discharged into rivers pose a risk to the environment and human health? An overview and UK case study. Journal of Hydrology 348, 167–175. https://doi.org/10.1016/j.jhydrol.2007.09.054

Kanjal, M.I., Muneer, M., Abdelhaleem, A., Chu, W., 2020. Degradation of methotrexate by UV/peroxymonosulfate: Kinetics, effect of operational parameters and mechanism. Chinese Journal of Chemical Engineering 28, 2658–2667. https://doi.org/10.1016/j.cjche.2020.05.033

Kidd, K.A., Blanchfield, P.J., Mills, K.H., Palace, V.P., Evans, R.E., Lazorchak, J.M., Flick, R.W., 2007. Collapse of a fish population after exposure to a synthetic estrogen. Proceedings of the National Academy of Sciences 104, 8897–8901. https://doi.org/10.1073/pnas.0609568104

Kiffmeyer, T., Götze, H.-J., Jursch, M., Lüders, U., 1998. Trace enrichment, chromatographic separation and biodegradation of cytostatic compounds in surface water. Fresenius' Journal of Analytical Chemistry 361, 185–191. https://doi.org/10.1007/s002160050859

Kleinert, C., Poirier-Larabie, S., Gagnon, C., André, C., Gagné, F., 2021. Occurrence and ecotoxicity of cytostatic drugs 5-fluorouracil and methotrexate in the freshwater unionid Elliptio complanata. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 244, 109027. https://doi.org/10.1016/j.cbpc.2021.109027

Kosjek, T., Negreira, N., de Alda, M.L., Barceló, D., 2015. Aerobic activated sludge transformation of methotrexate: Identification of biotransformation products. Chemosphere 119, S42–S50. https://doi.org/10.1016/j.chemosphere.2014.04.081

Kosjek, T., Perko, S., Žigon, D., Heath, E., 2013. Fluorouracil in the environment: Analysis, occurrence, degradation and transformation. Journal of Chromatography A 1290, 62–72. https://doi.org/10.1016/j.chroma.2013.03.046

Kosjek, T., Heath, E., 2011. Occurrence, fate and determination of cytostatic pharmaceuticals in the environment. TrAC Trends in Analytical Chemistry 30, 1065–1087. https://doi.org/10.1016/j.trac.2011.04.007

Kovalova, L., McArdell, C.S., Hollender, J., 2009. Challenge of high polarity and low concentrations in analysis of cytostatics and metabolites in wastewater by hydrophilic interaction chromatography/tandem mass spectrometry. Journal of Chromatography A 1216, 1100–1108. https://doi.org/10.1016/j.chroma.2008.12.028

Kümmerer, K., 2007. Sustainable from the very beginning: rational design of molecules by life cycle engineering as an important approach for green pharmacy and green chemistry. Green Chemistry 9, 899. https://doi.org/10.1039/b618298b

Kümmerer, K., 2001. Pharmaceuticals in the Environment. Springer Berlin Heidelberg, Berlin, Heidelberg. https://doi.org/10.1007/978-3-662-04634-0

Kümmerer, K., Haiß, A., Schuster, A., Hein, A., Ebert, I., 2016. Antineoplastic compounds in the environment—substances of special concern. Environmental Science and Pollution Research, 14791–14804. https://doi.org/10.1007/s11356-014-3902-8

Kümmerer, K., Al-Ahmad, A., Bertram, B., Wießler, M., 2000. Biodegradability of antineoplastic compounds in screening tests: influence of glucosidation and of stereochemistry. Chemosphere 40, 767–773. https://doi.org/10.1016/S0045-6535(99)00451-8

Kümmerer, K., Al-Ahmad, A., 1997. Biodegradability of the Anti-tumour Agents 5-Fluorouracil, Cytarabine, and Gemcitabine: Impact of the Chemical Structure and Synergistic Toxicity with Hospital Effluent. Acta Hydrochimica et Hydrobiologica. 25, 166–172. https://doi.org/10.1002/aheh.19970250402

Lai, W.W.-P., Hsu, M.-H., Lin, A.Y.-C., 2017. The role of bicarbonate anions in methotrexate degradation via UV/TiO 2 : Mechanisms, reactivity and increased toxicity. Water Research 112, 157–166. https://doi.org/10.1016/j.watres.2017.01.040

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

Lapworth, D.J., Baran, N., Stuart, M.E., Ward, R.S., 2012. Emerging organic contaminants in groundwater: A review of sources, fate and occurrence. Environmental Pollution 163, 287–303. https://doi.org/10.1016/j.envpol.2011.12.034

Lee, W., Li, Z.-H., Vakulenko, S., Mobashery, S., 2000. A Light-Inactivated Antibiotic. Journal of Medicinal Chemistry 43, 128–132. https://doi.org/10.1021/jm980648a

Lecomte V., Bertrand-Krajewski J.-L., Bouchez A., Cournoyer B., Dagot C., et al. SIPIBEL : un site pilote pour l'étude des effluents hospitaliers et urbains. Les grands enseignements après cinq ans de suivi et de recherche. Environnement, Risques et Santé, 2018, 17 (S1), pp.59-74.

Legrini, O., Oliveros, E., Braun, A.M., 1993. Photochemical processes for water treatment. Chemical Reviews. 93, 671–698. https://doi.org/10.1021/cr00018a003

Li, D., Chen, H., Liu, H., Schlenk, D., Mu, J., Lacorte, S., Ying, G.-G., Xie, L., 2021. Anticancer drugs in the aquatic ecosystem: Environmental occurrence, ecotoxicological effect and risk assessment. Environment International 153, 106543. https://doi.org/10.1016/j.envint.2021.106543

Li, W., Nanaboina, V., Chen, F., Korshin, G.V., 2016. Removal of polycyclic synthetic musks and antineoplastic drugs in ozonated wastewater: Quantitation based on the data of differential spectroscopy. Journal of Hazardous Materials 304, 242–250. https://doi.org/10.1016/j.jhazmat.2015.10.035

Lin, A.Y.-C., Lin, Y.-C., Lee, W.-N., 2014. Prevalence and sunlight photolysis of controlled and chemotherapeutic drugs in aqueous environments. Environmental Pollution 187, 170–181. https://doi.org/10.1016/j.envpol.2014.01.005

Lin, A.Y.-C., Yu, T.-H., Lateef, S.K., 2009. Removal of pharmaceuticals in secondary wastewater treatment processes in Taiwan. Journal of Hazardous Materials 167, 1163–1169. https://doi.org/10.1016/j.jhazmat.2009.01.108

Litter, M.I., 2005. Introduction to Photochemical Advanced Oxidation Processes for Water Treatment, in: Boule, P., Bahnemann, D.W., Robertson, P.K.J. (Eds.), Environmental Photochemistry Part II, The Handbook of Environmental Chemistry. Springer-Verlag, Berlin/Heidelberg, pp. 325–366. https://doi.org/10.1007/b138188

Llewellyn, N., Lloyd, P., Jürgens, M.D., Johnson, A.C., 2011. Determination of cyclophosphamide and ifosfamide in sewage effluent by stable isotope-dilution liquid chromatography–tandem mass spectrometry. Journal of Chromatography A 1218, 8519–8528. https://doi.org/10.1016/j.chroma.2011.09.061

Lloyd, M.E., 1999. The effects of methotrexate on pregnancy, fertility and lactation. QJM: An International Journal of Medicine 92, 551–563. https://doi.org/10.1093/qjmed/92.10.551

Lopez, B., Ollivier, P., Togola, A., Baran, N., Ghestem, J.-P., 2015. Screening of French groundwater for regulated and emerging contaminants. Science of The Total Environment 518–519, 562–573. https://doi.org/10.1016/j.scitotenv.2015.01.110

López-Serna, R., Jurado, A., Vázquez-Suñé, E., Carrera, J., Petrović, M., Barceló, D., 2013. Occurrence of 95 pharmaceuticals and transformation products in urban groundwaters underlying the metropolis of Barcelona, Spain. Environmental Pollution 174, 305–315. https://doi.org/10.1016/j.envpol.2012.11.022

López-Serna, R., Petrović, M., Barceló, D., 2012. Occurrence and distribution of multi-class pharmaceuticals and their active metabolites and transformation products in the Ebro River basin (NE Spain). Science of The Total Environment 440, 280–289. https://doi.org/10.1016/j.scitotenv.2012.06.027

Love, N.G., Bott, C.B., 2002. Evaluating the role of microbial stress response mechanisms in causing biological treatment system upset. Water Science and Technology 46, 11–18. https://doi.org/10.2166/wst.2002.0449

Lucci, P., Pacetti, D., Nunez, O., G., N., 2012. Current Trends in Sample Treatment Techniques for Environmental and Food Analysis, in: Calderon, L. (Ed.), Chromatography - The Most Versatile Method of Chemical Analysis. InTech. https://doi.org/10.5772/47736

Luo, Y., Guo, W., Ngo, H.H., Nghiem, L.D., Hai, F.I., Zhang, J., Liang, S., Wang, X.C., 2014. A review on the occurrence of micropollutants in the aquatic environment and their fate and removal during wastewater treatment. Science of The Total Environment 473–474, 619–641. https://doi.org/10.1016/j.scitotenv.2013.12.065

Lutterbeck, C.A., Baginska, E., Machado, Ê.L., Kümmerer, K., 2015a. Removal of the anti-cancer drug methotrexate from water by advanced oxidation processes: Aerobic biodegradation and toxicity studies after treatment. Chemosphere 141, 290–296. https://doi.org/10.1016/j.chemosphere.2015.07.069

Lutterbeck, C.A., Kern, D.I., Machado, Ê.L., Kümmerer, K., 2015b. Evaluation of the toxic effects of four anti-cancer drugs in plant bioassays and its potency for screening in the context of waste water reuse for irrigation. Chemosphere 135, 403–410. https://doi.org/10.1016/j.chemosphere.2015.05.019

Lutterbeck, C., Kern, D., Machado, Ê., Kümmerer, K., 2014. Ecotoxicological evaluation of selected anticancer drugs. Toxicology Letters 229, S71–S72. https://doi.org/10.1016/j.toxlet.2014.06.280

Mahnik, S.N., Lenz, K., Weissenbacher, N., Mader, R.M., Fuerhacker, M., 2007. Fate of 5-fluorouracil, doxorubicin, epirubicin, and daunorubicin in hospital wastewater and their elimination by activated sludge and treatment in a membrane-bio-reactor system. Chemosphere 66, 30–37. https://doi.org/10.1016/j.chemosphere.2006.05.051

Mahnik, SusanneN., Rizovski, B., Fuerhacker, M., Mader, RobertM., 2004. Determination of 5-fluorouracil in hospital effluents. Analytical and Bioanalytical Chemistry, 380. https://doi.org/10.1007/s00216-004-2727-6

Markovic, M., Neale, P.A., Nidumolu, B., Kumar, A., 2021. Combined toxicity of therapeutic pharmaceuticals to duckweed, Lemna minor. Ecotoxicology and Environmental Safety 208, 111428. https://doi.org/10.1016/j.ecoenv.2020.111428

Martín, J., Camacho-Muñoz, D., Santos, J.L., Aparicio, I., Alonso, E., 2014. Occurrence and ecotoxicological risk assessment of 14 cytostatic drugs in wastewater. Water, Air, and Soil Pollution 225. https://doi.org/10.1007/s11270-014-1896-y

Martín, J., Camacho-Muñoz, D., Santos, J.L., Aparicio, I., Alonso, E., 2011. Simultaneous determination of a selected group of cytostatic drugs in water using high-performance liquid chromatography-triple-quadrupole mass spectrometry. Journal of Separation Science 34, 3166–3177. https://doi.org/10.1002/jssc.201100461

Martinez, J.L., 2009. Environmental pollution by antibiotics and by antibiotic resistance determinants. Environmental Pollution 157, 2893–2902. https://doi.org/10.1016/j.envpol.2009.05.051

Mater, N., 2014. Evaluation de l'impact (eco)toxicologique de residus medicamenteux presents dans les effluents hospitaliers, urbains et dans l'environnement a l'aide d'une batterie de bioessais et de biomarqueurs (Pathologie, Toxicologie, Génétique et Nutrition). Institut National Polytechnique de Toulouse. Thèse 286 pp.

Moldovan, Z., 2006. Occurrences of pharmaceutical and personal care products as micropollutants in rivers from Romania. Chemosphere 64, 1808–1817. https://doi.org/10.1016/j.chemosphere.2006.02.003

Mompelat, S., Thomas, O., Le Bot, B., 2011. Contamination levels of human pharmaceutical compounds in French surface and drinking water. Journal of Environmental Monitoring, 13, 2929. https://doi.org/10.1039/c1em10335k

Muneer, M., Kanjal, M.I., Iqbal, M., Saeed, M., Khosa, M.K., Ud Den, N.Z., Ali, S., Nazir, A., 2020. Gamma and UV radiations induced treatment of anti-cancer methotrexate drug in aqueous medium: Effect of process variables on radiation efficiency evaluated using bioassays. Applied Radiation and Isotopes 166, 109371. https://doi.org/10.1016/j.apradiso.2020.109371

Nair, M.G., Bridges, T.W., Henkel, T.J., Kisliuk, R.L., Gaumont, Y., Sirotnak, F.M., 1981. Folate analogs altered in the C9-N10 bridge region. 18. Synthesis and antitumor evaluation of 11-oxahomoaminopterin and related compounds. Journal of Medicinal Chemistry, 24, 1068–1073. https://doi.org/10.1021/jm00141a010

Nassour, C., Barton, S.J., Nabhani-Gebara, S., Saab, Y., Barker, J., 2020. Occurrence of anticancer drugs in the aquatic environment: a systematic review. Environmental Science and Pollution Research, 27, 1339–1347. https://doi.org/10.1007/s11356-019-07045-2

Negreira, N., López de Alda, M., Barceló, D., 2014. Study of the stability of 26 cytostatic drugs and metabolites in wastewater under different conditions. Science of The Total Environment 482–483, 389–398. https://doi.org/10.1016/j.scitotenv.2014.02.131

Negreira, N., Mastroianni, N., López de Alda, M., Barceló, D., 2013. Multianalyte determination of 24 cytostatics and metabolites by liquidchromatography–electrospray–tandem mass spectrometry and study of their stability and optimum storage conditions in aqueous solution. Talanta 116, 290–299. https://doi.org/10.1016/j.talanta.2013.04.070

Nikolaou, A., Meric, S., Fatta, D., 2007. Occurrence patterns of pharmaceuticals in water and wastewater environments. Analytical and Bioanalytical Chemistry, 1225–1234. https://doi.org/10.1007/s00216-006-1035-8

Oaks, J.L., Gilbert, M., Virani, M.Z., Watson, R.T., Meteyer, C.U., Rideout, B.A., Shivaprasad, H.L., Ahmed, S., Iqbal Chaudhry, M.J., Arshad, M., Mahmood, S., Ali, A., Ahmed Khan, A., 2004. Diclofenac residues as the cause of vulture population decline in Pakistan. Nature 427, 630–633. https://doi.org/10.1038/nature02317

OECD, 2019. Recommendations for the management of pharmaceuticals in freshwater, in: Pharmaceutical Residues in Freshwater, OECD Studies on Water. OECD, pp. 117–133. https://doi.org/10.1787/14ed3c18-en

OECD, 2016. Water Governance in Cities, OECD Studies on Water. OECD. https://doi.org/10.1787/9789264251090-en

OECD, 1992. OECD Guidelines for the Testing of Chemicals. Ready Biodegradability 301D: Closed Bottle Test; OECD Pub.,

Olalla, A., Negreira, N., López de Alda, M., Barceló, D., Valcárcel, Y., 2018. A case study to identify priority cytostatic contaminants in hospital effluents. Chemosphere 190, 417–430. https://doi.org/10.1016/j.chemosphere.2017.09.129

Oliferova, L.A., Statkus, M.A., Tsisin, G.I., Wang, J., Zolotov, Yu.A., 2006. On-line coupling of sorption preconcentration to liquid-chromatographic methods of analysis. J Anal Chem 61, 416–441. https://doi.org/10.1134/S1061934806050029

Onesios, K.M., Yu, J.T., Bouwer, E.J., 2009. Biodegradation and removal of pharmaceuticals and personal care products in treatment systems: a review. Biodegradation 20, 441–466. https://doi.org/10.1007/s10532-008-9237-8

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

Parrella, A., Kundi, M., Lavorgna, M., Criscuolo, E., Russo, C., Isidori, M., 2014a. Toxicity of exposure to binary mixtures of four anti-neoplastic drugs in Daphnia magna and Ceriodaphnia dubia. Aquatic Toxicology 157, 41–46. https://doi.org/10.1016/j.aquatox.2014.09.012

Parrella, A., Lavorgna, M., Criscuolo, E., Russo, C., Fiumano, V., Isidori, M., 2014b. Acute and chronic toxicity of six anticancer drugs on rotifers and crustaceans. Chemosphere 115, 59–66. https://doi.org/10.1016/j.chemosphere.2014.01.013

Pavlović, D.M., Babić, S., Horvat, A.J.M., Kaštelan-Macan, M., 2007. Sample preparation in analysis of pharmaceuticals. TrAC Trends in Analytical Chemistry 26, 1062–1075. https://doi.org/10.1016/j.trac.2007.09.010

Petrovic, M., Petrovic, M., Barceló, D., 2007. LC-MS for identifying photodegradation products of pharmaceuticals in the environment. TrAC Trends in Analytical Chemistry 26, 486–493. https://doi.org/10.1016/j.trac.2007.02.010

Petrović, M., Hernando, M.D., Díaz-Cruz, M.S., Barceló, D., 2005. Liquid chromatography–tandem mass spectrometry for the analysis of pharmaceutical residues in environmental samples: a review. Journal of Chromatography A 1067, 1–14. https://doi.org/10.1016/j.chroma.2004.10.110

Phillips, P.J., Smith, S.G., Kolpin, D.W., Zaugg, S.D., Buxton, H.T., Furlong, E.T., Esposito, K., Stinson, B., 2010. Pharmaceutical Formulation Facilities as Sources of Opioids and Other Pharmaceuticals to Wastewater Treatment Plant Effluents. Environmental Science & Technology, 44, 4910–4916. https://doi.org/10.1021/es100356f

Pieczyńska, A., Fiszka Borzyszkowska, A., Ofiarska, A., Siedlecka, E.M., 2017. Removal of cytostatic drugs by AOPs: A review of applied processes in the context of green technology. Critical Reviews in Environmental Science and Technology 47, 1282–1335. https://doi.org/10.1080/10643389.2017.1370990

Pomiès, M., Choubert, J.-M., Wisniewski, C., Coquery, M., 2013. Modelling of micropollutant removal in biological wastewater treatments: A review. Science of The Total Environment 443, 733–748. https://doi.org/10.1016/j.scitotenv.2012.11.037

Quintana, J., Weiss, S., Reemtsma, T., 2005. Pathways and metabolites of microbial degradation of selected acidic pharmaceutical and their occurrence in municipal wastewater treated by a membrane bioreactor. Water Research 39, 2654–2664. https://doi.org/10.1016/j.watres.2005.04.068

Rabii, F.W., Segura, P.A., Fayad, P.B., Sauvé, S., 2014. Determination of six chemotherapeutic agents in municipal wastewater using online solid-phase extraction coupled to liquid chromatography-tandem mass spectrometry. Science of The Total Environment 487, 792–800. https://doi.org/10.1016/j.scitotenv.2013.12.050

Rastogi, T., Leder, C., Kümmerer, K., 2015a. A sustainable chemistry solution to the presence of pharmaceuticals and chemicals in the aquatic environment – the example of re-designing β-blocker Atenolol. RSC Advances 5, 27–32. https://doi.org/10.1039/C4RA10294K

Rastogi, T., Leder, C., Kümmerer, K., 2015b. Re-Designing of Existing Pharmaceuticals for Environmental Biodegradability: A Tiered Approach with β-Blocker Propranolol as an Example. Environmental Science & Technology, 49, 11756–11763. https://doi.org/10.1021/acs.est.5b03051

Ren, S., 2004. Assessing wastewater toxicity to activated sludge: recent research and developments. Environment International 30, 1151–1164. https://doi.org/10.1016/j.envint.2004.06.003

Richardson, S.D., Kimura, S.Y., 2016. Water Analysis: Emerging Contaminants and Current Issues. Analytical Chemistry, 88, 546–582. https://doi.org/10.1021/acs.analchem.5b04493

Roberts, P., Thomas, K., 2006. The occurrence of selected pharmaceuticals in wastewater effluent and surface waters of the lower Tyne catchment. Science of The Total Environment 356, 143–153. https://doi.org/10.1016/j.scitotenv.2005.04.031

Roig, B., Marquenet, B., Delpla, I., Bessonneau, V., Sellier, A., Leder, C., Thomas, O., Bolek, R., Kummerer, K., 2014. Monitoring of methotrexate chlorination in water. Water Research 57, 67–75. https://doi.org/10.1016/j.watres.2014.03.008

Rowney, N.C., Johnson, A.C., Williams, R.J., 2009. Cytotoxic drugs in drinking water: a prediction and risk assessment exercise for the thames catchment in the United Kingdom. Environmental Toxicology and Chemistry, 28, 2733. https://doi.org/10.1897/09-067.1

Rubino, F.M., 2001. Separation methods for methotrexate, its structural analogues and metabolites. Journal of Chromatography B: Biomedical Sciences and Applications 764, 217–254. https://doi.org/10.1016/S0378-4347(01)00402-9

Sabry, S.M., Abdel-Hady, M., Elsayed, M., Fahmy, O.T., Maher, H.M., 2003. Study of stability of methotrexate in acidic solution Spectrofluorimetric determination of methotrexate in pharmaceutical preparations through acid-catalyzed degradation reaction. Journal of Pharmaceutical and Biomedical Analysis 32, 409–423. https://doi.org/10.1016/S0731-7085(03)00239-5

Sanderson, H., 2003. Probabilistic hazard assessment of environmentally occurring pharmaceuticals toxicity to fish, daphnids and algae by ECOSAR screening. Toxicology Letters 144, 383–395. https://doi.org/10.1016/S0378-4274(03)00257-1

Santos, M.S.F., Franquet-Griell, H., Alves, A., Lacorte, S., 2018. Development of an analytical methodology for the analysis of priority cytostatics in water. Science of The Total Environment 645, 1264–1272. https://doi.org/10.1016/j.scitotenv.2018.07.232

Sauvé, S., Desrosiers, M., 2014. A review of what is an emerging contaminant. Chemistry Central Journal 8, 15. https://doi.org/10.1186/1752-153X-8-15

Schmitt-Jansen, M., Bartels, P., Adler, N., Altenburger, R., 2007. Phytotoxicity assessment of diclofenac and its phototransformation products. Analytical and Bioanalytical Chemistry, 387, 1389–1396. https://doi.org/10.1007/s00216-006-0825-3

Schwarz, S., Gildemeister, D., Hein, A., Schröder, P., Bachmann, J., 2021. Environmental fate and effects assessment of human pharmaceuticals: lessons learnt from regulatory data. Environmental Sciences Europe, 33, 68. https://doi.org/10.1186/s12302-021-00503-0

Schymanski, E.L., Jeon, J., Gulde, R., Fenner, K., Ruff, M., Singer, H.P., Hollender, J., 2014. Identifying Small Molecules via High Resolution Mass Spectrometry: Communicating Confidence. Environmental Science & Technology, 48, 2097–2098. https://doi.org/10.1021/es5002105

Secrétan, P.-H., Karoui, M., Levi, Y., Sadou-Yayé, H., Tortolano, L., Solgadi, A., Yagoubi, N., Do, B., 2018. Pemetrexed degradation by photocatalytic process: Kinetics, identification of transformation products and estimation of toxicity. Science of The Total Environment 624, 1082–1094. https://doi.org/10.1016/j.scitotenv.2017.12.182

Siedlecka, E.M., Ofiarska, A., Borzyszkowska, A.F., Białk-Bielińska, A., Stepnowski, P., Pieczyńska, A., 2018. Cytostatic drug removal using electrochemical oxidation with BDD electrode: Degradation pathway and toxicity. Water Research 144, 235–245. https://doi.org/10.1016/j.watres.2018.07.035

Steger-Hartmann, T., Kümmerer, K., Hartmann, A., 1997. Biological Degradation of Cyclophosphamide and Its Occurrence in Sewage Water. Ecotoxicology and Environmental Safety 36, 174–179. https://doi.org/10.1006/eesa.1996.1506

Steger-Hartmann, T., Kümmerer, K., Schecker, J., 1996. Trace analysis of the antineoplastics ifosfamide and cyclophosphamide in sewage water by twostep solid-phase extraction and gas chromatography-mass spectrometry. Journal of Chromatography A 726, 179–184. https://doi.org/10.1016/0021-9673(95)01063-7

Tauxe-Wuersch, A., De Alencastro, L.F., Grandjean, D., Tarradellas, J., 2006. Trace determination of tamoxifen and 5-fluorouracil in hospital and urban wastewaters. International Journal of Environmental Analytical Chemistry 86, 473–485. https://doi.org/10.1080/03067310500291502

Ternes, T.A., 1998. Occurrence of drugs in German sewage treatment plants and rivers1Dedicated to Professor Dr. Klaus Haberer on the occasion of his 70th birthday.1. Water Research 32, 3245–3260. https://doi.org/10.1016/S0043-1354(98)00099-2

Thomas, K.V., Dye, C., Schlabach, M., Langford, K.H., 2007. Source to sink tracking of selected human pharmaceuticals from two Oslo city hospitals and a wastewater treatment works. Journal of Environmental Monitoring, 9, 1410. https://doi.org/10.1039/b709745j

Tijani, J.O., Fatoba, O.O., Petrik, Leslie.F., 2013. A Review of Pharmaceuticals and Endocrine-Disrupting Compounds: Sources, Effects, Removal, and Detections. Water, Air, & Soil Pollution, 224, 1770. https://doi.org/10.1007/s11270-013-1770-3

Marchand, M., Tissier, C., 2007. L'analyse du risque chimique en milieu marin: l'approche méthodologique europeenne. Environnement, Risques & Santé 6, 127–141. https://doi.org/10.1684/ers.2007.0037

Togola, A., Lardy-Fonta, S., Lestremau, F., Soulier, C., 2015. Positionnement sur l'utilisation de la spectrometrie de masse haute resolution pour le criblage environnemental (No. Rapport final. BRGM/RP-65420-FR 75,65 p.,14 ill, 3 ann).

Togola, A., Baran, N., Coureau, C., 2014. Advantages of online SPE coupled with UPLC/MS/MS for determining the fate of pesticides and pharmaceutical compounds. Analytical and Bioanalytical Chemistry, 406, 1181–1191. https://doi.org/10.1007/s00216-013-7248-8

Toolaram, A.P., Kümmerer, K., Schneider, M., 2014. Environmental risk assessment of anti-cancer drugs and their transformation products: A focus on their genotoxicity characterization-state of knowledge and short comings. Mutation Research/Reviews in Mutation Research 760, 18–35. https://doi.org/10.1016/j.mrrev.2014.02.001

Tripathi, A.K., David, A., Govil, T., Rauniyar, S., Rathinam, N.K., Goh, K.M., Sani, R.K., 2020. Environmental Remediation of Antineoplastic Drugs: Present Status, Challenges, and Future Directions. Processes 8, 747. https://doi.org/10.3390/pr8070747

Turci, R., Sottani, C., Spagnoli, G., Minoia, C., 2003. Biological and environmental monitoring of hospital personnel exposed to antineoplastic agents: a review of analytical methods. Journal of Chromatography B 789, 169–209. https://doi.org/10.1016/S1570-0232(03)00100-4

Valcárcel, Y., González Alonso, S., Rodríguez-Gil, J.L., Gil, A., Catalá, M., 2011. Detection of pharmaceutically active compounds in the rivers and tap water of the Madrid Region (Spain) and potential ecotoxicological risk. Chemosphere 84, 1336–1348. https://doi.org/10.1016/j.chemosphere.2011.05.014

Verlicchi, P., Zambello, E., 2016. Predicted and measured concentrations of pharmaceuticals in hospital effluents. Examination of the strengths and weaknesses of the two approaches through the analysis of a case study. Science of The Total Environment 565, 82–94. https://doi.org/10.1016/j.scitotenv.2016.04.165

Verlicchi, P., Al Aukidy, M., Zambello, E., 2012. Occurrence of pharmaceutical compounds in urban wastewater: Removal, mass load and environmental risk after a secondary treatment—A review. Science of The Total Environment 429, 123–155. https://doi.org/10.1016/j.scitotenv.2012.04.028

Wayne, R.P., 2005. Basic Concepts of Photochemical Transformations, in: Boule, P., Bahnemann, D.W., Robertson, P.K.J. (Eds.), Environmental Photochemistry Part II, The Handbook of Environmental Chemistry. Springer-Verlag, Berlin/Heidelberg, pp. 1–47. https://doi.org/10.1007/b138178

Wang, X., Zhang, J., Chang, V.W.C., She, Q., Tang, C.Y., 2018. Removal of cytostatic drugs from wastewater by an anaerobic osmotic membrane bioreactor. Chemical Engineering Journal 339, 153–161. https://doi.org/10.1016/j.cej.2018.01.125

Weigel, S., Kallenborn, R., Hühnerfuss, H., 2004. Simultaneous solid-phase extraction of acidic, neutral and basic pharmaceuticals from aqueous samples at ambient (neutral) pH and their determination by gas chromatography–mass spectrometry. Journal of Chromatography A 1023, 183–195. https://doi.org/10.1016/j.chroma.2003.10.036

Widemann, B.C., Sung, E., Anderson, L., Salzer, W.L., Balis, F.M., Monitjo, K.S., McCully, C., Hawkins, M., Adamson, P.C., 2000. Pharmacokinetics and metabolism of the methotrexate metabolite 2, 4-diamino-N(10)-methylpteroic acid. The Journal of Pharmacology and Experimental Therapeutics, 294, 894–901.

Wormington, A.M., De María, M., Kurita, H.G., Bisesi, J.H., Denslow, N.D., Martyniuk, C.J., 2020. Antineoplastic Agents: Environmental Prevalence and Adverse Outcomes in Aquatic Organisms. Environmental Toxicology and Chemistry, 39, 967–985. https://doi.org/10.1002/etc.4687

Xu, Y., Yuan, Z., Ni, B.-J., 2016. Biotransformation of pharmaceuticals by ammonia oxidizing bacteria in wastewater treatment processes. Science of The Total Environment 566–567, 796–805. https://doi.org/10.1016/j.scitotenv.2016.05.118

Yao, Y., Lu, Z., Min, H., Gao, H., Zhu, F., 2012. The effect of tetrahydrofuran on the enzymatic activity and microbial community in activated sludge from a sequencing batch reactor. Ecotoxicology 21, 56–65. https://doi.org/10.1007/s10646-011-0765-3

Yin, J., Niu, Y., Shao, B., 2017. Products of methotrexate during chlorination. Journal of Environmental Sciences 55, 100–108. https://doi.org/10.1016/j.jes.2016.06.024

Yin, J., Shao, B., Zhang, J., Li, K., 2010. A Preliminary Study on the Occurrence of Cytostatic Drugs in Hospital Effluents in Beijing, China. Bulletin of Environmental Contamination and Toxicology 84, 39–45. https://doi.org/10.1007/s00128-009-9884-4

Załęska-Radziwiłł, M., Affek, K., Rybak, J., 2014. Ecotoxicity of chosen pharmaceuticals in relation to micro-organisms—risk assessment. Desalination and Water Treatment 52, 3908–3917. https://doi.org/10.1080/19443994.2014.887503

Zaviska, F., Drogui, P., Mercier, G., Blais, J.-F., 2009. Procédés d'oxydation avancée dans le traitement des eaux et des effluents industriels: Application à la dégradation des polluants réfractaires. Revue des sciences de l'eau 22, 535. https://doi.org/10.7202/038330ar

Zhang, S., Ye, C., Li, J., Yu, X., Feng, M., 2021. Treatment-driven removal efficiency, product formation, and toxicity evolution of antineoplastic agents: Current status and implications for water safety assessment. Water Research 206, 117729. https://doi.org/10.1016/j.watres.2021.117729

Zounkova, R., Kovalova, L., Blaha, L., Dott, W., 2010. Ecotoxicity and genotoxicity assessment of cytotoxic antineoplastic drugs and their metabolites. Chemosphere 81, 253–260. https://doi.org/10.1016/j.chemosphere.2010.06.029

Zounková, R., Odráška, P., Dolealová, L., Hilscherová, K., Maršálek, B., Bláha, L., 2007. Ecotoxicity and genotoxicity assessment of cytostatic pharmaceuticals. Environmental Toxicology and Chemistry 26, 2208. https://doi.org/10.1897/07-137R.1

Zuccato, E., Castiglioni, S., Fanelli, R., 2005. Identification of the pharmaceuticals for human use contaminating the Italian aquatic environment. Journal of Hazardous Materials 122, 205–209. https://doi.org/10.1016/j.jhazmat.2005.03.001

Zuccato, E., Calamari, D., Natangelo, M., Fanelli, R., 2000. Presence of therapeutic drugs in the environment. The Lancet 355, 1789–1790. https://doi.org/10.1016/S0140-6736(00)02270-4

Chapitre 3. Cinétiques de photodégradation, évolution des TPs (Identification et évaluation de leur cytotoxicité)

Ce troisième chapitre présente l'étude de photodégradation des deux molécules, le MTX et son analogue éco-conçu, le OMTX. Les objectifs sont de comparer leurs cinétiques de dégradation mais aussi de produire une large variété de TPs pour les identifier, enfin, suivre l'évolution de la cytotoxicité du mélange afin de s'assurer de la perte du mode d'action initiale de la part des TPs.

En accord avec la littérature présentée en **Chapitre 2**, la molécule MTX est connue pour être sensible à la photodégradation. Deux types de photolyse directes ont été menées, une irradiation sous 300-450 nm, représentative d'une irradiation solaire et une autre à 254 nm proche des conditions de potabilisation. Le MTX a été étudié sous deux conditions de pH afin de comparer sa dégradation selon sa forme ionique, quant au OMTX un pH environnemental de 7 a été choisi.

Le chapitre est divisé en deux sous parties, la première concerne uniquement le méthotrexate, et correspond à un article publié dans Environmental Science and Pollution Research (ESPR) en aout 2021. La deuxième sous partie a donné lieu à un article en cours de révision pour Chemosphere et est un travail collaboratif avec l'ICMMO, porteur du projet EDIFIS. Il présente la synthèse des différents analogues éco-conçus et la sélection de l'analogue OMTX en tant que candidat prometteur, cette première étape a été réalisée par l'ICMMO et l'étude de cet analogue OMTX lors de sa photodégradation, en comparaison au MTX, menée dans le cadre de la thèse.

D'autres types de dégradations abiotiques ont été envisagées, la dégradation par procédé Fenton a été étudiée mais l'instabilité du OMTX dans le milieu ne permettait pas d'obtenir des témoins T0 répétables et rendait la comparaison MTX/OMTX compliquée. Les résultats ne sont pas présentés dans ce manuscrit. Comme vu en **Chapitre 2**, ce procédé requiert un pH acide. L'instabilité du OMTX à un pH acide a été révélée *via* une expérimentation présentée en annexe 1 du manuscrit.

3.1. Photodégradation du methotrexate en solution aqueuse

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"Photodegradation of methotrexate in aqueous solution: degradation kinetics and identification of transformation products"

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Declarations

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3.1.1. Abstract

Methotrexate is an antineoplastic folate analog of high environmental concern, due to its low biodegradability and toxicological properties. This study focused on its photodegradation under two irradiation conditions, aiming to be representative of environment (300–450 nm) and drinking water treatment (254 nm). The photodegradation experiments were conducted at two pH, to vary the methotrexate ionization state and to produce a large variety of transformation products (TPs). The degradation kinetics determined through LC-UV monitoring were contrasted according to pH and irradiation wavelength. However, the quantum yields were independent of ionization state at 254 nm and the changes in kinetics at higher wavelengths were attributed to a change in the degradation mechanism. The TPs formed during the reactions were identified by UHPLC-MS/MS, using both the positive and negative modes. Among the eleven proposed structures, five were described as methotrexate TPs for the first time. The TPs result from N-demethylation, glutamic acid oxidation and C-N cleavage, all of them leading to further degraded photoproducts presenting modified or lost glutamic acid part. This was made possible thanks to the negative mode, which allowed the exploration of the glutamic acid moiety modifications. Cytotoxicity assessment on A549 cancer cells demonstrated that all photoproducts formed at pH 7 were less toxic than the parent compound.

3.1.2. Introduction

Pharmaceuticals and their degradation products are detected in surface waters, wastewater and groundwater at a range of ng/L to mg/L (aus der Beek et al. 2016; Morasch et al. 2010) and even in drinking waters (Benotti et al. 2009; Mompelat et al. 2011). Because more than 2,000 active ingredients are approved in Europe, Perazzolo et al. (2010) proposed a priority list of 57 compounds for pharmaceuticals risks assessment. This list was based on screening of their potential harm, analytical feasibility, and previous detection in water. These occurrences are mainly due to their pharmacokinetics since pharmaceuticals are excreted by patients either as the parent compound (60%-95% - Turci et al. 2003) or as metabolites before entering domestic wastewaters. Discharges from municipal sewage effluents are considered as the main source of pharmaceuticals in the environment (Garcia-Ac et al. 2009a) since they represent 80% of their release against 20% for hospital waste waters (Besse et al. 2012). Castiglioni et al. (2005) detected 19 pharmaceuticals in concentrations ranging from 0.5 to 2000 ng/L in Waste Water Treatment Plant (WWTP) and (Vergili et al. 2019) detected 21 and 32 compounds in urban wastewaters from Turkey and Germany, respectively. Parent compounds, metabolites and transformation products (TPs) formed by oxidative techniques such as ozonation, UV/hydrogen peroxide treatment and chlorination can enter in surface waters due to their incomplete degradation during

wastewater treatment. A lack of knowledge remains about the ecotoxicity of certain pharmaceuticals (Orias and Perrodin 2013). In addition, Escher and Fenner (2011) and González-Burciaga et al. (2021) have shown that awareness about TP and metabolites can be relevant for environmental risk assessment because they can be more persistent, more mobile or have a higher toxicity than the parent compound.

Among various classes of pharmaceuticals, antineoplastic agents are of particular environmental concern due to their highly potent mechanism of action: they are cytotoxic, genotoxic, mutagenic and teratogenic (Zounková et al. 2007). They have been detected in source waters at pg/L range for oxazaphosphorines for example (Buerge et al. 2006). The most investigated anti-cancer drug is cyclophosphamide (Besse et al. 2012), but another anticancer drug: methotrexate ((2S)−2-[(4-{[(2,4-diaminopteridin-6-yl)methyl](methyl)amino}benzoyl)amino] pentanedioic acid, MTX) is one of the most widely prescribed antineoplastic agents worldwide (Kosjek and Heath 2011). It has been used for more than 50 years (Lai et al. 2017) in treatments of meningeal leukemia, psoriasis and rheumatoid arthritis (Sabry et al. 2003). It competitively inhibits dihydrofolate reductase, the enzyme that reduces dihydrofolates to tetrahydrofolates. The reduced folate derivatives are important coenzymes in the biosynthesis of amino acids and the purine and pyrimidine units of DNA (Breithaupt et al. 1982). Perazzolo et al. (2010) have selected MTX on its priority list for pharmaceutical risk assessment.

Methotrexate is mostly excreted unchanged (81%) after administration to the patient (Benet et al. 2011) and the main metabolites are the 7-hydroxymethotrexate (7-OH-MTX), the 2,4-diamino-N(10)-methylpteroic acid (DAMPA) and the polyglutamylated forms (Widemann et al. 2000). DAMPA is known as a minor and inactive metabolite of MTX since it does not inhibit human dihydrofolate reductase (Donehower et al. 1979; Widemann et al. 2000). However, 7-OH-MTX is included in the priority list for environmental assessment due to its persistence and toxicity (Besse et al. 2012; Jacobs et al. 1976).

Numerous studies detected MTX in municipal WWTP influents with concentrations ranging from 2.6 to 59 ng/L in Canada (Garcia-Ac et al. 2009b) and Spain (Martín et al. 2014; Negreira et al. 2014). Castiglioni et al. (2005) detected MTX at 12.6 ng/L in one of eight samples of WWTP effluent, showing partial removal of this contaminant. Around hospital areas, the level of concentration could be higher: in hospital effluent, maximum concentrations can reach concentration levels of 4,689 ng/L in China (Yin et al. 2010), around 4,756 ng/L in Spain (Olalla et al. 2018) 28,700 ng/L in France (Catastini et al. 2008) and 300 ng/L in Taiwan (Lin et al. 2014). These high concentrations are not only related to hospital with specialized oncology area, because MTX is also used for other treatments. Catastini et al. (2008) detected even 2,000 ng/L in WWTP influent, which was connected to hospitals and 30 ng/L in the WWTP effluent, showing incomplete removal by the wastewater treatment process. Thus, MTX and some known transformation products could enter in the environment, but there is a lack of knowledge about their occurrence in drinking and surface waters.

MTX can be partially degraded through various processes, particularly during wastewater or drinking water treatment. Kiffmeyer et al. (1998) reported the biodegradation of MTX in eight days by an activated sludge system with an efficiency of 95% and identified 7-OH-MTX as the main TP. The concentration of this TP increased with the same rate as the MTX concentration decreased, indicating that it did not undergo further biodegradation. Recently, Isidori et al. (2016) observed the transformation of MTX into 7-OH-MTX in hospital effluent transiting to a WWTP by potential biotic conversion. Kosjek et al. (2015) have shown that MTX was readily biodegradable by activated sludge and transformed into several TPs. They have identified 8 new TPs resulting from demethylation, oxidative cleavage of amine, cleavage of C–N bond, aldehyde to carboxylate transformation and hydroxylation. DAMPA was the most abundant and persistent TP. These results contrast with a previous study by Henschel et al. (1997) with OECD 301 biodegradability test, which classified MTX as not readily biodegradable. After using the same OECD standardized procedure, Lutterbeck et al. (2015) proceeded to photo-irradiation at 200–400 nm wavelengths on the biodegradation mixture. They identified nine products including 7-OH-MTX and DAMPA, indicating that they did not undergo further degradation. Calza et al. (2014) identified seven photoproducts resulting from irradiation at 360 nm, with transformation involving decarboxylation and hydroxylation or the molecule cleavage. The addition of titanium dioxide favoured a supplementary transformation pathway by the elimination of succinic acid from MTX. Kanjal et al. (2020) compared kinetics of photodegradation with and without peroxymonosulfate and identified common TPs with Calza et al. (2014). Lai et al. (2017) studied the role of bicarbonate anions in MTX photocatalysis by UV-TiO_2 treatment. Transformations included the addition of atomic oxygen, hydroxylation, deamination, C-C cleavage and C-N cleavage of the aniline moiety, this last one being proposed as the primary degradation pathway. Upon the 16 TPs identified in this study, six were new whereas the others were common with previous photodegradation studies (Calza et al. 2014; Lutterbeck et al. 2015; Kanjal et al. 2020). Hsu et al. (2019) studied the self-sensitized degradation mechanism and pathways through direct photolysis and identified 10 TPs common to the study by Lai et al. (2017). Roig et al. (2014) identified chlorination products during the treatment water process. Some other studies focussed on degradation kinetics without identifying TPs, using various processes as catalytic ozonation (Alinejad et al. 2019), UV and gamma irradiation (Muneer et al. 2020) or hydrolysis (Toński et al. 2021)

Regarding toxicity assessment, few studies report bioassays with *Vibrio Fischeri,* showing a decrease in toxicity under direct photolysis (Calza et al. 2014; Lutterbeck et al.2015), while an increase in toxicity was seen during photodegradation in presence of bicarbonate anions (Lai et al. 2017). Alinejad et al. (2019) reported a decrease in cytotoxicity as Muneer et al. (2020), who reported also a decrease in mutagenicity after gamma and UV treatments.

In summary, various studies focussed on the MTX photodegradation, but none compared its reactivity in terms of degradation kinetics and/or transformation pathways, according neither to the wavelength nor the pH. Furthermore, various TPs issuing from MTX through abiotic (photodegradation or various AOPs) and biotic processes were evidenced and presented a high variability according to the studies.

The goal of this research was to disclose the MTX photodegradation under conditions occurring 1) in natural surface waters, thus receiving solar light (using 300–450 nm emitting lamps, i.e. using the photoactive part of the solar spectrum) and 2) during drinking water treatment, thus using 254 nm irradiation. Focusing on direct photolysis processes, experiments were realised in pure water to allow the identification of TPs. The pH was varied (pH 3 and 7) to study changes in reactivity according to the two ionization states of MTX. In addition, cytotoxicity assessment of TPs mixture was conducted during the photodegradation experiment at pH 7 under both irradiation conditions. This study aimed to complement the existing knowledge since the photodegradation of MTX is not documented at low wavelengths, focussing on the degradation kinetics of MTX, the identification of TPs and the evolution of cytotoxicity following MTX photodegradation. Our analytical strategy included the usage of mass spectrometry in both positive and negative modes to have multiple arguments for structural identification of the TPs.

3.1.3. Experimental

3.1.3.1. Chemicals and reagents

MTX (MTX, CAS 59-05-2, > 98%) was obtained from Sigma Aldrich (St Quentin Fallavier, France) (see Table 3.1- S1), perchloric acid ($HClO_4$ 70%-72%) and NaOH 1 mol/L from Merk (Darmstadt, Germany). HCl 1 mol/L was prepared from a commercial 37% solution (Carlo Erba, Val de Reuil, France). Water was supplied by a Milli-Q water system (Millipore, Saint-Quentin-en-Yvelines, France) at a quality of resistivity > 18.2 MΩ.cm and total organic carbon < 3 $\mu g.L^{-1}$. The analyses were performed using formic acid (98%-100%, Sigma Aldrich), acetonitrile (RS plus gradient, Carlo Erba) and methanol (RPE, Carlo Erba).

3.1.3.2. Photodegradation experiments

The degradation experiments were conducted using an initial MTX concentration of 40 µM by dilution from an alkaline aqueous stock solution prepared every two weeks. Selecting a concentration greater than environmental ones allowed monitoring the parent evolution, detecting and identifying the

TPs without sample pre-concentration. The pH was set at 7 or 3 by the addition of 0.05 M $HClO_4$ or NaOH. The solutions were kept in the dark before the experiments. During reactions, samples were withdrawn at specified times and stored in the dark at 10°C until their analysis by liquid chromatography HPLC-UV.

Two wavelength conditions, representative of natural conditions in surface water (300–450 nm) and drinking water treatment (254 nm) were used. For the 300–450 nm treatment, 150 mL of solution was placed in a 3-cm internal diameter tubular pyrex-reactor at the centre of a cylindrical device equipped with six lamps emitting within 300 to 450 nm with a maximum emission at 365 nm (TLD 15 W, Philips). A fan located outside the bottom of the tube was used to remove heat from the reactor, thus allowing the experiments to be conducted at an ambient temperature of 20 − 22 °C. The fan and lamps were turned on for at least 30 minutes before the start of each experiment. Light intensity measured by a radiometer (VLX-3W, Vilber Lourmat, Marne-la-Vallée, France) was for each of the six lamps ca 0.1 mW/cm^2 at 312 nm and 1.2 mW/cm^2 at 365 nm. The MTX solution was bubbled by a 150 mL/min flow of air during 20 min before exposition and during the irradiation (**Erreur ! Source du renvoi introuvable.**). For the 254 nm treatment, a quartz cylindrical 2-cm internal diameter tubular photoreactor containing 60 mL solution was placed parallel to a monochromatic low-pressure mercury lamp (TUV 15 W, Philips) outputting a 254 nm light intensity of 1.5 mW/cm2 at the photoreactor location. The solution was mixed by magnetic stirring (**Erreur ! Source du renvoi introuvable.**). To determine the quantum yields (see calculations in SI), the UV spectra of initial solutions were recorded in quartz cuvettes using a spectrophotometer (UV-2401 PC, Shimadzu).

3.1.3.3. Analytical determinations

i. High Performance liquid chromatography with ultraviolet detection (HPLC-UV) for MTX monitoring

The reaction monitoring was performed by HPLC-UV to establish rapidly the MTX degradation kinetics, before HPLC-MS (/MS) to characterize the photodegradation mixture in terms of TPs. The HPLC-UV was carried out using a Waters Alliance (2690/5) system on a HPLC C18 Gravity-SB column 150 × 3 mm, 3 µm particle size (Macherey-Nagel)**.** The gradient profile started at 97% water with 0.05% formic acid and linearly progressed up to 50% in 30 min, the second solvent being acetonitrile, at a constant flow rate of 0.5 mL/min. The detection was performed using diode-array detector (TUV 2489, Waters) acquiring data in the 230–305 nm range. Data were acquired and treated using Empower software. Quantifications were performed at 305 nm, using the 40 µM initial solution (mean of 4 injections for each experiment) as 100% abundance. All the peak areas were expressed in percentage of initial MTX. The MTX kinetics of degradation were treated as a first order form. The apparent first-order rate

constants k' were evaluated according to $Ln(C/C_0) = - k't$, where C is the MTX concentration at time t and C_0 its initial concentration.

ii. UHPLC-ESI-MS for TPs monitoring

The TPs were monitored by Ultra High Performance Liquid Chromatography (UHPLC) –mass spectrometry (MS). The device consisted on an Acquity UPLC system (Waters, Milford, MA, USA) composed of a high pressure binary solvent manager, an autosampler and a column oven connected to a triple quadrupole mass spectrometer (TQD, Waters). Chromatographic separation was carried out on a UPLC C18 Gravity-SB Column, 100 x 2 mm, 1.8 µm particle size (Macherey-Nagel). The gradient profile started at 97% water with 0.05% formic acid for 5 min, linearly progressed to 50% at 30 min, with a flow rate of 0.3 mL/min. The UHPLC effluent was directed to an UV detector (TUV, Waters) programmed at 305 nm, prior to its introduction into the mass spectrometer via an electrospray source operating either in positive or negative mode. The electrospray conditions were: capillary voltage 3 kV, cone voltage 40 V and 54 V respectively under positive and negative modes, source temperature 150°C, desolvation temperature 450°C, extractor voltage 3 V, cone and desolvation gas flow (nitrogen) at 20 and 900 L/h, respectively. LC-MS studies were carried out in full scan MS over the mass range of m/z 120–550. For quantitation, the integrations were performed on pseudo-molecular ions and the limit of quantitation using a signal-to-noise (S/N) of 5 was estimated to be 200 $\mu g.L^{-1}$ corresponding to 1% of residual MTX.

iii. Pre-concentration by Solid Phase Extraction (SPE)

To perform the structural determinations of the TPs, a pre-concentration was necessary. Each reaction was stopped at an irradiation time selected as a compromise between the variety of observed TPs and their relative abundance. The obtained solutions were then submitted to Solid Phase Extraction (SPE), using various conditions to maximize the TP extraction on the lipophilic/hydrophilic balance Oasis HLB (200 mg, 6 mL) cartridge. Na_2EDTA was added to the samples with a suitable volume of a 0.1 M solution to achieve a final concentration of 0.1% (gram Na_2EDTA per gram solution) to improve the extraction efficiency of target compounds according to Ferrando-Climent et al. (2013). The elution was done with 4 mL of methanol.

iv. UHPLC-ESI-MS/MS for identification

Structural elucidation of TPs was achieved with an ultra-high performance liquid chromatograph Ultimate 3000 (Thermo Fisher Scientific) coupled to a Q Exactive hybrid quadrupole – Orbitrap™ (Thermo Fisher Scientific) mass spectrometer through a Heated Electrospray Ionization (HESI-II) probe. The

chromatographic separation of the samples was performed on the same UPLC column and the same gradient as previously in UHPLC-ESI-MS. The analyses were performed in positive and negative modes under the following specific conditions: spray voltage, ± 3 kV; sheath gas pressure: 35 psi, auxiliary gas flow rare: 10 (arbitrary units); ion transfer tube and vaporizer temperature 300°C. Nitrogen was employed as sheath and auxiliary gas.

MS data were acquired in full scan within mass-to-charge (m/z) range of 120–550 m/z at a mass resolution of 70000 and a scanning speed of 3.7 scan/s. It allowed detecting the pseudo-molecular ions $[M + H]^+$ and $[M - H]^-$ of the various compounds, and thus their elemental composition. The pseudo-molecular ions were fragmented by higher-energy collisional dissociation (HCD) with normalized collision energy of 35% and then diriged to the Orbitrap where they were analysed with a resolution of 35000. Xcalibur™ software (Thermo Fisher Scientific) was employed for instrumental control and data processing.

3.1.3.4. In vitro cell-proliferation assay

Lung carcinoma (A549) cells were grown in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% of fetal calf serum (FCS) and 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen) in a 5% CO2 and 95% hygrometry environment at 37 °C.

The cytotoxic activity of MTX was investigated using the MTS ((3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium)) method (Promega). 2.5 10^3 cells (100 µL) were seeded in 96-well plates and incubated overnight at 37 °C in the presence of 5% CO_2 and 95% hygrometry. Then, the cells were treated with 100 µL of MTX at different final concentrations (from 50 µM to 0.05 nM). After 72 h, the media was aspirated and 100 µL of a MTS solution (10% in media) was added in each well. After a 3 h incubation period at 37 °C in the presence of 5% CO_2 and 95% hygrometry, the optical density was measured at 490 nm wavelength using a microplate reader (Infinite M200 Pro, Tecan trading AG, Switzerland). Untreated cells were used as control. Each concentration was tested in six replicates and the experiment was fulfilled in triplicates. The concentration inhibiting 50% of the cell proliferation (IC_{50}) was determined using GraphPad Prism (GraphPad Software Inc, San Diego, CA, USA). To evaluate the cytotoxicity evolution along the MTX degradation at pH 7, different time-points were chosen for the two irradiation conditions: treatment at 254 nm, time-points: t = 0, 60, 180 and 360 min; treatment at 300-450nm, time-points: t = 0, 60, 120 and 180 min. Each sample was then evaluated using the method described above with 100 µL of each solution at the final concentration of 100 nM (close value of the IC_{50} determined for MTX).

3.1.4. Results and discussion

3.1.4.1. Kinetics of MTX degradation and evolution of TPs

When the MTX aqueous solutions at pH 7 or 3 were placed in the dark, they remained stable for at least a 24 h period. Irradiated at 254 nm during 3 h, the MTX degraded up to ca 60% and 73% at pH 7 and 3, respectively (Figure 3.1- 1a). The reactions being conducted in MilliQ water without any photo-inducer, they can be interpreted as direct photolysis. The kinetics followed a pseudo-first-order with regression coefficients greater than r^2 of 0.99, and the constants were determined as 8.8 ± 1.6 10^{-5} s^{-1} and 12.4 ± 0.7 10^{-5} s^{-1} (mean ± standard deviation, n = 3) at pH 7 and 3, respectively (Figure 3.1- 2a). The significantly faster reactivity at pH 3 resulted from a different ionic form, as MTX is a polyelectrolyte with 3 pK_a at 3.36, 4.70, and 5.71 (Rubino, 2001). The change in photoreactivity with the ionic form has been widely observed for numerous compounds, and frequently linked with a change of the quantum yield (Chowdhury et al. 2020). However, the MTX UV-absorption spectrum sharply changes between the two pH, presenting around 254 nm a minimum at pH 3 when MTX is a cation and a maximum at pH 7 when MTX is an anion (Figure 3.1- S1). As a result, the quantum yields appeared to be similar at pH 7 and 3, around 1.63 10^{-3} and 1.83 10^{-3}, respectively, because of the differences in molar absorptivity at 254 nm (Figure 3.1- 2b).

The MTX photodegradation was also performed at both pH 7 and 3 under 300–450 nm wavelength range. At pH 3, the MTX degradation under 300-450 nm irradiation was slower than at 254 nm for the same pH 3, with pseudo-kinetic constant (2.7 ± 0.9 10-5 s-1, n = 4) (Figure 3.1- 2a) determined with regression coefficients in the 0.94–0.98 (r2) range, and quantum yield (1.7 ± 0.7 10-4) significantly different (Figure 3.1- 2b). At pH 7, the overall degradation kinetics appeared as comparable to the 254 nm ones, with a pseudo-first-order kinetic constant calculated as 12.8 ± 0.9 10-5 s-1 (n = 5) and a quantum yield not significantly different from 254 nm pH 7 (1.05 ± 0.1 10-3) (Figure 3.1- 2a and b). However, the regression coefficients were lower than in the other conditions (0.86 to 0.92) (r2) because of an important initial lag phase more pronounced than at pH 3 (Figure 3.1- 1b). Chatterji and Gallelli (1977) observed the same lag phase and explained it by the accumulation of free radical produced by bicarbonates introduced in the solution, but in this study, such important free radical production could not be expected in Milli-Q water. At high initial concentration of MTX and with an irradiation wavelength comparable to ours, Hsu et al. (2019) also observed the initial lag phase. They demonstrated that it was due to a self-sensitized photodegradation mechanism: MTX enhanced its own direct photolysis, thus increasing the degradation rate through the production of the triplet-excited state of methotrexate (3MTX*) and the triplet-excited state of pteridine derivatives, whereas the produced singlet oxygen quenched degradation. This self-sensitized degradation mechanism probably explains why we observed a higher degradation rate (and thus quantum yield) at pH 7 than at pH 3 and two pteridine derivatives issuing for cleavage were identified, TP206 and TP208 (see below). Even if their structure is not strictly

identical to the compound evidenced by Hsu et al. (2019), these TPs could also be hypothesized to self-sensitize the reaction. This assumption is furthermore supported by the protonation site located on the diamino-pteridine and corresponding to the dissociation constant (pK_a) of 5.71 (Poe, 1977; Rubino, 2001).

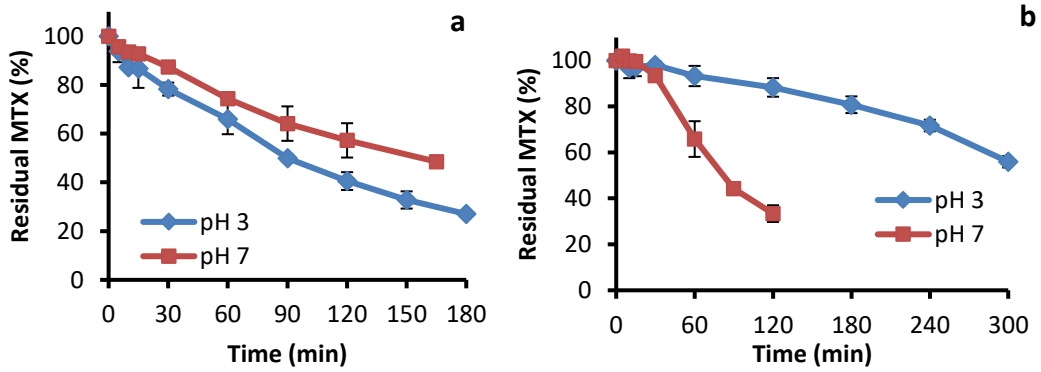


Figure 3.1- 1 Methotrexate degradation kinetics under 254 nm (a) and 300-450 nm (b) irradiation (40 µM aqueous solutions)

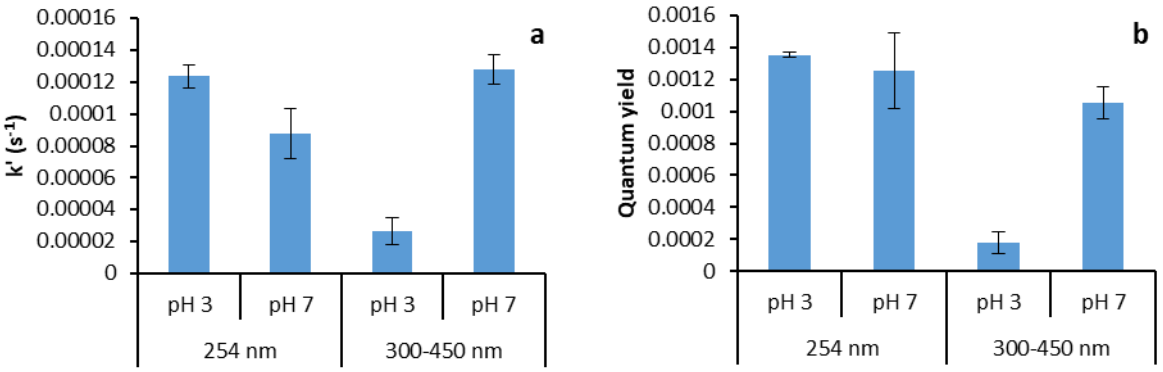


Figure 3.1- 2 Pseudo-first order kinetic constants (a) and quantum yield (b) of methotrexate photodegradation

3.1.4.2. Structural identification of photodegradation products

The elemental composition of the various compounds and their main fragment ions is summarized in Table 3.1- 1. Due to the lack of standards corresponding to most of the hypothesized photoproducts: 1) a comprehensive study of the MTX fragmentation was conducted and 2) most of the identifications corresponded to a level 2b (otherwise specified) according to the Schymanski et al. (2014) classification.

In the positive mode, the MTX fragmentation pathways have been found to be consistent with the literature (Calza et al. 2014; Lutterbeck et al. 2015; Lai et al. 2017; Hsu et al. 2019; Kosjek et al. 2015; Kanjal et al. 2020). The higher-energy collisional dissociation (HCD) of $[M + H]^+$ produced the ion m/z 308 by the cleavage of the amide bond between the glutamic and p-methylaminobenzoic acid residues. From this m/z 308 ion, the cleavage of the C–N bond between p-methylaminobenzoyl and diaminopteridinemethylene groups produced the ions m/z 175 and 134. In the negative mode, unexplored to our knowledge, three fragmentation pathways were distinguished (Figure 3.1- 3 and Figure 3.1- S2). The first fragmentation may correspond to the elimination of H2O to form m/z 435 or to the elimination of CO2 from the glutamic acid group, giving ion at m/z 409. This m/z 409 ion further evolved through elimination of H_2N-CN to form m/z 367, through N-C cleavage to form m/z 235 and then through CO_2 elimination to form m/z 191. An alternative and major MTX fragmentation pathway was interpreted through a rearrangement initiated by the hydroxyl group from the carboxylic acid linked to the benzamide derivative, forming a five-centered transition state before the elimination of 3-oxoaziridin-2-yl-propanoic acid as proposed by Secrétan et al. (2018) for pemetrexed. An alternative mechanism leading to the same m/z 324 ion was also proposed in the case of pralatrexate, including a N-C cleavage followed by rearrangement (Sastry et al. 2016). The obtained ion m/z 324, which presents the same structure as $[M - H]^-$ of DAMPA, may further eliminate CO_2 (to form m/z 280) or produce the radical ions m/z 149, 174 and 189 (through m/z 280 rearrangement) by heterolytic cleavage.

In the positive mode, most of the TPs (TP324, TP424a, TP424b, TP408a) presented the fragment ions m/z 308, 175 and 134, or at least for the lower mass TPs the ion m/z 134 (TP280, TP250). Being respectively characteristics of the diamino-pteridine and *N*-methylaminobenzoic acid parts of the original MTX, the ions m/z 175 and 134 suggested that those parts were unchanged, and m/z 308 comforted both were unchanged. In contrast, TP440 HCD spectrum presented m/z 175 ion (thus intact diamino pteridine group), but m/z 294 and 120 instead of m/z 308 and 134. The −14 Da shift was proved to be due to a CH_2 difference from MTX according to the elemental composition. This was thus due to the demethylation of the N-methyl bridge. Accordingly, TP266, which presents a 14 Da mass lower than TP280 and a m/z 120 ion, should be 2-(4-aminobenzamido)pentanedioic acid and results from N-demethylation of TP280 (Calza et al. 2014). The TP408b also presented the fragments m/z 294 and 120 showing its demethylation of the *N*-methyl bridge; its identification will be presented below. The lower mass TPs corresponding to pteridine derivatives, TP206 and TP208, were identified according to their positive mass spectra as corresponding to already known structures (Lutterbeck et al. 2015; Calza et al. 2014; Lai et al. 2017; Kosjek et al. 2015; Kanjal et al. 2020). Both spectra contained the same m/z 163 ion ($C_6H_7N_6^+$) resulting either from loss of CO_2 for TP206 or successive losses of H_2O (m/z 191) and CO for TP208. These two pteridine derivatives could explain the lag phase observed in the kinetic studies and explained by a self-sensitized degradation mechanism (Hsu et al. 2019). Indeed, in the first hour of MTX

degradation, the abundance of these two TPs was higher under 300-450 nm pH 7 than under 254 nm pH 7.

Only ESI-positive mode is generally studied, which appeared to be more sensitive and suitable for both the parent compound and for most of the photogenerated products (Calza et al. 2014). However, the fragmentation in negative mode gives useful information about the glutamic acid part. For instance, TP280, $C_{13}H_{16}N_2O_5$, could eliminate consecutively two CO_2 molecules, forming m/z 235 and then m/z 191 ions. As observed for MTX, a rearrangement can lead to the formation of ion m/z 150. These two fragmentation pathways indicate that the glutamic part of TP280 is unchanged, leading to its identification as 2-(4-(methylamino)benzamido)pentanedioic acid. TP250, whose chemical formula indicates that it differs from TP280 with a loss of CH_2O, can also be identified on the basis of its fragments in negative mode. The fragment m/z 149 indicates that the methylamino part was conserved, as this ion is 4-(methylamino)benzoylamide. The loss concerned the glutamic acid part, as proven by the ion 4-oxobut-2-enoate at m/z 99. Such ions m/z 99 and 149 were also observed in TP424a and TP424b spectra, indicating that both result from MTX oxidation, formally by loss of formaldehyde from the glutamic acid in α and γ position. The identification of TP250, TP424a and TP424b are of Schymanski level 3, as no indication is available to attribute α or γ position. Two isomers TP408a and TP408b were also identified at level 3. The compound TP408a resulted from MTX loss of HCOOH. Its isomer TP408b, N-demethylated according to the positive mode, presented the same m/z 363, 321, 189 and 174 ions as TP408a, but also as major ions m/z 309 and 267 (absent in the TP408a spectrum). Those ions indicate that the glutamic acid part of MTX was in TP408b transformed into amino-pentenoic acid (Figure 3.1- S3 and Figure 3.1-S4). Finally, TP324, which presented the fragments m/z 149 and 174 previously mentioned, was identified (at level 2b) as the 4-(((2,4-diaminopteridin-6-yl)methyl)(methyl)amino)benzamide.

Among these eleven identified TPs presented in (Figure 3.1- 4), only six have been evidenced previously (Calza et al. 2014; Kosjek et al. 2015; Lutterbeck et al. 2015; or Lai et al. 2017; Kanjal et al. 2020): TP206, TP208, TP280, TP266, TP324 and TP440. In summary, three degradation pathways can be distinguished: N-demethylation of the bridge and C-N cleavage between p-methylaminobenzoyl and diaminopteridinemethylene groups, previously known, and glutamic acid oxidation evidenced in this study.

Table 3.1- 1 Accurate mass measurements in the positive and negative modes determined by LC-Q-Orbitrap-MS in MS or MS/MS mode

	Positive mode					Negative mode				
compound	nominal ion mass	formula	$[M+H]^+$ accurate mass	$[M+H]^+$ theoretical	error (ppm)	nominal ion mass	formula	$[M-H]^-$ accurate mass	$[M-H]^-$ theoretical	error (ppm)
TP 206	207	$C_7H_7N_6O_2^+$	207.0621	207.0625	1.93					
	163	$C_6H_7N_6^+$	163.0724	163.0727	1.84					
TP 208	209	$C_7H_9N_6O_2^+$	209.0778	209.0782	1.91					
	191	$C_7H_7N_6O^+$	191.0673	191.0676	1.57					
	163	$C_6H_7N_6^+$	163.0724	163.0727	1.84					
TP 250	251	$C_{12}H_{15}N_2O_4^+$	251.1026	251.1026	0.00	249	$C_{12}H_{13}N_2O_4^-$	249.0881	249.0881	0.00
	134	$C_8H_8NO^+$	134.0600	134.0606	4.48	149	$C_8H_9N_2O^-$	149.0715	149.0720	3.35
						99	$C_4H_3O_3^-$	99.0082	99.0088	6.06
TP 266	267	$C_{12}H_{15}N_2O_5^+$	267.0968	267.0976	3.00	265	$C_{12}H_{13}N_2O_5^-$	265.0830	265.0830	0.00
	120	$C_7H_6NO^+$	120.0445	120.0444	-0.83	221	$C_{11}H_{13}N_2O_3^-$	221.0927	221.0926	-0.45
	92	$C_6H_6N^+$	92.0500	92.0495	-5.43	128	$C_5H_6NO_3^-$	128.0343	128.0348	3.91
						177	$C_{10}H_{13}N_2O^-$	177.1026	177.1028	1.13
						135	$C_7H_7N_2O^-$	135.0554	135.0558	2.96
						92	$C_6H_6N^-$	92.0493	92.0500	7.60
TP 280	281	$C_{13}H_{17}N_2O_5^+$	281.1124	281.1132	2.85	279	$C_{13}H_{15}N_2O_5^-$	279.0985	279.0987	0.72
	134	$C_8H_8NO^+$	134.0601	134.0600	-0.75	235	$C_{12}H_{15}N_2O_3^-$	235.1085	235.1088	1.28
						191	$C_{11}H_{15}N_2O^-$	191.1183	191.1190	3.66
						150	$C_8H_8NO_2^-$	150.0551	150.0561	6.66
TP 324	325	$C_{15}H_{17}N_8O^+$	325.1520	325.1520	-0.06	323	$C_{15}H_{15}N_8O^-$	323.1374	323.1374	0.09
	308	$C_{15}H_{14}N_7O^+$	308.1254	308.1254	0.10	281	$C_{14}H_{13}N_6O^-$	281.1151	281.1151	-0.07
	175	$C_7H_7N_6^+$	175.0727	175.0727	-0.17	174	$C_7H_6N_6^{-\bullet}$	174.0654	174.0654	-0.06
	160	$C_7H_6N_5^+$	160.0618	160.0618	-0.19	149	$C_8H_9N_2O^-$	149.0715	149.0720	3.62
	134	$C_8H_8NO^+$	134.0600	134.0600	0.30	133	$C_8H_7NO^{-\bullet}$	133.0528	133.0533	3.83
TP 408a	409	$C_{19}H_{21}N_8O_3^+$	409.1721	409.1731	2.44	407	$C_{19}H_{19}N_8O_3^-$	407.1588	407.1586	-0.49
	308	$C_{15}H_{14}N_7O^+$	308.1251	308.1254	0.97	321	$C_{17}H_{17}N_6O^-$	321.1473	321.1469	-1.25
	175	$C_7H_7N_6^+$	175.0727	175.0727	0.00	363	C18H19N8O-	363.1692	363.1687	-1.38
	134	$C_8H_8NO^+$	134.0602	134.0600	-1.49	189	$C_{11}H_{13}N_2O^-$	189.1028	189.1033	2.64
						174	C7H6N6•-	174.0654	174.0654	0.00
TP 408b	409	$C_{19}H_{21}N_8O_3^+$	409.1721	409.1731	2.44	407	$C_{19}H_{19}N_8O_3^-$	407.1588	407.1586	-0.49
	294	$C_{14}H_{12}N_7O^+$	294.1090	294.1098	2.72	363	$C_{18}H_{19}N_8O^-$	363.1689	363.1687	-0.55
	175	$C_7H_7N_6^+$	175.0722	175.0727	2.86	321	$C_{17}H_{17}N_6O^-$	321.1468	321.1469	0.31
	120	$C_7H_6NO^+$	120.0445	120.0444	-0.83	309	$C_{14}H_{13}N_8O^-$	309.1219	309.1218	-0.32
						267	$C_{13}H_{11}N_6O^-$	267.1001	267.1000	-0.37
						189	$C_{11}H_{13}N_2O^-$	189.1027	189.1033	3.17
						174	$C_7H_6N_6^{\bullet-}$	174.0653	174.0659	3.45
						99	$C_4H_3O_3^-$	99.0076	99.0088	12.12
TP 424a	425	$C_{19}H_{21}N_8O_4^+$	425.1667	425.1680	3.06	423	$C_{19}H_{19}N_8O_4^-$	423.1539	423.1535	-0.95
	308	$C_{15}H_{14}N_7O^+$	308.1249	308.1254	1.62	323	$C_{15}H_{15}N_8O^-$	323.1376	323.1374	-0.62
	175	$C_7H_7N_6^+$	175.0726	175.0727	0.57	281	$C_{14}H_{13}N_6O^-$	281.1158	281.1156	-0.71
	134	$C_8H_8NO^+$	134.0601	134.0600	-0.75	174	$C_7H_6N_6^{\bullet-}$	174.0653	174.0654	0.57
						149	$C_8H_9N_2O^-$	149.0712	149.0720	5.37
						99	$C_4H_3O_3^-$	99.0077	99.0088	11.11
TP424b	425	$C_{19}H_{21}N_8O_4^+$	425.1672	425.1680	1.88	423	$C_{19}H_{19}N_8O_4^-$	423.1539	423.1535	-0.95
	308	$C_{15}H_{14}N_7O^+$	425.1672	425.1680	1.88	323	$C_{15}H_{15}N_8O^-$	323.1375	323.1374	-0.31
	175	$C_7H_7N_6^+$	308.1248	308.1254	1.95	281	$C_{14}H_{13}N_6O^-$	281.1158	281.1156	-0.71
	134	$C_8H_8NO^+$	175.0725	175.0727	1.14	174	$C_7H_6N_6^{\bullet-}$	174.0653	174.0654	0.57
						149	$C_8H_9N_2O^-$	149.0711	149.0720	6.04
						99	$C_4H_3O_3^-$	99.0076	99.0088	12.12
TP 440	441	$C_{19}H_{21}N_8O_5^+$	441.1628	441.1629	0.23	439	$C_{19}H_{19}N_8O_5^-$	439.1476	439.1484	1.82

	294	$C_{14}H_{12}N_7O^+$	294.1092	294.1098	2.04	395	$C_{18}H_{19}N_8O_3^-$	395.1583	395.1586	0.76
	175	$C_7H_7N_6^+$	175.0723	175.0727	2.28	363	$C_{18}H_{19}N_8O^-$	363.1685	363.1687	0.55
	120	$C_7H_6NO^+$	120.0445	120.0444	-0.83	321	$C_{17}H_{17}N_6O^-$	321.1468	321.1469	0.31
						310	$C_{14}H_{12}N_7O_2^-$	310.1058	310.1058	0.00
						266	$C_{13}H_{12}N_7^-$	266.1159	266.1160	0.38
						221	$C_{11}H_{13}N_2O_3^-$	221.0926	221.0926	0.00
						189	$C_{11}H_{13}N_2O^-$	189.1026	189.1033	3.70
						177	$C_{10}H_{13}N_2O^-$	177.1024	177.1028	2.26
						128	$C_5H_6NO_3^-$	128.0343	128.0353	7.81
MTX	455	$C_{20}H_{23}N_8O_5^+$	455.1770	455.1786	3.52	453	$C_{20}H_{21}N_8O_5^-$	453.1634	453.1640	1.32
(454 Da)	308	$C_{15}H_{14}N_7O^+$	308.1249	308.1254	1.62	435	$C_{20}H_{19}N_8O_4^-$	435.1530	435.1535	1.15
	175	$C_7H_7N_6^+$	175.0725	175.0727	1.14	409	$C_{19}H_{21}N_8O_3^-$	409.1743	409.1742	-0.24
	134	$C_8H_8NO^+$	134.0600	134.0600	0.00	367	$C_{18}H_{19}N_6O_3^-$	367.1523	367.1524	0.27
						324	$C_{15}H_{14}N_7O_2^-$	324.1216	324.1215	-0.31
						280	$C_{14}H_{14}N_7^-$	280.1319	280.1316	-1.07
						235	$C_{12}H_{15}N_2O_3^-$	235.1087	235.1088	0.43
						191	$C_{11}H_{15}N_2O^-$	191.1185	191.1190	2.62
						189	$C_{11}H_{13}N_2O^-$	189.1028	189.1033	2.64
						174	$C_7H_6N_6^{\bullet-}$	174.0653	174.0654	0.57
						149	$C_8H_7NO_2^{\bullet-}$	149.0474	149.0482	5.37

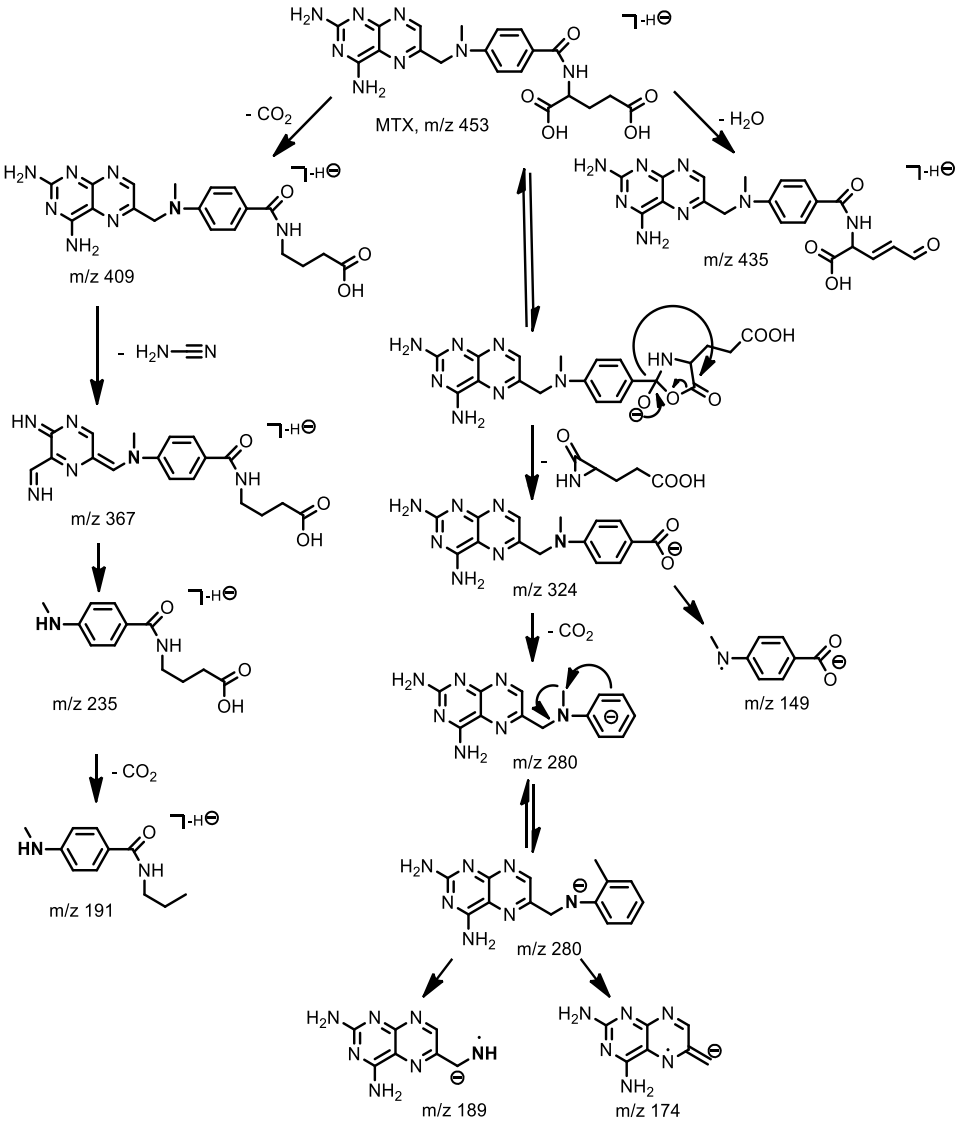


Figure 3.1- 3 Methotrexate fragmentation pattern from deprotonated parent ion

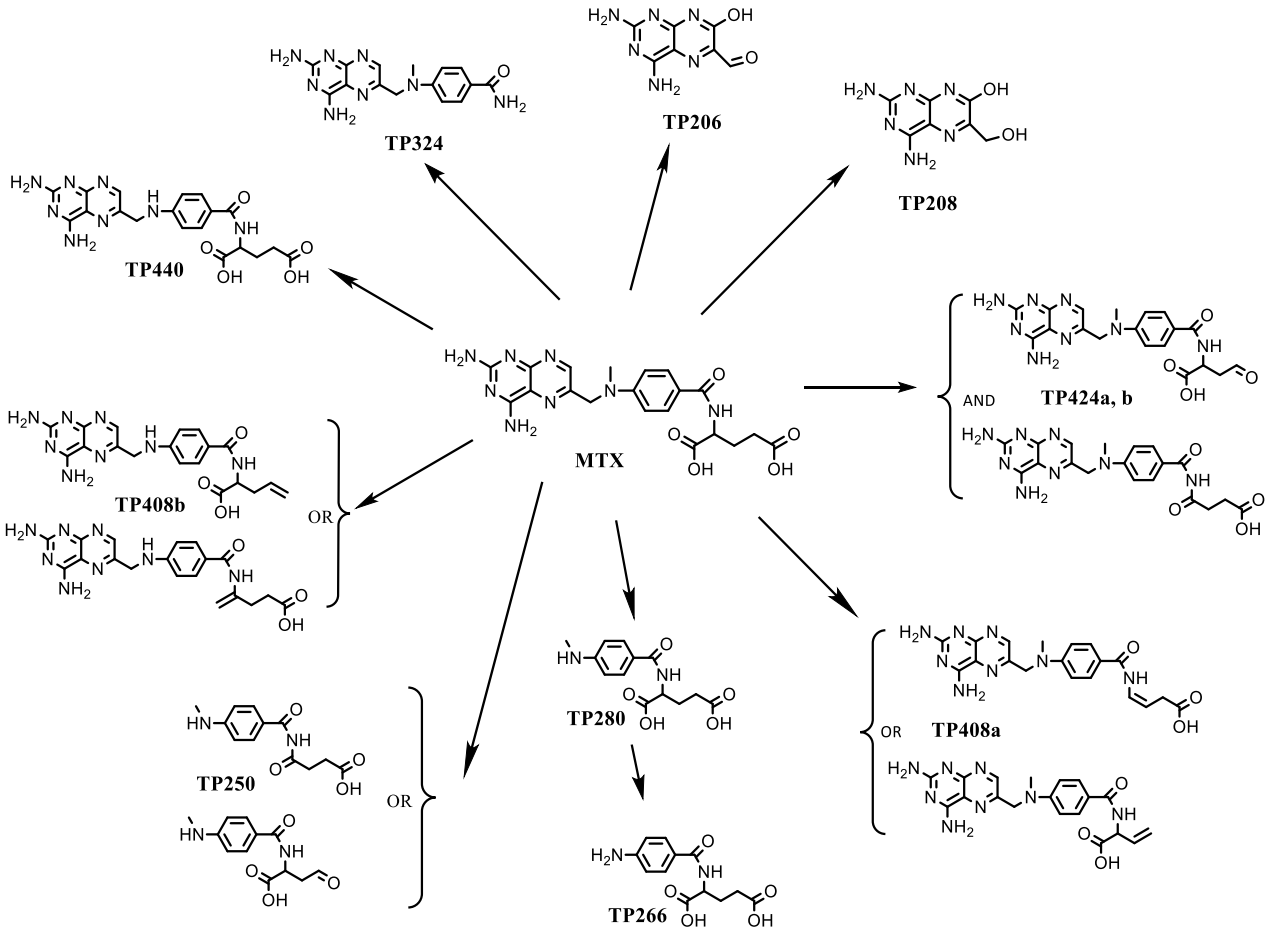


Figure 3.1- 4 Proposed structures of the transformation products of MTX under irradiation

3.1.4.3. Evolution of by-products and cytotoxicity at pH 7

The IC_{50} of MTX against A549 cancer cells was determined as 120 ± 20.0 nM. Consistently, at 100 nm and before photodegradation, the viability of cancer cells was close to 50% (Figure 3.1- 5). Then, for both irradiation wavelengths, the viability increased as the percentage of MTX decreased, indicating that TPs formed during irradiation at pH 7 were less cytotoxic than the parent compound. For the 300-450 nm treatment, the two pteridine derivatives, TP208 and TP206, were the main photoproducts reaching up to 31 and 92 % of residual MTX, respectively. While MTX fully disappeared after 3 h of irradiation, both TPs persisted in solution, even if TP208 decreased. Meanwhile, the viability reached 100%, showing the TPs innocuity on A549 cancer cells (Figure 3.1- 5a). At 254 nm, in addition to these same TP208 and TP206 appearing in a lower abundance than at 300-450 nm (both around 30%), TP424 also accumulated after 5 h of irradiation (with an equivalent of 18% of residual MTX). The slower degradation of MTX and the presence of TP424 could explain that complete viability was not retrieved (90.61 ± 4.18%) (Figure 3.1- 5b). TP280, TP266 and TP250 were not detected with the irradiation at pH 7, but this monitoring

was done without preconcentration step (by SPE) therefore their presence at trace level cannot be excluded.

Calza et al. (2014) demonstrated that TPs formed during photodegradation in pure water were less toxic than MTX and identified TP208 as a major photoproduct. Lutterbeck et al. (2015) concluded that the intermediates formed during the reaction were less toxic than the parent compound, which included TP206 and TP208. However, these results were obtained in *Vibrio Fischeri* bioassays and did not focus on the anticancer activity of MTX as proposed herein.

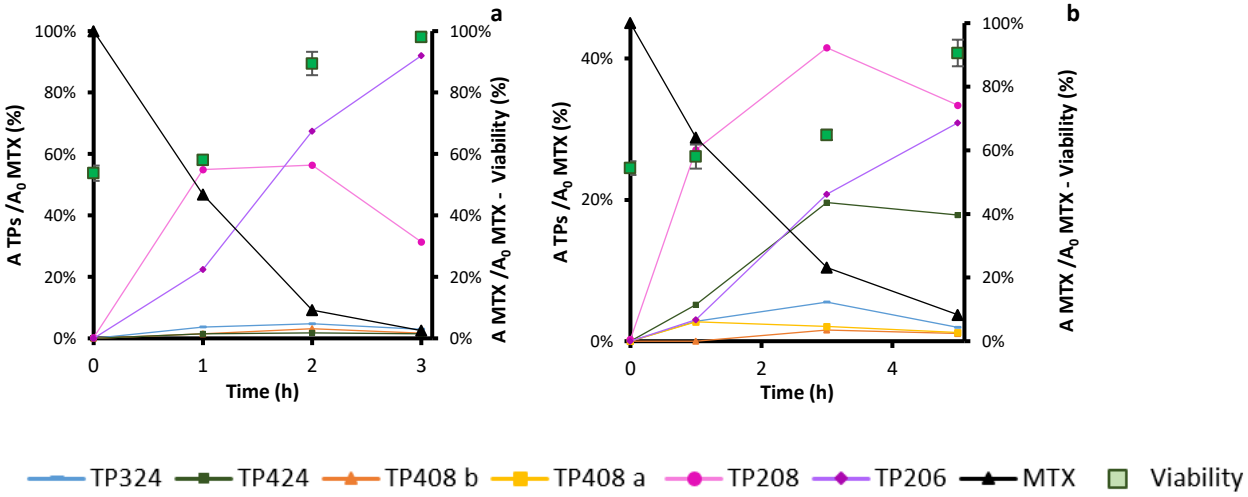


Figure 3.1- 5 Viability evolution (%) (green square) of cancer cells, against MTX (black line) and TPs (colored lines) evolution as function of the irradiation time: a) 300-450 nm pH 7 b) 254 nm pH 7

3.1.5. Conclusion

Under different irradiation conditions, MTX photodegradation can produce a large variety of TPs. The kinetics showed that photodegradation was the fastest under environmental conditions (300–450 nm, pH 7) with only 7% of residual MTX after 4 h of irradiation. Nevertheless, not only the residual MTX is of environmental concern and several TPs were monitored and characterized. LC-HRMS allowed identifying eleven photoproducts, including five compounds, which were not mentioned in previous studies. The identification of these uncommon TPs was allowed by the use of mass spectrometry in the negative mode and evidenced the important oxidation degradation pathway of the glutamic acid part. The decrease in cytotoxicity during MTX degradation demonstrated that TPs formed in pure water at pH 7 are less cytotoxic than the parent compound. Nevertheless, the well-known TPs of MTX, DAMPA and 7-OH-MTX, were not observed in our irradiation conditions.

Even though our experiments attempt to mimic the photodegradation that occurs in natural water or wastewater treatment, one needs to be aware that the formation of TPs highly depends on the composition of water, as well as the concentration of the parent compound. It is therefore well possible

that MTX phototransformation products identified in our study appeared only as transient species in other aqueous matrices than pure water. Therefore, further investigations are necessary to estimate the occurrence of the identified TPs and their effects in natural and waste waters. This implies to further develop analytical methods to identify and quantify these TPs in natural matrices.

3.1.6. Supplementary information

Table 3.1- S1 Physico-chemical and Biological properties of MTX

Property	Value	Reference*
Formula	$C_{20}H_{22}N_8O_5$	
CAS	59-05-2	
Molecular mass (g.mol-^{1})	454.44	
pKa	4.7	*PubChem**
	3.36 – 4.7 – 5.71	*Rubino et al. 2001*
	5.71 - 4.70 -3.36	*Poe et al. 1977*
Log Kow	-1,85	*PubChem**
Solubility (mg.L^{-1})	2600	*PubChem**
	409 *(pH 5)* – 9080 *(pH 7)***	*Rubino et al.2001*
Henry's law constant (atm-cu m.mol^{-1})	1.54 x 10^{-31}	*PubChem**
Vapor pressure (mm Hg)	2.1 x 10^{-19}	*PubChem**
Koc	1	*PubChem**
% Excretion unchanged	81	*Benet et al. 2011*

*https://pubchem.ncbi.nlm.nih.gov/compound/126941#section=Environmental-Fate-Exposure-Summary
** Calculated from µM values indicated in Rubino et al. (2001)

Irradiation conditions

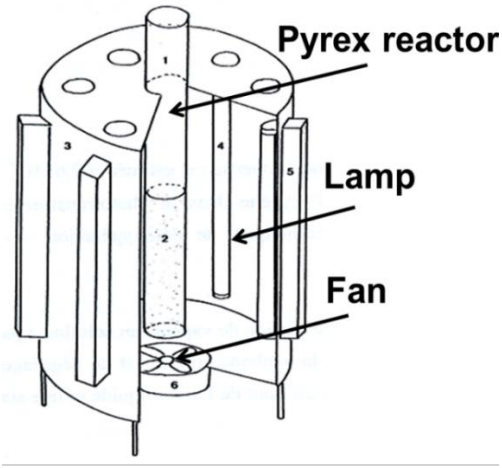


Figure 3.1- S1 Photoreactor setup used for 300-450 nm irradiations, showing the six Phillips TLD 15W lamps surrounding the Pyrex glass reactor.

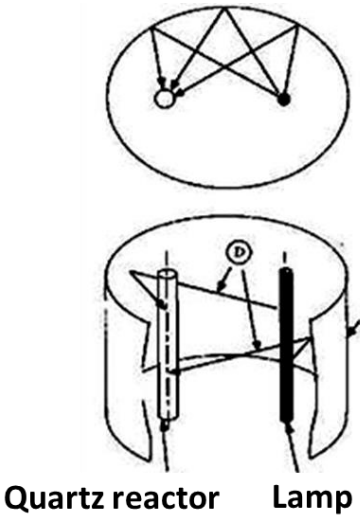


Figure 3.1- S2 Photoreactor setup used for 254 nm irradiations, showing the Phillips TUV 15W lamp and the quartz reactor located at the two focal points of the elliptical metallic device (and the potential trajectories of light rays

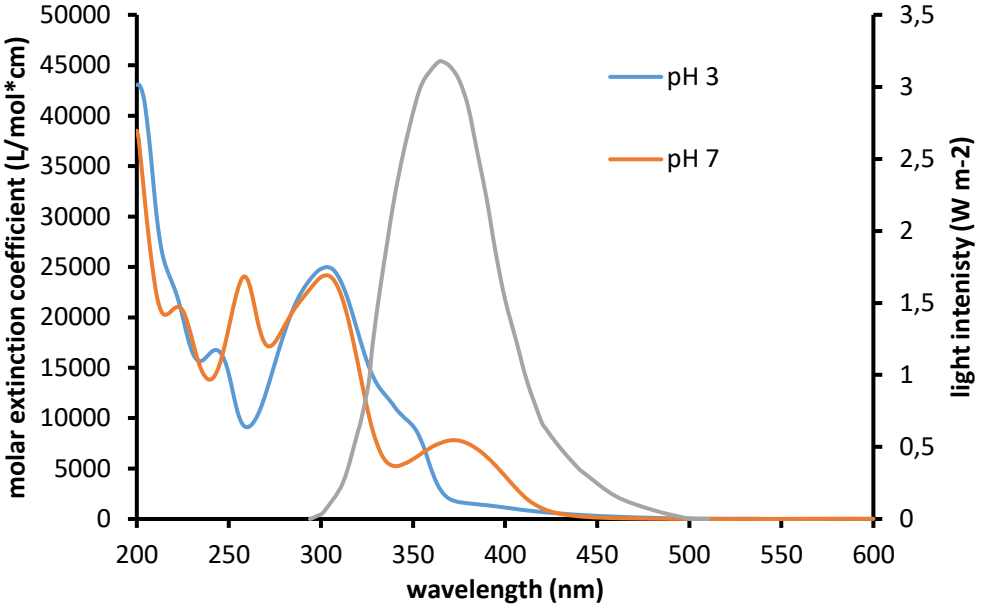


Figure 3.1- S1 Methotrexate absorption spectra at pH 7 and 3, and emission spectrum of the polychromatic lamps.

Calculation of the Quantum yields (ϕ): Equations S1 To S5:

- **For 254 nm**

At the single wavelength 254 nm, the quantum yields ‧(254) were calculated using the following formula S1-S3:

$$\phi(254) = L \,.\, \frac{\Delta C \,.\, \mathcal{N}a}{t \,.\, Io \,.\, (1-10^{-A})}$$ **Equation S1**

With:
L = the optical path length in cm (L = 2 cm in our system)
ΔC as the percentage of residual methotrexate (>25%)
$\mathcal{N}a$ = Number of Avogadro = 6.02 × 10^{23} molecules
t of this residual % (seconds)

A was given by Beer lambert law:

$$A = \varepsilon \,.\, L \,.\, C$$ **Equation S2**

With:
$\varepsilon_i(\lambda)$ = Methotrexate decadic molar extinction coefficient at wavelength 254 nm, in $mol^{-1}.L.cm^{-1}$ (Fig S.3)
L = the optical path length in cm
C = the initial concentration of methotrexate in $mol.L^{-1}$

I_o = the lamp irradiance, $I_0(\lambda)$ was measured at 254 nm in mW/cm^2 and converted in $photon.cm^{-2}.s^{-1}$ as follows:

$$I_0 = \frac{I_0\,(\lambda) measured \cdot \lambda}{h \cdot c}$$ **Equation S3**

With
h = the Plank constant (6.626 × 10^{-34} J.s)
c = the speed of light (3 × 10^8 $m.s^{-1}$)
λ = the wavelength in m (254 × 10^{-9} m)

- **For 300-450 nm**

Over the wavelength range 300-450 nm, the quantum yields ‧(‧) were calculated using the following formula S4-S6:

$$\phi(\lambda) = \frac{k}{k_a(\lambda)}$$ **Equation S4**

With k as the methotrexate pseudo first-order phototransformation rate constant obtained in the experiments, and $k_a(\lambda)$ as the specific rate of light absorption at a given wavelength λ, calculated according to the simplified equation proposed for shallow waters and applicable in the case of our experimental conditions (low absorption because of short path length (1.5 cm) and low solution absorption (Schwarzenbach et al. 2003):

$$k_a(\lambda) = 2.3 \cdot \frac{I_0}{\mathrm{N}a} \cdot \varepsilon(\lambda)$$ **Equation S5**

With 2.3 = approximation factor suggested by Schwarzenbach et al. (2003)

The lamp irradiance, $I_0(\lambda)$ was measured at 312 and 365 nm, in mW/cm^2, converted as previously (equation S3) and multiplied by the number of lamps (n=6). As expected, the value at 312 nm represented approximately 8% of the value at 365 nm. Therefore, the irradiance at the other wavelengths was estimated from the known spectrum of the lamp, represented as a relative proportion of the value obtained at 365 (set at 100%). As our lamp emitted between 290 and 450 nm, the $k_a(\lambda)$ spectrum was integrated by the trapezoidal rule over the range and divided by the wavelength range.

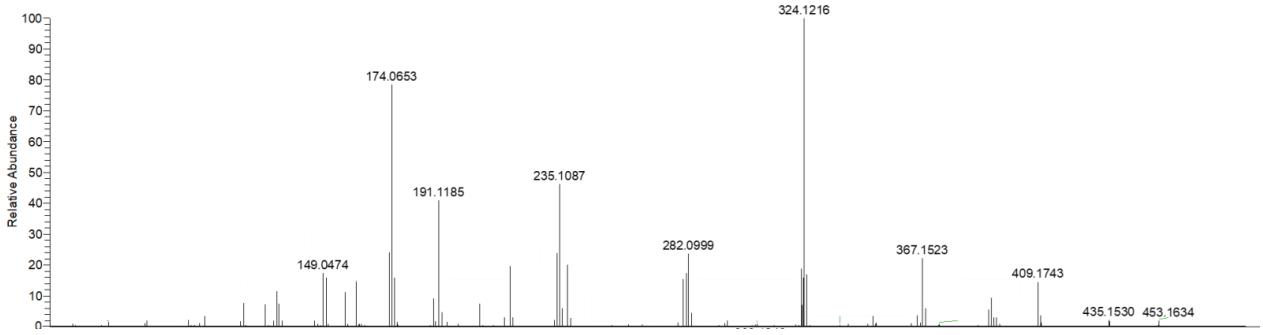
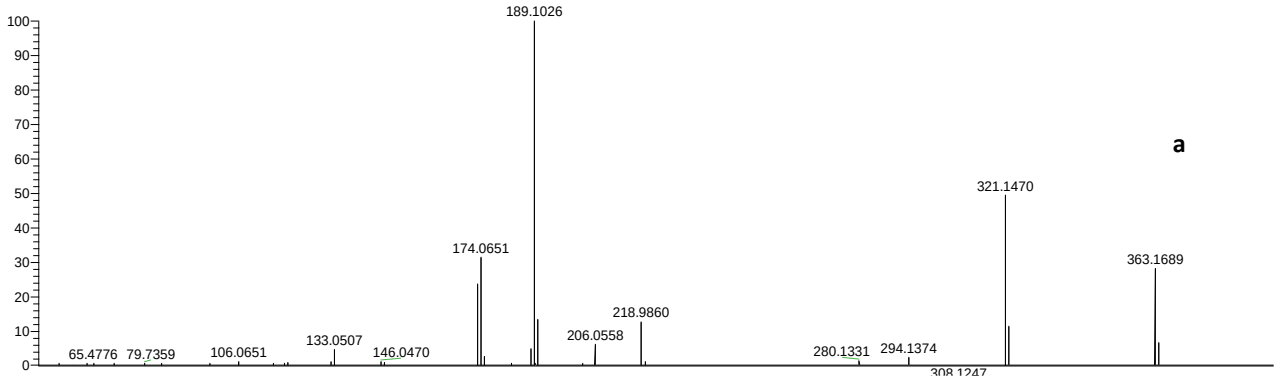


Figure 3.1- S2 Daughter ion spectrum (MS^2) of $[M - H]^-$ Methotrexate pseudo-molecular ion



b

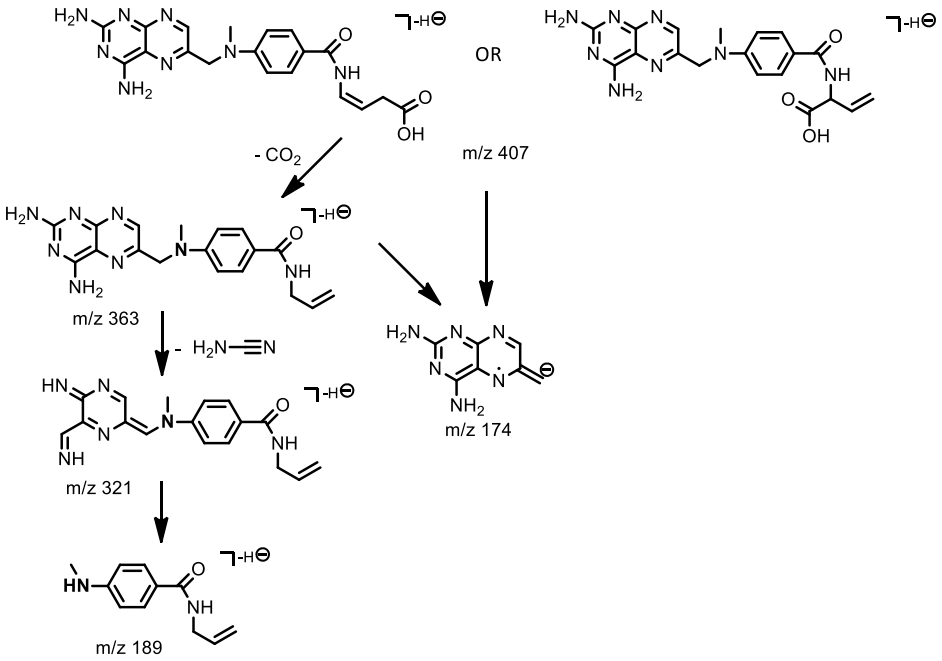


Figure 3.1- S3 Negative mode for the identification of TP 408a:

a) Daughter ion spectrum (MS^2) of $[M - H]^-$ TP408a pseudo-molecular ion (m/z 407)

b) Fragmentation pattern from deprotonated parent ion

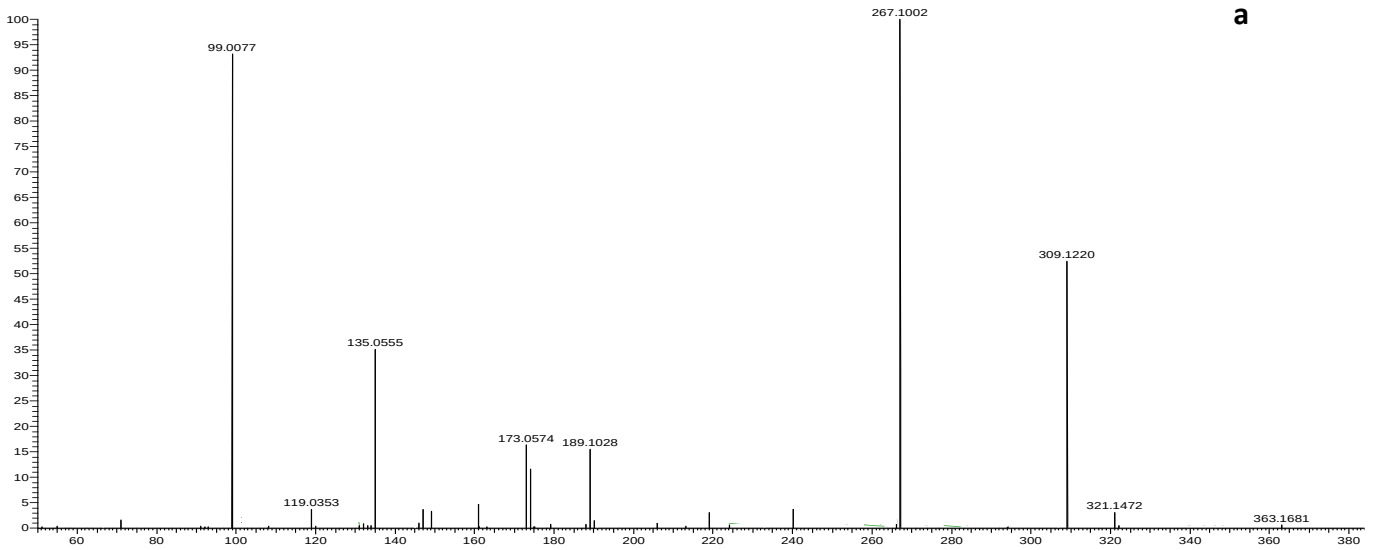


Figure 3.1- S4 Negative mode for the identification of TP 408b:

a) Daughter ion spectrum (MS^2) of $[M - H]^-$ TP408b pseudo-molecular ion (m/z 407)

b) Fragmentation pattern from deprotonated parent ion

3.1.7. References

Alinejad A, Akbari H, Ghaderpoori M, Jeihooni A.K, Adibzadeh A (2019) Catalytic ozonation process using a MgO nano-catalyst to degrade methotrexate from aqueous solutions and cytotoxicity studies in human lung epithelial cells (A549) after treatment. RSC Adv 9:8204-8214. https://doi.org/10.1039/C9RA00320G

aus der Beek T, Weber FA, Bergmann A, Hickmann S, Ebert I, Hein A, Küster A (2016) Pharmaceuticals in the environment-Global occurrences and perspectives: Pharmaceuticals in the global environment. Environ Toxicol Chem 35:823–835. https://doi.org/10.1002/etc.3339

Benet LZ, Broccatelli F, Oprea T (2011) BDDCS applied to over 900 drugs. The AAPS J 13:519-47. https://doi.org/10.1208/s12248-011-9290-9

Benotti MJ, Trenholm RA, Vanderford BJ, Holady JC, Stanford BD, Snyder SA (2009) Pharmaceuticals and Endocrine Disrupting Compounds in U.S. Drinking Water. Environ Sci Technol 43:597–603. https://doi.org/10.1021/es801845a

Besse JP, Latour JF, Garric J (2012) Anticancer drugs in surface waters. Environ Internat 39:73–86. https://doi.org/10.1016/j.envint.2011.10.002

Breithaupt H, Küenzlen E, Goebel G (1982) Rapid high-pressure liquid chromatographic determination of methotrexate and its metabolites 7-hydroxymethotrexate and 2,4-diamino-N10-methylpteroic acid in biological fluids. Anal Biochem 121:103–113. https://doi.org/10.1016/0003-2697(82)90562-0

Buerge IJ, Buser HR, Poiger T, Müller MD (2006) Occurrence and Fate of the Cytostatic Drugs Cyclophosphamide and Ifosfamide in Wastewater and Surface Waters. Environ Sci Technol 40:7242–7250. https://doi.org/10.1021/es0609405

Calza P, Medana C, Sarro M, Rosato V, Aigotti R, Baiocchi C, Minero C (2014) Photocatalytic degradation of selected anticancer drugs and identification of their transformation products in water by liquid chromatography–high resolution mass spectrometry. J Chromatogr A 1362:135–144. https://doi.org/10.1016/j.chroma.2014.08.035

Castiglioni S, Bagnati R, Calamari D, Fanelli R, Zuccato E (2005) A multiresidue analytical method using solid-phase extraction and high-pressure liquid chromatography tandem mass spectrometry to measure pharmaceuticals of different therapeutic classes in urban wastewaters. J Chromatogr A 1092:206–215. https://doi.org/10.1016/j.chroma.2005.07.012

Catastini C, Mullot JU, Boukari S, Mazellier P, Levi Y, Cervantes P, Ormsby JN (2008) Identification de molécules anticancéreuses dans les effluents hospitaliers. Eur J Water Qual 39:171–180. https://doi.org/10.1051/water/2008004

Chatterji DC, Gallelli JF (1977) High-pressure liquid chromatographic analysis of methotrexate in presence of its degradation products. J Pharm Sci 66:1219–1222. https://doi.org/10.1002/jps.2600660904

Chowdhury P, Sarathy SR, Das S, Li J, Ray AK, Ray MB (2020) Direct UV photolysis of pharmaceutical compounds: Determination of pH-dependent quantum yield and full-scale performance. Chem Eng J 380:122460. https://doi.org/10.1016/j.cej.2019.122460

Donehower RC, Hande KR, Drake JC, Chabner BA (1979) Presence of 2,4-diamino-N ^{10} -methylpteroic acid after high-dose methotrexate. Clin Pharmacol Ther 26:63–72. https://doi.org/10.1002/cpt197926163

Escher BI, Fenner K (2011) Recent Advances in Environmental Risk Assessment of Transformation Products. Environ Sci Technol 45:3835–3847. https://doi.org/10.1021/es1030799

Ferrando-Climent L, Rodriguez-Mozaz S, Barceló D (2013) Development of a UPLC-MS/MS method for the determination of ten anticancer drugs in hospital and urban wastewaters, and its application for the screening of human metabolites assisted by information-dependent acquisition tool (IDA) in sewage samples. Anal Bioanal Chem 405:5937–5952. https://doi.org/10.1007/s00216-013-6794-4

Garcia-Ac A, Segura PA, Gagnon C, Sauvé S (2009a) Determination of bezafibrate, methotrexate, cyclophosphamide, orlistat and enalapril in waste and surface waters using on-line solid-phase extraction liquid chromatography coupled to polarity-switching electrospray tandem mass spectrometry. J Environ Monit 11:830-838. https://doi.org/10.1039/b817570e

Garcia-Ac A, Segura PA, Viglino L, Fürtös A, Gagnon C, Prévost M, Sauvé S (2009b) On-line solid-phase extraction of large-volume injections coupled to liquid chromatography-tandem mass spectrometry for the quantitation and confirmation of 14 selected trace organic contaminants in drinking and surface water. J Chromatogr A 1216:8518–8527. https://doi.org/10.1016/j.chroma.2009.10.015

González-Burciaga LA, García-Prieto JC, García-Roig M, Lares-Asef I, Núñez-Núñez CM, Proal-Nájera JB (2021) Cytostatic drug 6-mercaptopurine degradation on pilot scale reactors by advanced oxidation processes: UV-C/H2O2 and UV-C/TiO2/H2O2 kinetics. Catalysts 11:567. https://doi.org/10.3390/catal11050567

Henschel KP, Wenzel A, Diedrich M, Fliedner A (1997) Environmental Hazard Assessment of Pharmaceuticals. Regul Toxicol Pharmacol 25:220–225. https://doi.org/10.1006/rtph.1997.1102

Hsu MH, Tsai CJ, Lin AYC (2019) Mechanism and pathways underlying the self-sensitized photodegradation of methotrexate under simulated solar irradiation. J Hazard Mater 373:468–475. https://doi.org/10.1016/j.jhazmat.2019.03.095

Isidori M, Lavorgna M, Russo C, Kundi M, Žegura B, Novak M, Filipič M, Mišík M, Knasmueller S, de Alda ML, Barceló D, Žonja B, Česen M, Ščančar J, Kosjek T, Heath E (2016) Chemical and toxicological characterisation of anticancer drugs in hospital and municipal wastewaters from Slovenia and Spain. Environ Pollut 219:275–287. https://doi.org/10.1016/j.envpol.2016.10.039

Jacobs SA, Stoller RG, Chabner BA, Johns DG (1976) 7-Hydroxymethotrexate as a urinary metabolite in human subjects and rhesus monkeys receiving high dose methotrexate. J Clin Invest 57:534–538. https://doi.org/10.1172/JCI108308

Kanjal MI, Muneer M, Abdelhaleem A, Chu W (2020) Degradation of methotrexate by UV/Peroxymonosulfate: kinetics, effect of operational parameters and mechanism. Chin J Chem Eng 28:2658-2667. https://doi.org/10.1016/j.cjche.2020.05.033

Kiffmeyer T, Götze HJ, Jursch M, Lüders U (1998) Trace enrichment, chromatographic separation and biodegradation of cytostatic compounds in surface water. Fresenius J Anal Chem 361:185–191. https://doi.org/10.1007/s002160050859

Kosjek T, Heath E (2011) Occurrence, Fate and determination of cytostatic pharmaceuticals in the environment. Trends Anal Chem TrAC 30:1065-1087. https://doi.org/10.1016/j.trac.2011.04.007

Kosjek T, Negreira N, de Alda ML, Barceló D (2015) Aerobic activated sludge transformation of methotrexate: Identification of biotransformation products. Chemosphere 119:S42–S50. https://doi.org/10.1016/j.chemosphere.2014.04.081

Lai WWP, Hsu MH, Lin AYC (2017) The role of bicarbonate anions in methotrexate degradation via UV/TiO_2: Mechanisms, reactivity and increased toxicity. Water Res 112:157–166. https://doi.org/10.1016/j.watres.2017.01.040

Lin AYC, Lin YC, Lee WN (2014) Prevalence and sunlight photolysis of controlled and chemotherapeutic drugs in aqueous environments. Environ Pollut 187:170–181. https://doi.org/10.1016/j.envpol.2014.01.005

Lutterbeck CA, Baginska E, Machado ÊL, Kümmerer K (2015) Removal of the anti-cancer drug methotrexate from water by advanced oxidation processes: Aerobic biodegradation and toxicity studies after treatment. Chemosphere 141:290–296. https://doi.org/10.1016/j.chemosphere.2015.07.069

Martín J, Camacho-Muñoz D, Santos JL, Aparicio I, Alonso E (2014) Occurrence and ecotoxicological risk assessment of 14 cytostatic drugs in wastewater. Water Air Soil Pollut 225:1896. https://doi.org/10.1007/s11270-014-1896-y

Mompelat S, Thomas O, Le Bot B (2011) Contamination levels of human pharmaceutical compounds in French surface and drinking water. J Environ Monit 13:2929-2939. https://doi.org/10.1039/c1em10335k

Morasch B, Bonvin F, Reiser H, Grandjean D, de Alencastro LF, Perazzolo C, Chèvre N, Kohn T (2010) Occurrence and fate of micropollutants in the Vidy Bay of Lake Geneva, Switzerland. Part II: micropollutant removal between wastewater and raw drinking water. Environ Toxicol Chem 29:1658-1668. https://doi.org/10.1002/etc.222.

Muneer M, Kanjal MI, Iqbal M, Saeed M, Khosa MK, Ud Den NZ, Ali S, Nazir A (2020) Gamma and UV radiations induced treatment of anti-cancer methotrexate drug in aqueous medium: effect of process variables on radiation efficiency evaluated using bioassays . Appl Radiat Isot 166:109371. https://doi.org/10.1016/j.apradiso.2020.109371.

Negreira N, de Alda ML, Barceló D (2014) Cytostatic drugs and metabolites in municipal and hospital wastewaters in Spain: Filtration, occurrence, and environmental risk. Sci Tot Environ 497–498:68–77. https://doi.org/10.1016/j.scitotenv.2014.07.101

Olalla A, Negreira N, López de Alda M, Barceló D, Valcárcel Y (2018) A case study to identify priority cytostatic contaminants in hospital effluents. Chemosphere 190:417-430. https://doi.org/10.1016/j.chemosphere.2017.09.129.

Orias F, Perrodin Y (2013) Characterisation of the ecotoxicity of hospital effluents: a review. Sci Tot Environ 454-455:250-276. https://doi.org/10.1016/j.scitotenv.2013.02.064.

Perazzolo C, Morasch B, Kohn T, Magnet A, Thonney D, Chèvre N (2010) Occurrence and fate of micropollutants in the Vidy Bay of Lake Geneva, Switzerland. Part I: Priority list for environmental risk assessment of pharmaceuticals. Environ Toxicol Chem. 29:1649-1657. https://doi.org/10.1002/etc.221.

Poe M (1977) Acidic dissociation constants of folic acid, dihydrofolic acid, and methotrexate. J Biol Chem 252:3724–3728. https://doi.org/10.1016/S0021-9258(17)40312-7

Roig B, Marquenet B, Delpla I, Bessonneau V, Sellier A, Leder C, Thomas O, Bolek R, Kummerer K (2014) Monitoring of methotrexate chlorination in water. Water Res 57:67–75. https://doi.org/10.1016/j.watres.2014.03.008

Rubino FM (2001) Separation methods for methotrexate, its structural analogues and metabolites. J Chromatogr B Biomed Sci App 764:217–254. https://doi.org/10.1016/S0378-4347(01)00402-9

Sabry SM, Abdel-Hady M, Elsayed M, Fahmy OT, Maher HM (2003) Study of stability of methotrexate in acidic solution Spectrofluorimetric determination of methotrexate in pharmaceutical preparations through acid-catalyzed degradation reaction. J Pharm Biomed Anal 32:409–423. https://doi.org/10.1016/S0731-7085(03)00239-5

Sastry RVRP, Venkatesan CS, Sastry BS, Mahesh K (2016) Identification and characterization of forced degradation products of pralatrexate injection by LC-PDA and LC–MS. J Pharm Biomed Anal 131:400–409. https://doi.org/10.1016/j.jpba.2016.08.023

Schwarzenbach RP, Gschwend PM, Imboden DM. (2003) Environmental Organic Chemistry, 2nd edn; John Wiley & Sons, Hoboken, pp 611-654.

Schymanski EL, Jeon J, Gulde R, Fenner K, Ruff M, Singer HP, Hollender J (2014) Identifying Small Molecules via High Resolution Mass Spectrometry: Communicating Confidence. Environ Sci Technol 48:2097–2098. https://doi.org/10.1021/es5002105

Secrétan PH, Karoui M, Levi Y, Sadou-Yayé H, Tortolano L, Solgadi A, Yagoubi N, Do B (2018) Pemetrexed degradation by photocatalytic process: Kinetics, identification of transformation products and estimation of toxicity. Sci Tot Environ 624:1082–1094. https://doi.org/10.1016/j.scitotenv.2017.12.182

Toński M, Dołżonek J, Stepnowski P, Białk-Bielińska A (2021) Hydrolytic stability of anticancer drugs and one metabolite in the aquatic environment. Environ Sci Pollut Res. https://doi.org/10.1007/s11356-021-14360-0.

Turci R, Sottani C, Spagnoli G, Minoia C (2003) Biological and environmental monitoring of hospital personnel exposed to antineoplastic agents: a review of analytical methods. J Chromatogr B 789:169–209. https://doi.org/10.1016/S1570-0232(03)00100-4

Vergili I, Kaya Y, Gönder ZB, Boergers A, Tuerk J (2019) Occurence and prioritization of pharmaceutical active compounds in domestic/municipal wastewater treatment plants. Bull Environ Contam Toxicol 102: 252-258. https://doi.org/10.1007/s00128-019-02550-z.

Widemann BC, Sung E, Anderson L, Salzer WL, Balis FM, Monitjo KS, McCully C, Hawkins M, Adamson PC (2000) Pharmacokinetics and metabolism of the methotrexate metabolite 2, 4-diamino-N(10)-methylpteroic acid. J Pharmacol Exp Ther 294:894–901.

Yin J, Shao B, Zhang J, Li K (2010) A Preliminary Study on the Occurrence of Cytostatic Drugs in Hospital Effluents in Beijing, China. Bull Environ Contam Toxicol 84:39–45. https://doi.org/10.1007/s00128-009-9884-4

Zounková R, Odráška P, Dole□alová L, Hilscherová K, Maršálek B, Bláha L (2007) Ecotoxicity and genotoxicity assessment of cytostatic pharmaceuticals. Environ Toxicol Chem 26:2208-2214. https://doi.org/10.1897/07-137R.1

3.2. Photodégradation du OMTX en solution aqueuse

Cette deuxième sous partie présente les résultats soumis dans Chemosphere. Le manuscrit présente la synthèse des différents analogues éco-conçus réalisée par l'ICMMO. L'OMTX est sélectionné pour son étude en photodégradation en comparaison au MTX.

Le format imposé par la revue conduit à un manuscrit court, qui se concentre sur l'irradiation 254 nm, la majorité des informations sur l'ensemble de l'étude est placé en Supplementary Information.

Le Supporting Information est présenté en partie 3.2.3 et comporte :

-La synthèse des analogues réalisée par l'ICMMO et placée en annexe 2, car elle ne fait pas partie du travail de la thèse.

-Le matériel et méthodes de l'ensemble des expérimentations, des informations complémentaires concernant les études de métabolisme hépatiques *in vitro* (menées par l'ICMMO), ainsi qu'une partie des résultats de la photodégradation sous 300-450 nm.

"Re-designing Environmentally Persistent Pharmaceutical Pollutant through programmed inactivation: the case of methotrexate"

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Keywords: Eco-design - Pharmaceutical pollutants – Photodegradation - Retro catabolic drug design - Structural identification.

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3.2.1. Abstract

Environmental emission of pharmaceutical pollutants notably causes the contamination of aquatic ecosystems and drinking water. Typically, reduction of these pollutants in the environment is mostly managed by ameliorated wastewater treatments. Here, we report a method for the eco-design of drugs through the introduction within the molecular structure of a sensitive chemical group responsive to water treatments. The new drugs are thus programmed to fragment more easily and quickly than the original drugs. In this "retro catabolic drug design" strategy, methotrexate was used as drug model and an ether analog displaying a similar pharmacological profile was selected. Using photo-irradiation experiments at 254 nm, a representative drinking water treatment process, the identified transformation products were predominantly obtained from the expected molecular scission. Moreover, a faster kinetics of degradation was measured for the ether analog as compared to methotrexate and its transformation products were completely devoid of the initial cytotoxic activity.

3.2.2. Article Manuscript

The presence of Active Pharmaceutical Ingredients (APIs) in the environment has been largely demonstrated, including in soil and soil water (Edwards et al., 2009; Wu et al., 2010; Gottschall et al., 2012), in surface water (ter Laak et al., 2010; Hughes et al., 2013), in groundwater (Lopez et al., 2015) potentially used to produce drinking water (Benotti et al., 2009; Fram and Belitz, 2011) and drinking water itself (Fick et al., 2009; Vulliet et al., 2011). Current approaches directed at reducing API levels in the environment have focused on pollution control, particularly improved wastewater treatment and take-back collection of unused medications. Even though API manufacturing processes are now greener in term of solvent and energy uses, pharmaceutical companies do not control the entire life cycle of their APIs. K. Kümmerer was the first to propose the better biodegradability in the environment of APIs called the "begnin by design" concept (Kümmerer, 2007; Kümmerer et al., 2018; Kümmerer, 2017; Kümmerer, 2019).

It implies the design of chemicals that will fragment after use in the environment into innocuous and non-persistent transformation products. Recently, β-blockers such as metoprolol and propranolol were subject to a method based on the nontargeted photogeneration of hydroxylated drug analogs since oxygenated compounds were shown to carry better aerobic biodegradability (Rastogi et al., 2014; Leder et al., 2015; Rastogi et al., 2015b; Rastogi et al., 2015a).

Herein, we propose to go one step further by implementing a strategy for the programmable inactivation of drugs. This new approach of "retro catabolic drug design" for the programmed fragmentation of transformation products relies on an inbuilt sensitive moiety within the ecodesigned drugs. Water treatment processes will trigger the self-disassembly of the oxidized molecule with loss of original activity (Figure 3.2- 1)

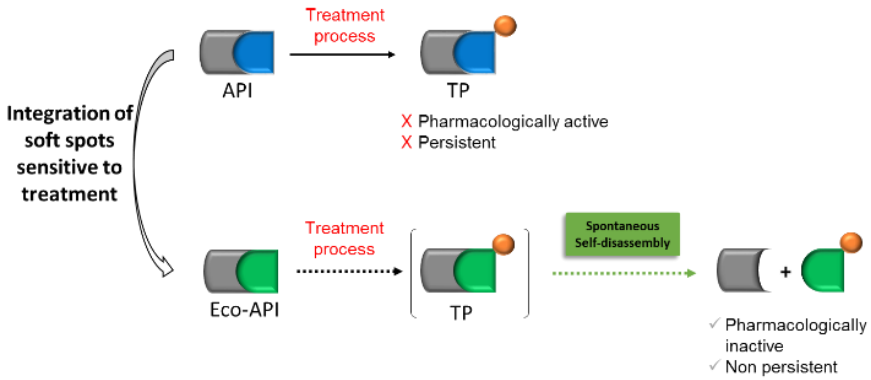


Figure 3.2- 1 Retro catabolic drug design" by integration of water treatment-responsive soft spots into API structures. The original API leads to transformation products (TPs) that can be persistent and still active. Depending on the TPs obtained after a particular treatment process, the newly designed eco-API would bear an easily breakable bond triggered by this treatment. The majority of the new TPs should then undergo facilitated disassembly leading to fragments anticipated as inactive.

The new APIs should be (i) stable toward human metabolism and (ii) soft spots should be incremented at specific molecular positions of the drug candidates. Therore, these "benign by design" APIs must capitalize on the larger transformation capacity of the treatment processes such as UV treatment compared to the human metabolism. The UV treatment of water is recommended for its strong effectiveness at breaking molecules apart in a short process time (Kim and Tanaka, 2009). Furthermore, phase I oxidation of human metabolism should be restricted by either its non- or very slow-capacity to oxidize a particular position of an API within its given residence time in the body.

We recently studied the photodegradation of methotrexate (MTX) (Espinosa et al., 2021), an antimetabolite and antifolate drug in prioritized lists for environmental risk assessment (Besse et al., 2012; Booker et al., 2014).

Methotrexate is schematically made of three moieties: a pteridine ring, a p-aminobenzoic acid and a glutamic acid (Figure 3.2- 2). Upon UV irradiation at both 254 nm and 300-450 nm, we mainly observed the transformation products originating from the fragmentation of the amine bond of methotrexate to afford non-cytotoxic pteridine and aminobenzoyl derivatives (~ 80% at 254 nm and ~

100% at 300-450 nm) (Espinosa et al., 2021). This disassembly can be attributed to well-known mechanisms of either α-oxidation of amines (Vel Leitner et al., 2002; Largeron, 2013), or even to self-immolation leading to spontaneous elimination (Alouane et al., 2015; Roth et al., 2016) (Figure 3.2- 1a).

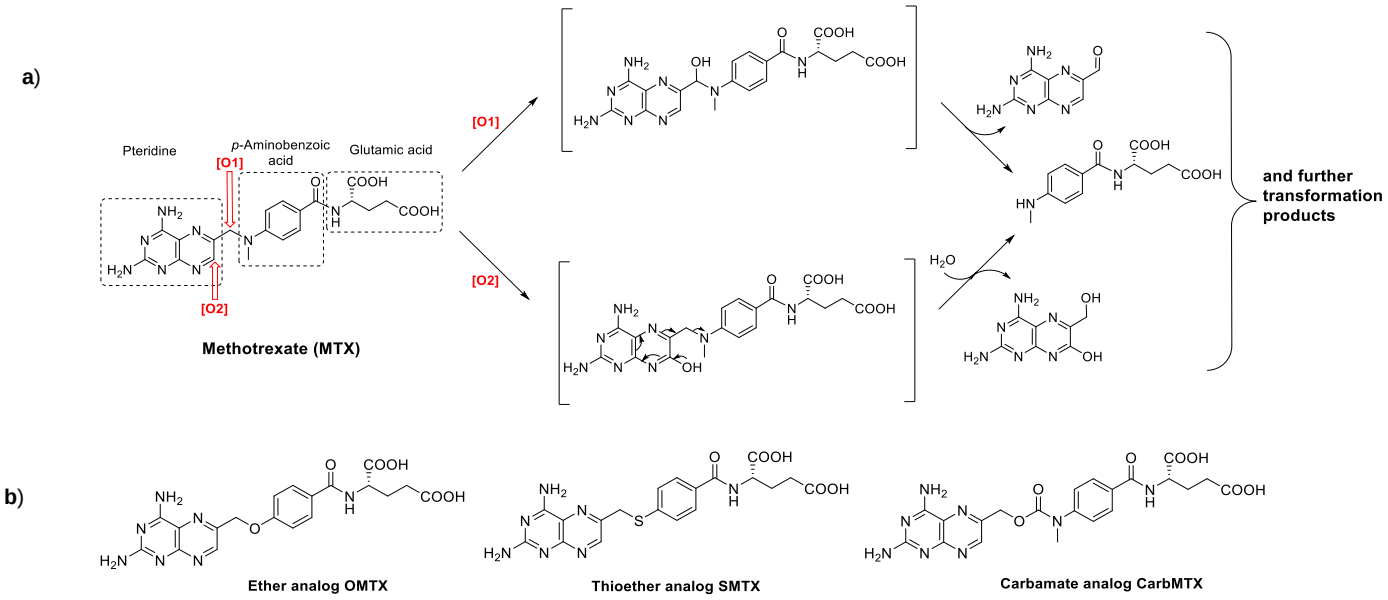


Figure 3.2- 2 Structure of methotrexate (MTX) and proposed degradation mechanisms by irradiation at 254 nm. Oxidations [O1] and [O2] are associated to the hydroxylation of the amine bond and the C7 of the pteridine nucleus respectively leading to spontaneous disassembly. (b) Structures of the ether, thioether and carbamate analogs of MTX designed to potentially disassemble faster than the parent compound.

We thus considered methotrexate as a good starting point for the introduction of our approach of eco-designed drugs. In this context, we decided to modify the amine bond within the methotrexate molecule in order to compare the degradability of the new entities with the parent drug. First, we reasoned that the hydrogen abstraction of the C-H bond adjacent to N-methyl group would be facilitated by its replacement with a stronger electron withdrawing atom or function during photodegradation. In regards to the self-immolation mechanism, this process would be accelerated by the presence of good leaving groups in contrast to an aniline motif (Alouane et al., 2015). Thus, three analogs of methotrexate containing either an ether, thioether or carbamate bond between the pteridine nucleus and the benzoyl-glutamyl part were then developed (Figure 3.2- 2b). Advantageously, the oxygenated and, to a lesser extent, the thiolated analogs were already shown to display strong anticancer activity in vitro and in vivo (Montgomery et al., 1979; Sirotnak et al., 1980; Nair et al., 1981).

We have redesigned the synthesis of the oxygenated analog OMTX (Montgomery et al., 1979) with several modifications in order to up-scale the synthetic route (Figure 3.2- 3) 4-Benzyloxybenzoic

acid 1 was coupled to the tert-butyl ester-protected glutamate 2 through an amide bond formation using the peptide coupling agent N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide (EDC) and hydroxybenzotriazol (HOBt). In the second step, removal of the benzyl group of 3 was carried out using hydrogen in presence of palladium on charcoal in excellent yield (98%). The coupling of the pteridine derivative 5 with the previously obtained phenol 4 was performed in dry DMF at room temperature and in the presence of a strong base (NaH). The desired OMTX was finally synthesized by the deprotection of the tert-butyl groups of 6 with TFA in 90% yield. The preparation of the analogs containing either a thioether or a carbamate bond were prepared following closely related synthetic pathways [5]

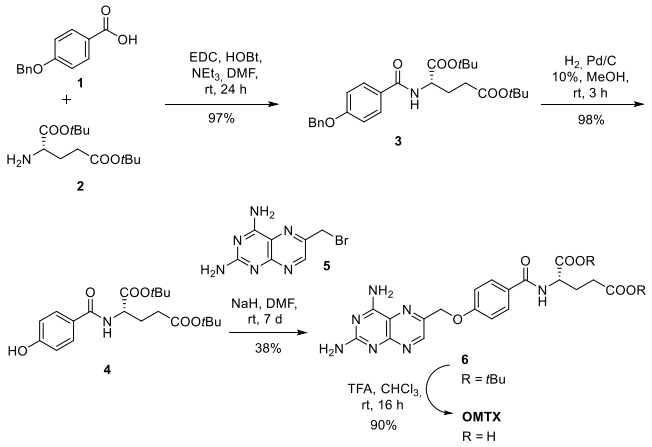


Figure 3.2- 3 Synthetic pathway to ether analog

In order to evaluate the pharmacological activity of the methotrexate analogs, we first tested their cytotoxic activity against A549 cancer cells (see SI, § 3.2.3.1). In regards to methotrexate (IC_{50} ~ 0.1 µM), the oxygenated analog OMTX exihibited a similar activity (IC_{50} ~ 0.3 µM) and thiolated analog SMTX was less cytotoxic (IC_{50} ~ 2.6 µM), in good agreement with the literature. The carbamate analog CarbMTX was however completely inactive (IC_{50} > 500 µM) and was not further taken into account.

In vitro hepatic metabolism was then assayed on methotrexate and the bioactive analogs. Methotrexate is subject to liver metabolism with the formation of 7-hydroxymethotrexate under aldehyde oxidase action (Breithaupt and Küenzlen, 1982;Moriyasu et al., 2006). Using partially purified aldehyde oxidase from rabbit liver (Johns et al., 1966; Johns and Loo, 1967) the 7-hydroxymethotrexate was obtained as the sole metabolite as expected (Figure 3.2- S1). We further examined the analogs OMTX and SMTX and similarly observed the formation of one hydroxylated metabolite of the parent

[5] Annexe 2 : Schémas de synthèse Figure S1 à S3

compounds in each case. Then, metabolization of MTX, OMTX and SMTX was compared in cytosol from human liver and initial rate of hydroxylation of the different compounds was determined (**Figure 3.2- S2**). After 24 h of incubation, 4% of methotrexate was metabolized whereas 7-hydroxymethotrexate was formed at a rate of 1.3 min^{-1}. In the same conditions, thioether analog SMTX was hydroxylated ~ 20 times faster (21.6 min^{-1}) than methotrexate whereas ether analog OMTX was slowly converted into the corresponding metabolite (0.06 min^{-1}). From this experiment, we noted that thioether SMTX was quickly metabolized up to 34% in 24 h which is detrimental for its potential pharmacological activity as its dose in the organism would hardly reach the effective concentration. However, ether OMTX metabolization was slow and we further studied the structure of its metabolite by UHPLC-MS/MS analysis. We concluded that hydroxylation occurred at the 7-position of the pteridine nucleus as in methotrexate (Figure 3.2- S3; Figure 3.2- S4; Figure 3.2-S5).

Owing to the aforementioned results and since its pharmacological activity was already demonstrated in vivo (Nair et al., 1981), we then selected the OMTX analog for a thorough comparative photodegradation study with methotrexate. Aqueous 40 µM solutions at pH 7 were submitted to irradiation, either at 254 nm or 300-450 nm wavelength samples were taken regularly and analyzed by HPLC-UV (material and methods are described in SI). The aqueous solutions of MTX and its analog OMTX appeared as stable for at least 24 h in the dark, at pH 7. When irradiated at 300-450 nm, i.e. the wavelength range photoactive in sunlight, the first order kinetic constants of both compounds are of same order of magnitude, 12.8 ± 0.9 10-5 s-1 and 16.2 ± 0.2 10-5 s-1 for MTX and OMTX respectively (Figure 3.2- S6). The reactivity difference between the two compounds is much more marked when the irradiation was performed at 254 nm, the OMTX presenting a first order kinetic constant 32-fold higher than MTX (281 ± 4 10-5 s^{-1} and 8.8 ± 1.6 10-5 s^{-1} for OMTX and MTX, respectively; presented in SI Figure 3.2- S6). Considering the same degradation state (around 20% of residual parent compounds) at this wavelength, this advancement corresponded to only 15 min for OMTX whereas 3 h was necessary for MTX as presented below, Figure 3.2- 4a). As the process to produce drinking water often includes a 254 nm irradiation step which must be as short as possible in order to avoid the blockage of important volumes of water for a long period, OMTX would thus be more efficiently removed than MTX.

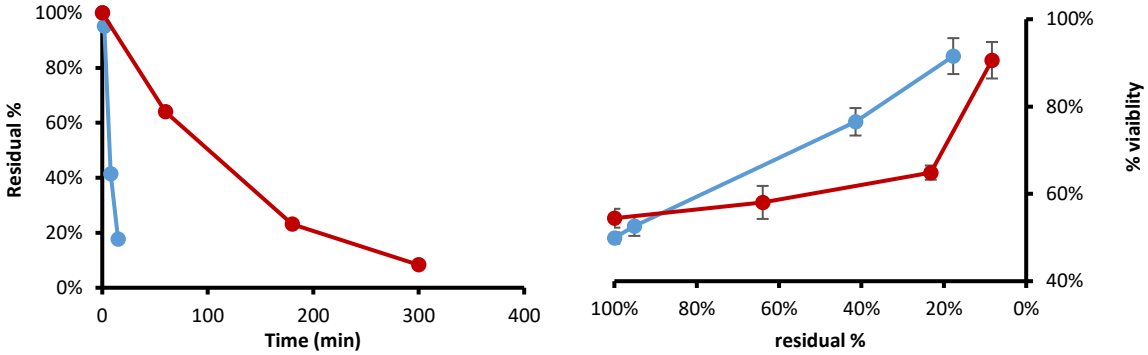


Figure 3.2- 4 (a) Photodegradation kinetics under 254 nm irradiation of 40 µM aqueous solutions of MTX (red) and OMTX (blue). (b) Viability of the A549 cancer cells in function of residual percentage of MTX (red) and OMTX (blue) after irradiation at 254 nm.

The structural determinations and the reaction monitoring of the transformation products (TPs) were carried out as described by Espinosa et al. (2021) via UHPLC-MS/MS using a high resolution equipment for the identifications and a triple quadripole programmed under multiple reaction monitoring adapted to perform quantitation (see SI). The elemental composition of the pseudo-molecular ions and their main fragment ions are summarized in Table 3.2- S2. Due to the lack of standards corresponding to the hypothesized photoproducts, a comprehensive study of the OMTX fragmentation was conducted and presented in Figure 3.2- S7. Identifications of a majority of TPs (Figure 3.2- S8) corresponded to a level 2b according to Schymanski et al. (2014). Nonetheless, TP267 corresponded to a level 1 as this compound was synthesized and used as chemical standard. TP411a and b, and TP413 coincided to a level 3 because of the lack of stricking information to propose only one structure and finally TP441 to a level 4 due to its similar degradation pathway as OMTX which prevented elucidation of its structure. Monitoring of TPs along photodegradation experiments gave indication about mechanisms of degradation (Figure 3.2- S9). Focusing on 254 nm condition after 3h of irradiation, 18% of residual OMTX were observed and the major photoproducts (> 5%) were TP192, TP441, and also TP208 and TP411b (26%, 15%, 17% and 23% respectively) (Figure 3.2- 5 and Table 3.2- S3). In regards to the expected fragmentation of the ether bond between the pteridine nucleus and the hydroxybenzoyl derivative, we observed that a conversion of ~ 60% with the transformation products TP192, TP206, TP208 and TP267 corresponded to this envisioned scission (Figure 3.2- 2 and Table 3.2- S3). In regards to the stability of 7-hydroxy metabolites observed in the cytosol, it appears that self-immolation may not be the main mechanism of scission of both MTX and OMTX upon UV treatment. Therefore, cleavage could mostly occur by direct scission of the ether (OMTX) or amine (MTX) bond and hydroxylation of

the pteridine nucleus would then occur before or after this specified fragmentation. Nonetheless, the UV treatment at 254 nm could also promote self-immolation by the generation of the aryloxy radical (pteridine-O·) (Zhao et al., 2010). Indeed, neither 7-OH-MTX nor 7-OH-OMTX remained observable at the end of irradiation. Lastly, considering the TPs resulting from modifications of the glutamyl part, the sum was higher for OMTX (41%, including TP411a, TP441, TP411b, Figure 3.2- S8) than for MTX (30%) (Table 3.2- S3)(Espinosa et al., 2021).

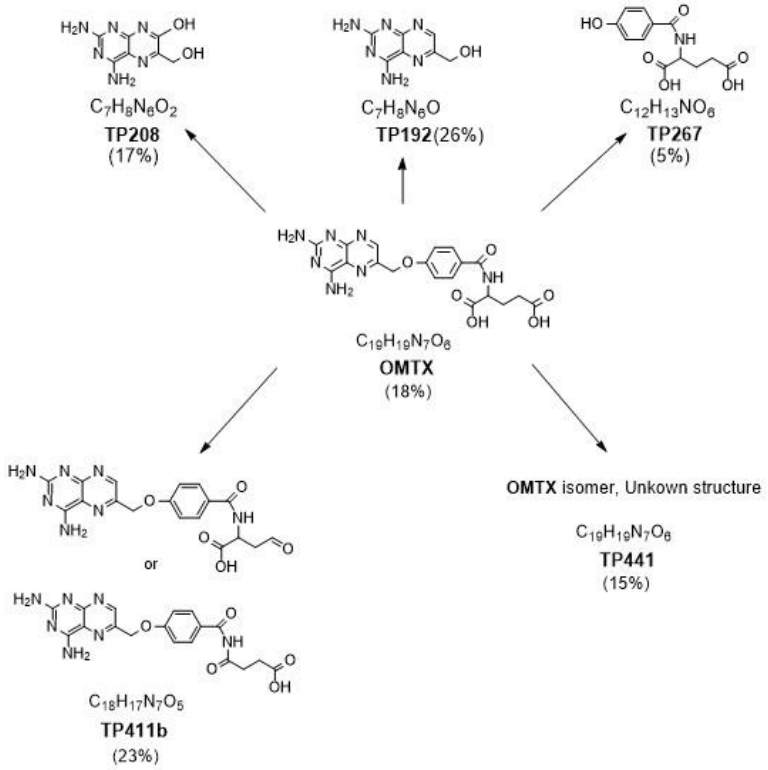


Figure 3.2- 5 Proposed structures of the transformation products (> 5%) of OMTX under irradiation at 254 nm

Finally, the viability of A549 cancer cells was assessed on all the samples of OMTX photodegradation (Figure 3.2- S9) and compared to the previously described results obtained starting from MTX (Espinosa et al., 2021). Only 65% of cell viability was restored after 3h of MTX irradiation whereas the cell viability was almost completely retrieved (~ 90%) in 15 min of OMTX irradiation at 254 nm (Figure 3.2- 4b). At the time point where the amount of residual parent compounds are approximately equivalent: specifically 23% residual MTX and 18% residual OMTX, the cytotoxicity of the TPs obtained from OMTX is greatly weaker than these generated from MTX even though the number of

TPs generated and identified from OMTX (= 9) was higher than that of MTX (= 6). Viability evolution under irradiation at 300-450 nm followed a similar trend (Figure 3.2- S9a).

Herein, we have described a methodology for the programmed inactivation of environmentally problematic drugs upon typical water treatment. This strategy relies on the spontaneous disassembly of a dual or multipartite drug triggered by the applied process. The detachments of at least two moieties of the original drug lead to anticipated loss of the pharmacological activity. Using methotrexate as a drug model, we demonstrated the feasibility of this strategy with the development of a pharmacologically potent analog that is mostly and promptly disassembled by UV treatment into two parts displaying no cytotoxicity. Likewise, several other environmentally problematic drugs of major interest could benefit from a similar re-design. As of today, pharmaceutical companies do not survey the persistence of APIs or their metabolites in the environment even though the Food and Drug Administration (FDA) and European Medicines Agency (EMA) request a pre-marketing environmental risk assessment (ERA) before new drug approval. Undoubtedly, laws imposing the innocuousness of new APIs toward the environment will be adopted in the future. In the longer term, methods accompanying medicinal chemists to choose compounds that will be harmless to the environment have to be envisioned. Our approach and others emanating from this study could be integrated into the design of new drug candidates.

3.2.3. Supplementary information

The first part (from 3.2.3.1 to 3.2.3.3) is related to material and methods, and determination of the cytotoxic activity of parent compounds.

The second part (from 3.2.3.4) presents results of *in vitro* hepatic metabolism, identification of the hydroxylated metabolites, photodegradation experiment of both 254 nm and 300-450 nm (the 254 irradiation was already discussed in the manuscript), identification of TPs and their monitoring in regard to cytotoxicity evolution.

3.2.3.1. Cytotoxicity experiments on A549 cancer cells

i. Determination of the IC_{50}s of methotrexate and analogs

A549 cells were grown in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% of fetal calf serum (FCS) and 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen) in a 5% CO_2 and 95% hygrometry environment at 37 °C.

The cytotoxic activity of methotrexate and analogs has been investigated using the MTS ((3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium)) method (Promega). 2.5 10^3 cells (100 µL) were seeded in 96-well plates and incubated overnight at 37 °C in presence of 5% CO_2 and 95% hygrometry. Then, the cells were treated with 100 µL of each compound (MTX, OMTX, SMTX and CarbMTX) at different final concentrations (from 50 µM to 0.05 nM). After 72 h, the media was aspirated and 100 µL of a MTS solution (10% in media) were added in each well. After a 3 h incubation period at 37 °C in presence of 5% CO_2 and 95% hygrometry, the optical density was measured at 490 nm wavelength using a microplate reader (Infinite M200 Pro, Tecan trading AG, Switzerland). Untreated cells were used as control. Each concentration was tested in six replicates and the experiment was fulfilled in triplicates. The concentration inhibiting 50% of the cell proliferation (IC_{50}) was determined using GraphPad Prism software (GraphPad Software Inc, San Diego, CA, USA) and presented Table 3.2- S1 IC50 of studied compounds

Table 3.2- S1 IC_{50} of studied compounds

Compounds	IC_{50} (nM)
MTX	120 ± 20.0
OMTX	310 ± 30
SMTX	2600 ± 500
CarbMTX	> 500 .10^3

ii. Evolution of OMTX transformation products cytoxicity

To evaluate the cytotoxicity evolution along the OMTX degradation at pH 7, different time-points were chosen for the two irradiation conditions: treatment at 254 nm, time-points: t = 0, 2, 8 and 15 min; treatment at 300-450 nm, time-points: t = 0, 2, 8 and 15 min. Each sample was then evaluated using the method described above with 100 µL of each solution at the final concentration of 100 nM (close value of the IC_{50} determined for MTX and OMTX).

3.2.3.2. Metabolisation and photodegradation of methotrexate and analogs

i. Metabolisation by aldehyde oxidase from rabbit liver

The livers were collected at the slaughterhouse from freshly sacrificed rabbits. Aldehydes oxidase was partially purified according to literature with modifications(Johns et al., 1966; Johns and Loo, 1967). Three rabbit livers (≈ 300 g) were homogenized (electric blender) in 250 mL of cold distilled water. The homogenate was centrifuged for 30 minutes at 13000 x *g* and the supernatant fraction was heated at 60 °C for 10 minutes under slow magnetic stirring in an Erlenmeyer. The suspension was immediately cooled in an ice-bath and was centrifuged for 10 minutes at 13000 x *g*. The supernatant was mixed with 120 mL of ammoniacal ammonium sulfate (AAS) solution[6]. This mixture was centrifuged for 10 minutes at 13000 x *g* and the precipitate was saved. The supernatant was stirred with 10 mL of AAS solution and centrifuged for 10 minutes at 13000 x *g*. The precipitate was then dissolved in 18 mL of distilled water and 2 mL of AAS solution. Enzymatic activity was determined spectrophotometrically (420 nm, quartz cuvette with one cm optical path length, Varian Cary 300-bio spectrophotometer) at 37°C in 10 mM Tris-HCl, pH 8.5 containing 20 mM $MgCl_2$ using benzaldehyde as substrate and potassium ferricicyanide as electron acceptor (Mahro et al., 2011).

Methotrexate or its analogs were then solubilized in 100 mL of 10 mM Tris-HCl, pH 8.5 containing 20 mM $MgCl_2$. 80 mL of distilled water were added to the previously prepared (20 mL) enzyme solution. The solution of methotrexate or derivatives was added over 2 hours to the diluted enzyme solution. The mixture was gently stirred at room temperature for 4 days and was cooled at 4 °C for 15 hours. The observed suspension was centrifuged for 10 minutes at 13000 x *g* and the supernatant was discarded. The precipitate was suspended in 20 mL of distilled water and was heated at 100 °C for 5 minutes and was centrifuged for 10 minutes at 13000 x *g*. The supernatant was acidified with 2 mL of glacial acetic acid and was then evaporated to afford the hydroxylated metabolites.

ii. Metabolisation by cytosol from human liver

Methotrexate or analogs solutions at 800 µM were prepared in 10 mM Tris-HCl, pH 7.4 containing 20 mM $MgCl_2$ and 1% DMSO (v/v). 400 µL of the compound solution were mixed with 400 µL of prewarmed human liver cytosol at a final concentration of 10 mg of total protein/mL. The mixture was gently shaken in an incubator at 37 °C and samples (80 µL) were taken up at different time intervals up

[6] AAS solution was prepared with 60 mL of ammoniun hydroxide (29%) poured in 940 mL of saturated ammonium sulfate solution.

to 28h of reaction. Each sample was filtered (PTFE pore size 0.2 µm) before being analyzed by HPLC using the method described above.

iii. Photodegradation

The degradation experiments were carried out as described by Espinosa et al. (2021). Briefly, 40 µM solutions of MTX or OMTX, prepared in Milli-Q water with pH adjusted to pH 7, were exposed either to wavelength conditions representative of natural conditions in surface water (300–450 nm) or drinking water treatment (254 nm). For the 300-450 nm treatment, the solution was placed in a tubular pyrex reactor at the center of a cylindrical device equipped with six lamps emitting within 300 to 450 nm with a maximum emission at 365 nm (TLD 15 W, Philips). The temperature was maintained at 20-22°C by a fan located outside the bottom of the tube. Mixing and oxygenation were assured by air bubbling. For the 254 nm treatment, the solution was placed in a tubular quartz reactor parallel to a monochromatic low-pressure mercury lamp (TUV 15 W, Philips) and mixed by magnetic stirring. At photoreactor location, the light intensity measured by a radiometer (VLX-3W, Vilber Lourmat, Marne-la-Vallée, France) was for each of the six 300-450 nm lamps ca 0.1 mW/cm^2 at 312 nm and 1.2 mW/cm^2 at 365 nm, and for the 254 nm lamp 1.5 mW/cm^2 at 254 nm.

3.2.3.3. Analytical methods for biotic and abiotic degradations

i. Monitoring of hepatic metabolism

Analyses were performed on Agilent 1260 Infinity II LC System coupled to an Agilent mass spectrometry MS SQ 6120 (ESI/Q). UV detection was primarily made at 280 nm. Elutions were performed using a Zorbax Eclipse Plus C18 column (3.5 µm; 4.6 x 100 mm). Mobile phases consisted of solution A: acetonitrile/formic acid 0.1% (v/v) and solution B water/formic acid 0.1% (v/v). The column was eluted with a gradient linearly increasing from 2 to 85% of solution A during 19 minutes. The flow rate was 1 mL/min.

ii. Identification of hepatic metabolites

The structure of the hepatic metabolite issuing from the OMTX analog was studied after incubation with cytosol for 5h (according to the protocol described above), by Ultra High Performance Liquid Chromatography (UHPLC)–mass spectrometry (MS). The device consisted on an Acquity UPLC

system (Waters, Milford, MA, USA) composed of a high pressure binary solvent manager, an autosampler and a column oven connected to a triple quadrupole mass spectrometer (TQD, Waters). Chromatographic separation was carried out on a UPLC C18 Gravity-SB Column, 100 x 2 mm, 1.8 µm particle size (Macherey-Nagel). The gradient profile started at 97% water with 0.05% formic acid for 5 min, linearly progressed to 50% at 30 min, with a flow rate of 0.3 mL/min. The UHPLC effluent was directed to an UV detector (TUV, Waters) programmed at 305 nm, prior to its introduction into the triple-quadripole mass spectrometer (TQD, Waters) via an electrospray source operating either in positive or negative mode. The electrospray conditions were: capillary voltage 3 kV, cone voltage 52 V and 42 V respectively under positive and negative modes, source temperature 150°C, desolvation temperature 450°C, extractor voltage 3 V, cone and desolvation gas flow (nitrogen) at 20 and 900 L/h, respectively. In MS-MS, the pseudo-molecular ions were fragmented at higher-energy collisional dissociation (HCD) of 20 and 35eV with 3.4×10^{-3} mbar of argon. Data were acquired and treated using MassLynx software v4.1 (Waters).

iii. Monitoring of photoproducts

The reaction monitoring and the structural determination of the transformation products (TPs) were realized as described by Espinosa et al. (2021).

The degradation kinetics of the parent compounds (MTX and OMTX) were established using the HPLC-UV Waters Alliance (2690/5) system on a HPLC C18 Gravity-SB column 150 × 3 mm, 3 µm particle size (Macherey-Nagel). The gradient profile started at 97% water with 0.05% formic acid and linearly progressed up to 50% in 30 min, the second solvent being acetonitrile, at a constant flow rate of 0.5 mL/min. The detection was performed using diode-array detector (TUV 2489, Waters) acquiring data in the 230–305 nm range. Data were acquired and treated using Empower software. Quantifications were performed at 290 nm, using the 40 µM initial solution (mean of 4 injections for each experiment) as 100% abundance. All the peak areas were expressed in percentage of initial OMTX. The OMTX kinetics of degradation were treated as a first order form. The apparent first-order rate constants k' were evaluated according to $Ln(C/C_0) = - k't$, where C is the OMTX concentration at time t and C_0 its initial concentration.

The TPs were monitored by UHPLC-MS/MS using the Acquity UPLC system (Waters) connected to the triple quadrupole mass spectrometer (TQD, Waters) as described above for the identification of hepatic metabolites. The acquisitions were performed under MS full scan mode and the quantifications using the pseudo-molecular ions in positive mode.

iv. Identification of photoproducts

The structural determinations of the TPs were performed at the end of irradiation and after pre-concentration by solid-phase extraction on Oasis HLB cartridges, with an ultra-high performance liquid chromatograph Ultimate 3000 (Thermo Fisher Scientific) coupled to a Q Exactive hybrid quadrupole – Orbitrap™ (Thermo Fisher Scientific) mass spectrometer through a Heated Electrospray Ionization (HESI-II) probe. The chromatographic separation of the sample was performed on a UPLC C18 Gravity-SB Column, 100 x 2 mm, 1.8 µm particle size (Macherey-Nagel). The gradient profile started at 97% water with 0.05% formic acid for 5 min, linearly progressed to 50% at 30 min, with a flow rate of 0.3 mL/min. The analyses were performed in positive and negative modes under the following specific conditions: spray voltage, ± 3 kV; sheath gas pressure: 35 psi, auxiliary gas flow rare: 10 (arbitrary units); ion transfer tube and vaporizer temperature 300°C. Nitrogen was employed as sheath and auxiliary gas. MS data were acquired in full scan within mass-to-charge (m/z) range of 120–550 m/z at a mass resolution of 70000 and a scanning speed of 3.7 scan/s. It allowed detecting the pseudo-molecular ions $[M + H]^+$ and $[M - H]^-$ of the compounds, and thus their elemental composition. The pseudo-molecular ions were fragmented by higher-energy collisional dissociation (HCD) with normalized collision energy of 35% and then directed to the Orbitrap where they were analyzed with a resolution of 35000. Xcalibur™ software (Thermo Fisher Scientific) was employed for instrumental control and data processing.

3.2.3.4. Results from metabolisation and photodegradation

i. Rabbit and human hepatic metabolism: monitoring and hydroxylation

HPLC analysis of samples issuing from metabolization by aldehyde oxidase from rabbit liver revealed a total conversion of the methotrexate and its analogs into one major hydroxylated metabolites (see the chromatograms in Figure 3.2- S1). The retention times was 14.24, 13.82 and 14.28 min respectively for the metabolites of MTX, OMTX and SMTX. Methotrexate afforded the 7-hydroxymethotrexate 7-OH-MTX (*m/z* = 470 $[M+H]^+$) as expected (Moriyasu et al., 2006). Oxygenated and thiolated analogs afforded hydroxylated metabolites (*m/z* = 459 $[M+H]^+$ and *m/z* = 474 $[M+H]^+$ respectively) which were tentatively analyzed as the corresponding 7-OH metabolites of the parent structures.

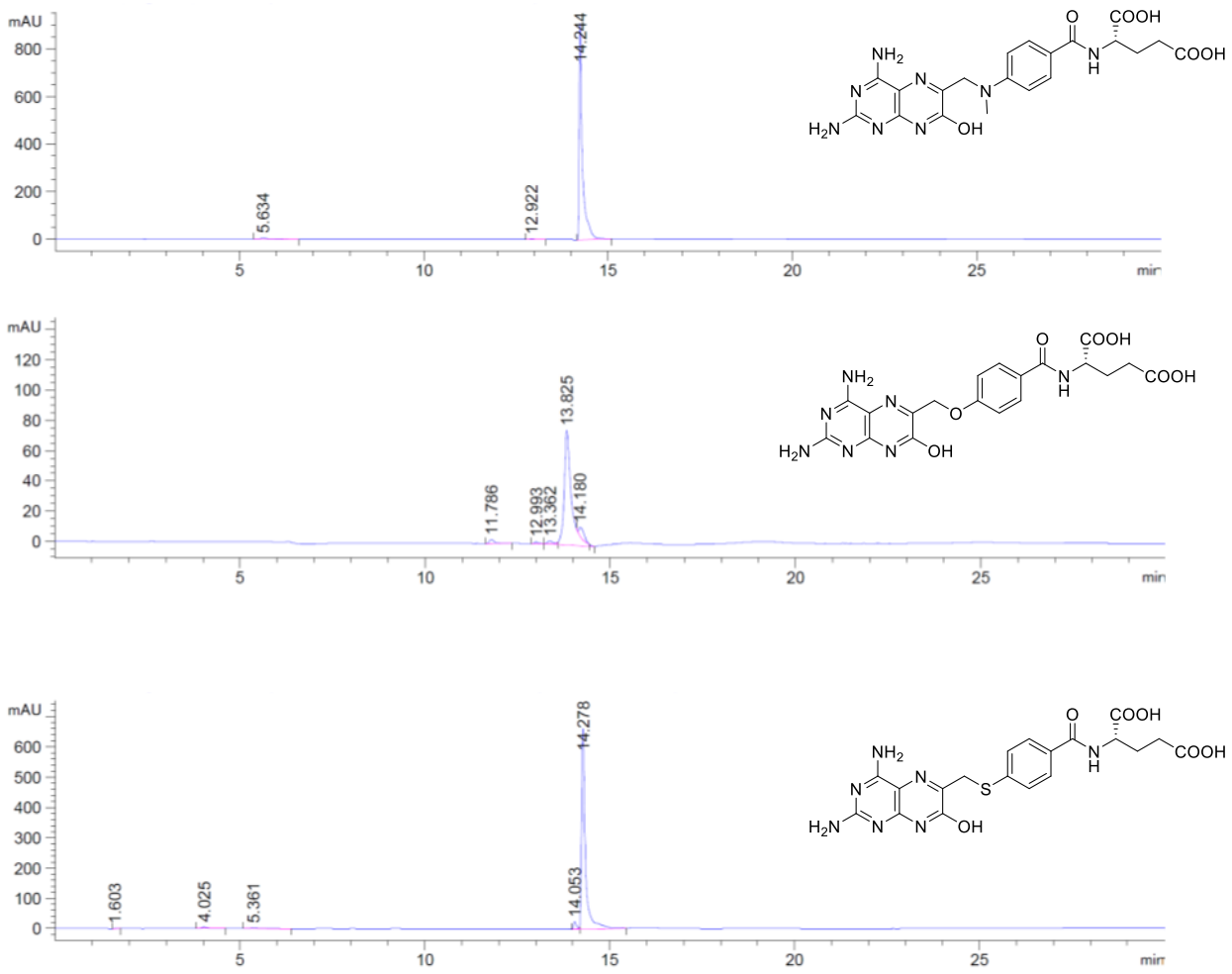


Figure 3.2- S1 Chromatograms of metabolized MTX, OMTX and SMTX.

After metabolization by cytosol from human liver, again, the hydroxylated metabolite was observed as the sole metabolite formed in all cases. The temporal evolution of each metabolite was plotted and allowed us to extract the initial rate of hydroxylation reactions (see the kinetics in Figure 3.2-S2). As the substrates (MTX, OMTX and SMTX) were in excess and at the same concentration, we expressed the initial rates of hydroxylation in min^{-1}.

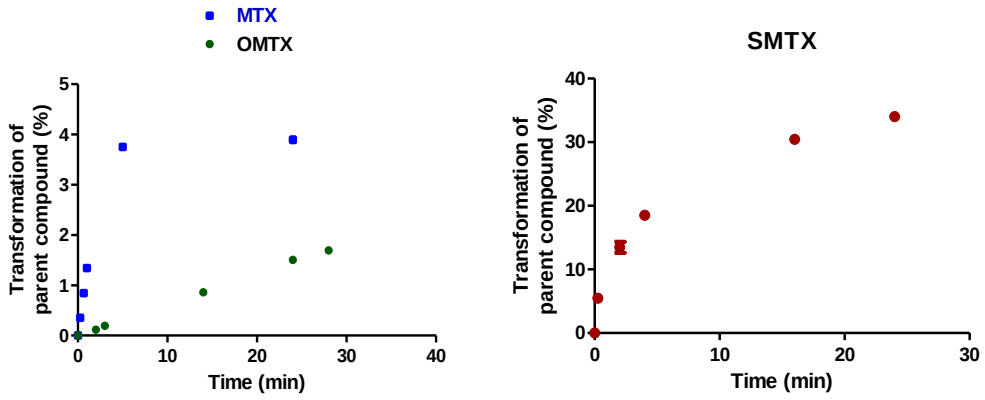


Figure 3.2- S2 Kinetics of formation of the hydroxylated metabolites starting from (a) MTX, OMTX or (b) SMTX in human liver cytosol.

The rate of hydroxylation of thiolated analog (21.6 min^{-1}) was 20 times faster than the methotrexate hydroxylation (1.3 min^{-1}). However, the oxygenated analog was the slowest metabolized compound (0.06 min^{-1}).

To assess the structure of the potential hydroxylated metabolite of the OMTX analog, a first injection carried out in full scan MS over the mass range of m/z 120–550 allowed detecting the pseudo-molecular ions $[M + H]^+$ and $[M - H]^-$ (Figure 3.2- S3).

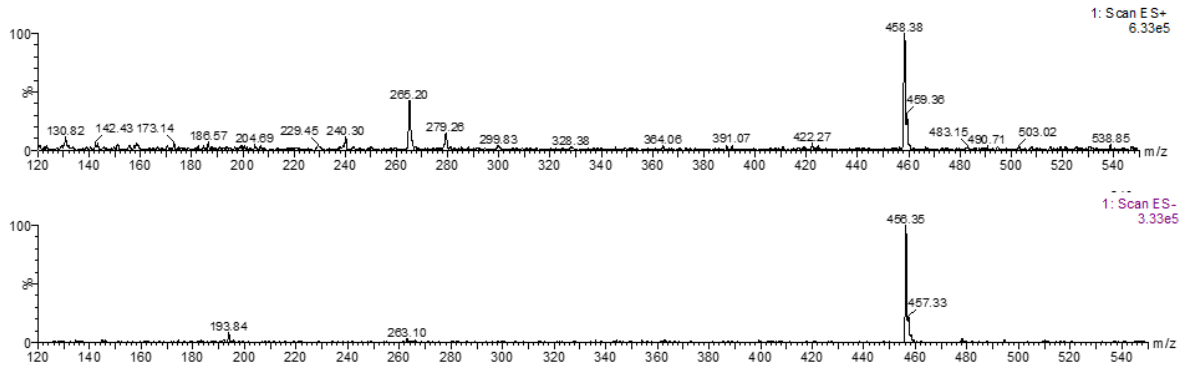


Figure 3.2- S3 Pseudo-molecular ions $[M + H]^+$ and $[M - H]^-$ of 7-OH-OMTX in full scan LC-MS.

In a second step, the MS/MS analysis was performed (Figure 3.2- S4) and the fragments were identified as presented in Figure 3.2-S5.

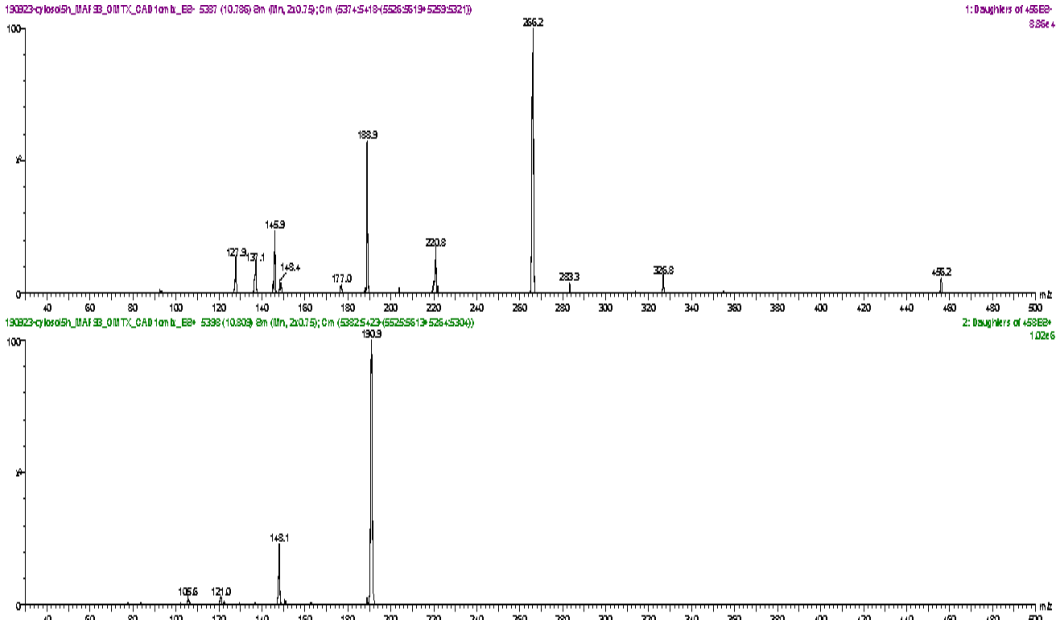


Figure 3.2- S4 Daughter ion spectrum (MS^2) of the potential 7-OH-OMTX in positive and negative mode.

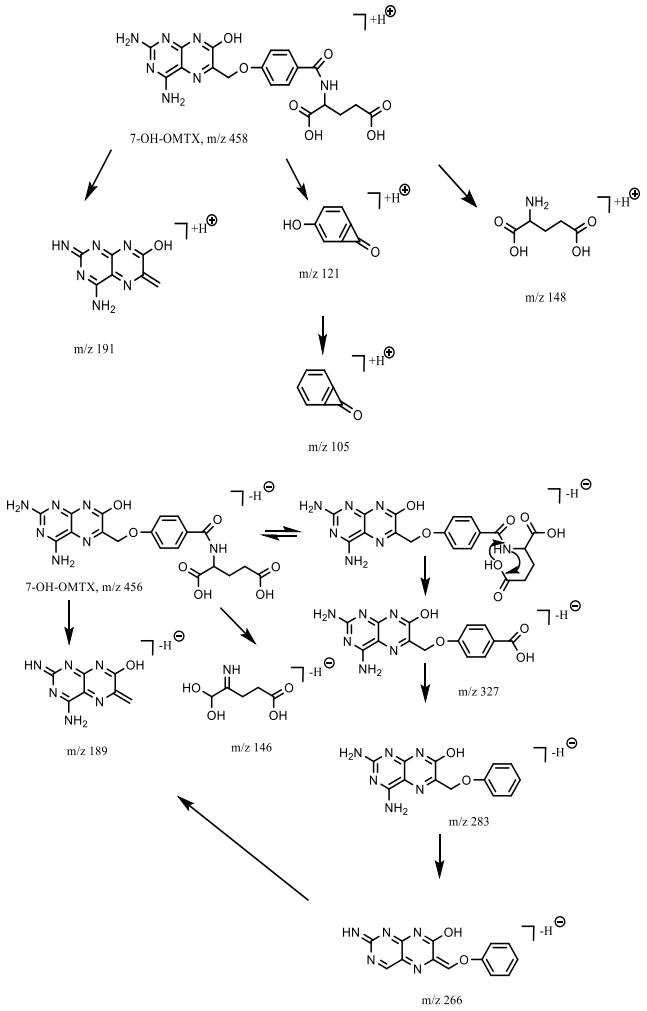


Figure 3.2-S5 Daughter ion spectrum (MS^2) and fragmentation pathways of pseudo-molecular ions at (a) 35eV for [M + H]+ and (b) 20eV for [M - H]-.

ii. Photodegradation kinetics

The MTX and OMTX photoreactivities were compared both in terms of pseudo-first order kinetic constants (Figure 3.2- S6a) and quantum yields (Figure 3.2- S6b). At 300-450 nm, the kinetic constants were not significantly different ($12.8 \pm 0.9\ 10^{-5}\ s^{-1}$ and $16.2 \pm 0.2\ 10^{-5}\ s^{-1}$ for MTX and OMTX respectively), whereas considering the quantum yields of MTX was significantly lower, according to a Mann and Withney test, than that of its analog OMTX: $1.05 \pm 0.1\ 10^{-3}$ and $3.88 \pm 1\ 10^{-3}$, respectively). As previously mentioned in the manuscript, at 254 nm, OMTX appeared much more reactive than MTX, with a kinetic constant 32-fold higher ($281 \pm 4\ 10^{-5}\ s^{-1}$ and $8.8 \pm 1.6\ 10^{-5}\ s^{-1}$ for OMTX and MTX, respectively). A 26-

fold factor was observed when quantum yields measured at 254 nm were considered: 32.4 ± 3 10^{-3} and 1.26 ± 0.2 10^{-3} for OMTX and MTX respectively.

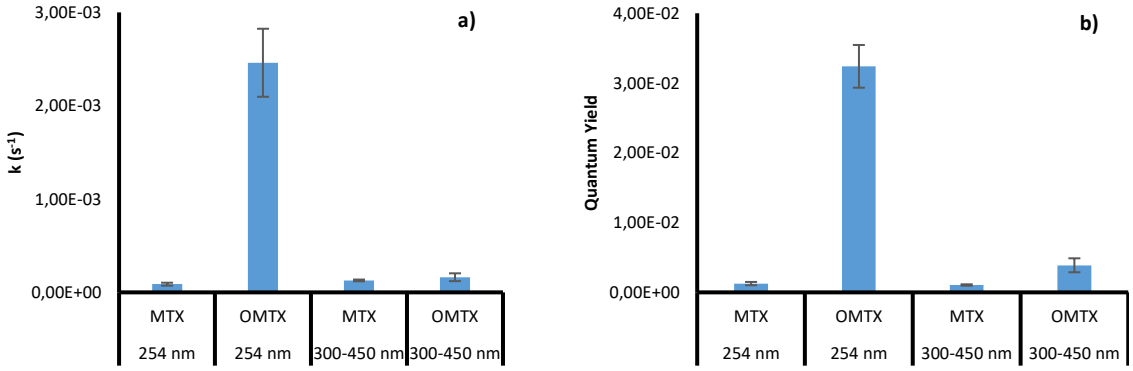


Figure 3.2- S6 Pseudo-first order kinetic constants (a) and quantum yield (b) of MTX and OMTX photodegradations.

iii. Structural identification of photodegradation products

Fragmentation pathway of OMTX:

In the first step of fragmentation in positive mode (Figure 3.2- S7a), OMTX may lose HCOOH leading to m/z 396 (and then m/z 269 by $C_5H_5NO_3$ loss). OMTX could also undergo a rearrangement initiated by the hydroxyl group of the α-carboxylic acid bore in the glutamyl part, forming a five-center transition state before the elimination of 3-oxoaziridin-2-yl-propanoic acid as proposed by Secrétan et al. (2018) for pemetrexed. An alternative mechanism was proposed in the case of pralatrexate, including a N-C cleavage followed by a rearrangement (Sastry et al., 2016). This rearrangement was also observed in the case of MTX (Espinosa et al., 2021) leading to a similar structure as the 2,4-diamino-N(10)-methylpteroic acid (DAMPA) which could be the oxygenated counterpart, the ODAMPA (fragment m/z 313). The next fragmentation steps are also similar to the MTX ones, with H_2O loss forming m/z 295 and then cleavage of the C-O bond leading to the complementary fragments m/z 175 and 121.

In the negative mode (Figure 3.2- S7b), the m/z 440 pseudo-molecular ion may form the fragment m/z 311 after the same rearrangement as observed in positive mode. The fragment m/z 266 was obtained from the cleavage of the C-O bond between the diamino-pteridine nucleus and the phenol moiety; the latter derivative could further evolve to the fragments m/z 222 by decarboxylation of the α-

carboxylic acid from glutamyl chain and then m/z 204 by loss of H_2O. In parallel, the fragment m/z 128 may correspond to the glutamyl part after elimination of H_2O.

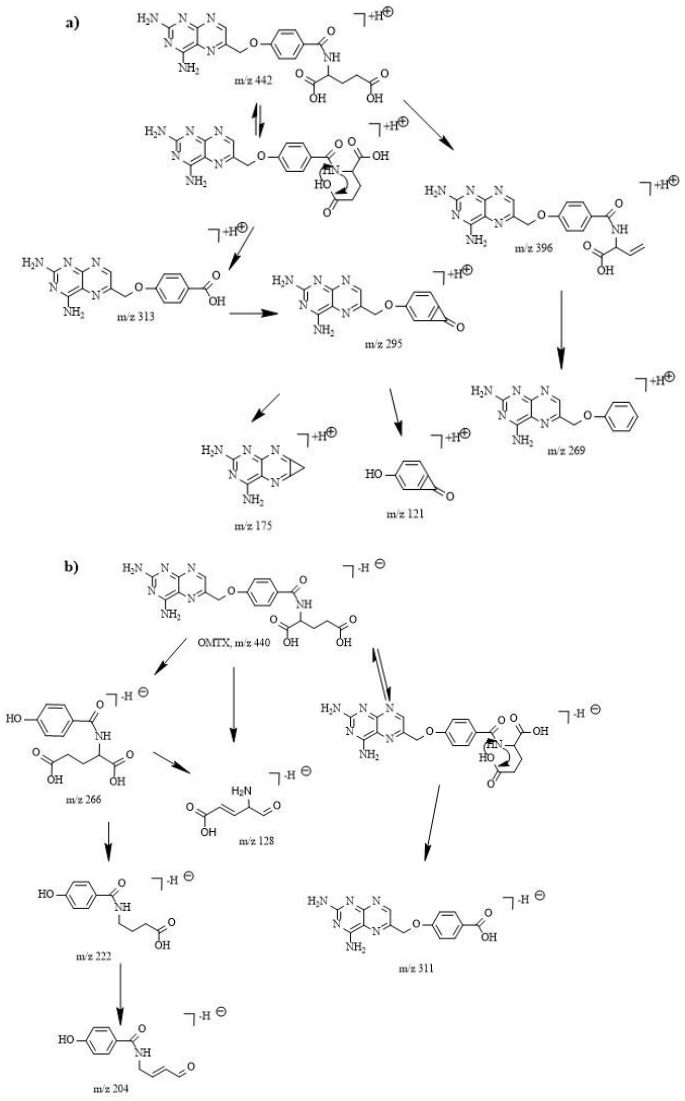


Figure 3.2- S7 OMTX fragmentation pattern from (a) protonated parent ion and (b) deprotonated parent ion.

Elucidation of OMTX's TPs structures:

The elemental composition of the pseudo-molecular ions and their main fragment ions are summarized in Table 3.2- S2. The elucidation of OMTX fragmentation pathways allowed assumptions for the identification of TPs structures. As seen for OMTX, m/z 396, 313 and 295 indicate that the molecules containing the pteridine ring linked to the benzoyl part were present in the TPs, fragments m/z 312 and 340 also support this assumption corresponding to $C_{14}H_{14}N_7O_2^+$ and $C_{15}H_{14}N_7O_3^+$ formula, respectively.

These fragments are common to most of the TPs (TP443a or b, TP441, TP429a and b, TP413, TP411a and b, TP312). Furthermore, the fragments mentioned above can be fragmented to smaller ones: m/z 175 or its radical equivalent m/z 176 (diamino-pteridine moities) and m/z 121 and 133 (*p*-hydroxybenzoyl moieties). These last ones are absent from the fragmentation of TP192 and TP208, supporting that these three TPs are pteridine derivatives, while the m/z 121 fragment is observed for TP267 in accordance with the structure of the synthesized standard, the 4-(hydroxybenzoyl)-L-glutamic acid[7] TP206 was not confirmed by high resolution but was previously identified for MTX degradation (Lutterbeck et al., 2015; Lai et al., 2017;)

In negative mode, the negative fragments containing the pteridine nucleus and the benzoyl-glutamyl part were consistent with the structures hypothesized in positive mode. Additionally, few fragments brought additional information about the glutamic acid part. This negative mode was determinant for the elucidation of structures, as already seen for MTX (Espinosa et al., 2021). It was the case of the fragments m/z 116 for TP411a and m/z 99 for both TP411a and b, which indicated an OMTX oxidation, formally by loss of formaldehyde from the glutamyl chain in α and γ positions. As no indication is available to attribute the position, these two isomers were identified at the level 3 of Schymanski. This same pathway was highlighted in the MTX study (Calza et al., 2014). Furthermore, the fragment m/z 99, formed by the TP411a, TP411b and TP429b fragmentation, indicates the presence of a four carbon-chain, while the fragment m/z 82 in TP429a was determinant for attributing the structure of the chain linked to the nitrogen of the amide function. These two isomers, TP429a and TP429b, result from the loss of formaldehyde and hydroxylation of the glutamic acid part. The TP441 is an isomer of OMTX, with similar formula and fragmentation pattern.

Figure 3.2- S8 shows hypothetical structures of TPs, no major difference of mechanism was highlighted under the two wavelengths, with formation of two class of TPs, common with MTX ones (Espinosa et al., 2021). They result from: (i) modifications of the glutamyl part: TP429 a and b, TP413, TP411 a and b, and TP312 displaying almost complete loss of the glutamyl chain; (ii) a cleavage between the benzoyl-glutamyl part and the diamino-pteridine moiety. The pteridines derivatives TP208 and TP206 which are obtained in both MTX and OMTX photodegradation pathways (these two TPs were previously identified by other groups studying MTX degradation) (Calza et al., 2014; Lutterbeck et al., 2015; Lai et al., 2017; Kanjal et al., 2020), and TP192 which was not seen for MTX and then the benzoyl counterparts TP267 and TP281.

[7] Annexe 2 : Schémas de synthèse Figure S1 à S3

b) **Table 3.2- S2** Accurate mass measurements in the positive and negative modes determined by LC-Q-Orbitrap-MS in MS or MS/MS mode.1

compound	Positive mode					Negative mode				
	nominal ion mass	formula	$[M+H]^+$ accurate mass	$[M+H]^+$ theoretical	error (ppm)	nominal ion mass	formula	$[M-H]^-$ accurate mass	$[M-H]^-$ theoretical	error (ppm)
TP192	193	$C_7H_9N_6O^+$	193.0830	193.0832	1.04					
			175.0724	175.0727	1.71					
TP206	207	$C_7H_7N_6O_2^+$		*207.0625*						
TP208	209	$C_7H_9N_6O_2^+$	209.0779	209.0781	0.96					
		$C_7H_9N_6O^+$	193.0830	193.0832	1.04					
TP267	268	$C_{12}H_{14}NO_6^+$	268.081	268.0816	2.24	266	$C_{12}H_{12}NO_6^-$	266.0668	266.0670	0.75
			121.0286	121.0295	7.44					
TP281	282	$C_{13}H_{16}NO_6^+$	282.0973	282.0972		280	$C_{13}H_{14}NO_6^-$	280.0829	280.0827	
		$C_{12}H_{16}NO_4^+$	238.1068	238.1073			$C_{12}H_{10}NO_5^-$	248.0565	248.05645	
		$C_{11}H_{12}NO_3^+$	206.0813	206.0812			$C_{11}H_{10}NO_3^-$	204.0658	204.0666	
		$C_5H_8NO_3^+$	130.0499	130.0499						
		$C_7H_5O_2^+$	121.0285	121.0284						
TP311	312	$C_{14}H_{14}N_7O_2^+$	312.1195	312.1203	2.56	310	$C_{14}H_{12}N_7O_2^-$	310.1061	310.1058	-0.97
		$C_{14}H_{11}N_6O_2^+$	295.0930	295.0938	2.71		$C_{13}H_{10}N_5O_2^-$	268.0830	268.0840	3.73
		$C_7H_8N_6^{\bullet+}$	176.0801	176.0805	2.27		$C_{12}H_9N_4O^-$	225.0774	225.0782	3.55
							$C_6H_5N_6^-$	161.0572	161.0581	5.71
							$C_7H_6NO_2^-$	136.0393	136.0404	8.09
							$C_5H_3N_4^-$	119.0351	119.0363	10.08
TP411a or b	412	$C_{18}H_{18}N_7O_5^+$	412.1356	412.1364	1.94	410	$C_{18}H_{16}N_7O_5^-$	410.1213	410.1218	1.22
		$C_{15}H_{14}N_7O_3^+$	340.1159	340.1153	-1.76		$C_{14}H_{12}N_7O_2^-$	310.1056	310.1053	-1.13
		$C_{14}H_{14}N_7O_2^+$	312.1196	312.1203	2.24		$C_{14}H_9N_6O_2^-$	293.0799	293.0792	-2.39
		$C_{14}H_{11}N_6O_2^+$	295.0921	295.0938	5.76		$C_{13}H_{11}N_6O^-$	267.0998	267.0994	-1.39
		$C_{13}H_{11}N_6O^+$	267.0984	267.0994	3.86		$C_{12}H_9N_4O^-$	225.0774	225.0776	1.07
		$C_7H_7N_6^+$	175.0725	175.0727	0.97		$C_7H_6NO_2^-$	136.0394	136.0399	3.31
		$C_8H_5O_2^+$	133.0280	133.0284	3.31		$C_4H_6NO_3^-$	116.0340	116.0353	11.38
							$C_4H_3O_3^-$	99.0075	99.00822	7.27
TP TP411a or b	412	$C_{18}H_{18}N_7O_5^+$	412.1355	412.1364	2.18	410	$C_{18}H_{16}N_7O_5^-$	410.1213	410.1218	1.22
		$C_{15}H_{14}N_7O_3^+$	340.1143	340.1153	2.82		$C_{14}H_{12}N_7O_2^-$	310.1057	310.1053	-1.45
		$C_{14}H_{14}N_7O_2^+$	312.1195	312.1203	2.56		$C_{14}H_9N_6O_2^-$	293.0782	293.0792	3.41
		$C_{14}H_{11}N_6O_2^+$	295.0918	295.0938	6.78		$C_{13}H_{11}N_6O^-$	267.0996	267.0994	-0.64
		$C_7H_7N_6^+$	175.0725	175.0727	0.97		$C_6H_4NO_4^-$	154.0115	154.1016	-0.64
		$C_7H_5O_2^+$	121.0289	121.0284	-4.05		$C_7H_6NO_2^-$	136.0393	136.0399	4.04
							$C_5H_3N_4^-$	119.0352	119.0363	8.40
							$C_4H_3O_3^-$	99.0075	99.00822	7.27
TP413	A2	$C_{18}H_{20}N_7O_5^+$	414.1520	414.1511	-2.17		$C_{18}H_{18}N_7O_5^-$	412.1367	412.1375	1.94
		$C_{18}H_{18}N_7O_4^+$	396.1417	396.1415	-0.50					
		$C_{14}H_{14}N_7O_2^+$	312.1196	312.1203	2.24					
		$c_{14}H_{11}N_6O_2^+$	295.0905	295.0938	11.18					
		$C_7H_7N_6^+$	175.0724	175.0727	1.49					
TP429a	430	$C_{18}H_{20}N_7O_6^+$	430.1459	430.1470	2.56	428	$C_{18}H_{18}N_7O_6^-$	428.1316	428.1324	1.87
		$C_{18}H_{18}N_7O_4^+$	396.1404	396.1415	2.78		$C_{18}H_{16}N_7O_4^-$	394.1287	394.1269	-4.49
		$C_{15}H_{14}N_7O_3^+$	340.1144	340.1153	2.65		$C_{17}H_{16}N_7O_2^-$	350.1368	350.1371	0.86
		$C_{14}H_{14}N_7O_2^+$	312.1196	312.1203	2.24		$C_{14}H_{12}N_7O_2^-$	310.1055	310.1058	0.97
		$C_{14}H_{11}N_6O_2^+$	295.0929	295.0938	3.05		$C_{14}H_9N_6O_2^-$	293.0784	293.0792	2.73
		$C_7H_7N_6^+$	175.0724	175.0727	1.54		$C_{13}H_{11}N_6O^-$	267.0998	267.1000	0.67
		$C_8H_5O_2^+$	133.0283	133.0284	0.75		$C_{12}H_9N_4O^-$	225.0776	225.0782	2.67
							$C_8H_7N_4O^-$	175.0620	175.0625	3.03
							$C_7H_6NO_2^-$	136.0393	136.0404	8.09
							$C_4H_4NO^-$	82.0286	82.0298	15.12

TP429b	430	$C_{18}H_{20}N_7O_6^+$	430.1460	430.1470	2.32	428	$C_{18}H_{18}N_7O_6^-$	428.1316	428.1324	1.87
		$C_{18}H_{18}N_7O_4^+$	396.1401	396.1415	3.53		$C_{18}H_{16}N_7O_4^-$	394.1239	394.1269	7.69
		$C_{15}H_{14}N_7O_3^+$	340.1143	340.1153	2.82		$C_{17}H_{16}N_7O_2^-$	350.1370	350.1371	0.29
		$C_{14}H_{14}N_7O_2^+$	312.1196	312.1203	2.24		$C_{14}H_{12}N_7O_2^-$	310.1058	310.1058	0.00
		$C_{14}H_{11}N_6O_2^+$	295.0924	295.0938	4.74		$C_{14}H_9N_6O_2^-$	293.0788	293.0792	1.36
		$C_7H_7N_6^+$	175.0724	175.0727	1.54		$C_{13}H_{11}N_6O^-$	267.0999	267.1000	0.30
		$C_8H_5O_2^+$	133.0507	133.0509	1.28		$C_{12}H_9N_4O^-$	225.0776	225.0782	2.67
							$C_8H_7N_4O^-$	175.0625	175.0625	0.17
							$C_7H_6NO_2^-$	136.0393	136.0404	8.09
							$C_5H_3N_4^-$	119.0353	119.0363	-2.69
							$C_4H_3O_3^-$	99.0075	99.0088	12.83
OMTX	442	$C_{19}H_{20}N_7O_6^+$	442.1461	442.1470	1.95	440	$C_{19}H_{18}N_7O_6^-$	440.1316	440.1324	1.82
		$C_{18}H_{18}N_7O_4^+$	396.1380	396.1415	8.84		$C_{14}H_{11}N_6O_3^-$	311.0899	311.0898	-0.29
		$C_{14}H_{13}N_6O_3^+$	313.1035	313.1049	4.50		$C_{12}H_{12}NO_6^-$	266.0669	266.0670	0.41
		$C_{14}H_{11}N_6O_2^+$	295.0931	295.0938	2.37		$C_{11}H_{12}NO_4^-$	222.0766	222.0772	2.61
		$C_{13}H_{13}N_6O^+$	269.1128	269.1145	6.47		$C_{11}H_{10}NO_3^-$	204.0655	204.0666	5.39
		$C_7H_7N_6^+$	175.0725	175.0727	0.91		$C_5H_6NO_3^-$	128.0341	128.0353	9.53
		$C_7H_5O_2^+$	121.0287	121.0284	-2.40					
TP441	442	$C_{19}H_{20}N_7O_6^+$	442.1457	442.1467	2.85	440	$C_{19}H_{18}N_7O_6^-$	440.1330	440.1324	-1.36
		$C_{18}H_{18}N_7O_4^+$	396.1397	396.1415	4.54		$C_{14}H_{11}N_6O_3^-$	311.0895	311.0898	1.00
		$C_{14}H_{13}N_6O_3^+$	313.1033	313.1049	5.14		$C_{13}H_{11}N_6O^-$	267.0998	267.0100	0.67
		$C_{14}H_{11}N_6O_2^+$	295.0935	295.0938	1.02		$C_5H_8NO_4^-$	146.0448	146.0459	7.39
		$C_7H_7N_6^+$	175.0726	175.0727	0.34		$C_5H_6NO_3^-$	128.0342	128.0348	4.45
		$C_6H_5N_4^+$	133.0283	133.0284	0.75		$C_7H_5NO^{\bullet -}$	119.0352	119.0377	20.67
							$C_4H_4NO^-$	82.0285	82.0298	16.34

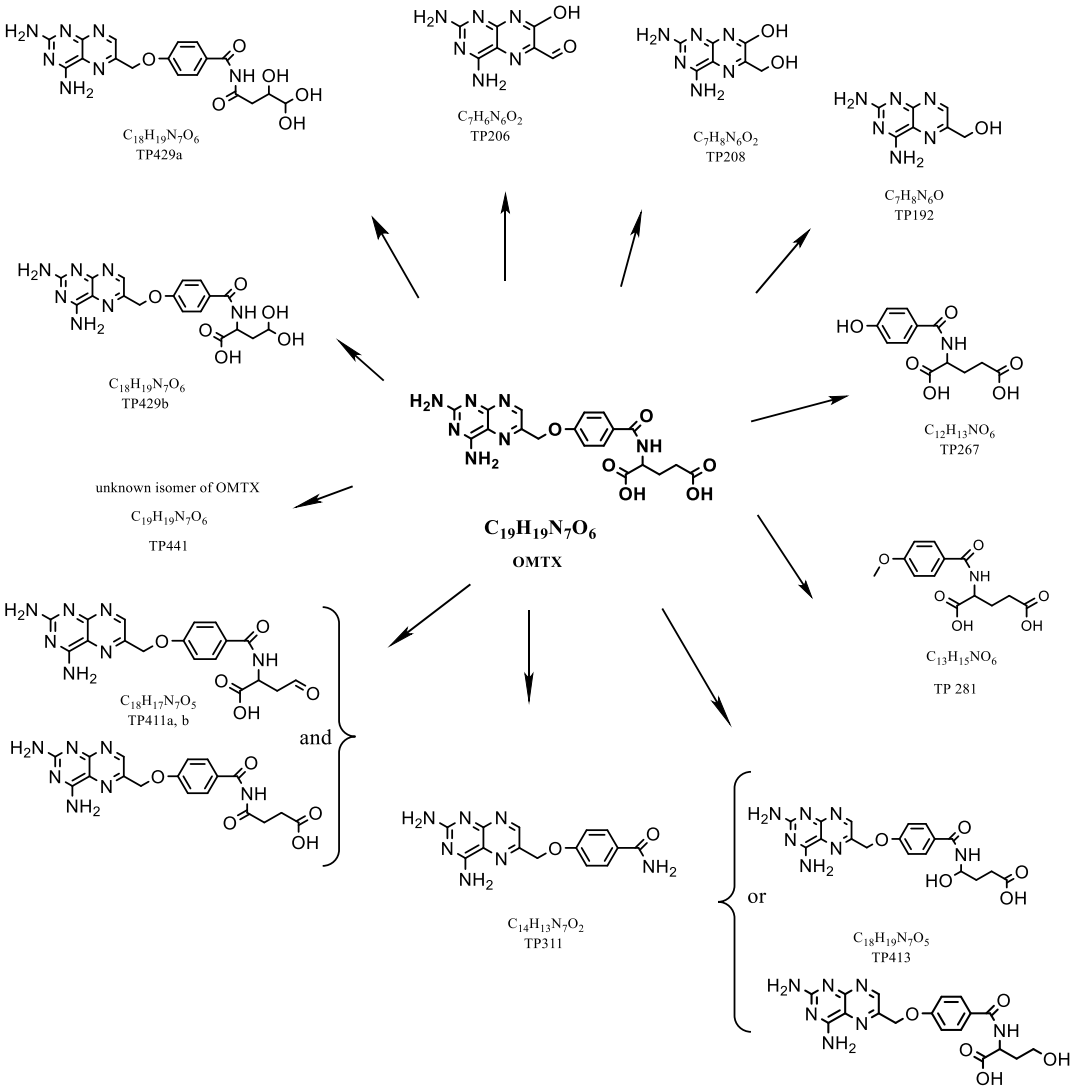
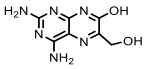
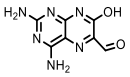
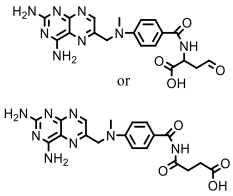
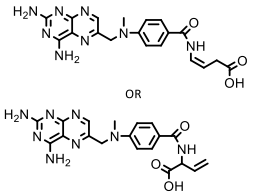
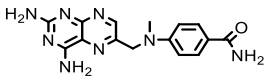
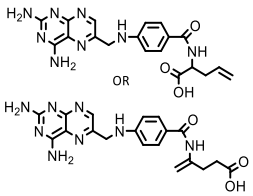


Figure 3.2- S8 Proposed structures of the transformation products of OMTX under irradiation.

Table 3.2- S3 Comparison of TPs abundance at similar residual % of parent compound under 254 nm photo-irradiation

MTX (254 nm / 3h)		**OMTX (254 nm / 15 min)**	
residual **MTX**	23%	residual **OMTX**	18%
		TP192	26%
		TP208	17%
TP208 	42%	TP267	5%
TP206 	21%	TP206	1%
TP424 	20%	TP411b	23%
TP408a 	2%	TP411a	3%
TP324 	6%	TP441	15%
TP408b 	2%		

iv. Monitoring of photodegradation products and cytotoxicity

Under 300-450 nm irradiation conditions, the major photoproducts from OMTX were its isomer TP441 and the pteridine derivative TP192 along the 0-3 h period and TP206 became also predominant at 3 h (Figure 3.2- S9a). At 3 h, the residual OMTX corresponded to 5% of its initial concentration and the TPs abundance (expressed in percentage of the detected area relatively to the OMTX initial area) were 29%, 22% and 15% for TP192, TP441 and TP206 respectively, while all other photoproducts remain under 10 %. In our previous study on MTX (Espinosa et al., 2021) under similar reaction conditions and after 3 h, the MTX residual amount was comparable (3% vs 5% for OMTX as previously mentioned). Its two main photoproducts also corresponded to the pteridine moieties issuing from a bipartite cleavage: the same TP206 with an aldehyde group and its alcohol equivalent TP208 but this cleavage represented almost 100% of the converted MTX while for OMTX the conversion was around 60% (sum of TP192, TP206, TP208 and TP267), while the ~ 40% remaining were attributed to modified or loss of the glutamyl part.

The results obtained with the samples irradiated at 254 nm are discussed in the Main Text and supported by Figure 3.2- S9b. Referring again to our previous study on MTX (Espinosa et al., 2021), the viability reached 100% for both MTX and OMTX irradiated 3 h at 300-450 nm demonstrating the TPs innocuousness on A549 cancer cells. A slight difference may be seen at an earlier degradation state: the viability seems to ride up slowly for MTX in comparison to OMTX: At 1h of irradiation, residual MTX reached up to 47% and the viability attained 58% whereas residual OMTX remains above 60% associated to a viability of 94%. This difference could result from the parent compound OMTX being slightly less cytotoxic or from the generation of MTX's transient TPs being cytotoxic.

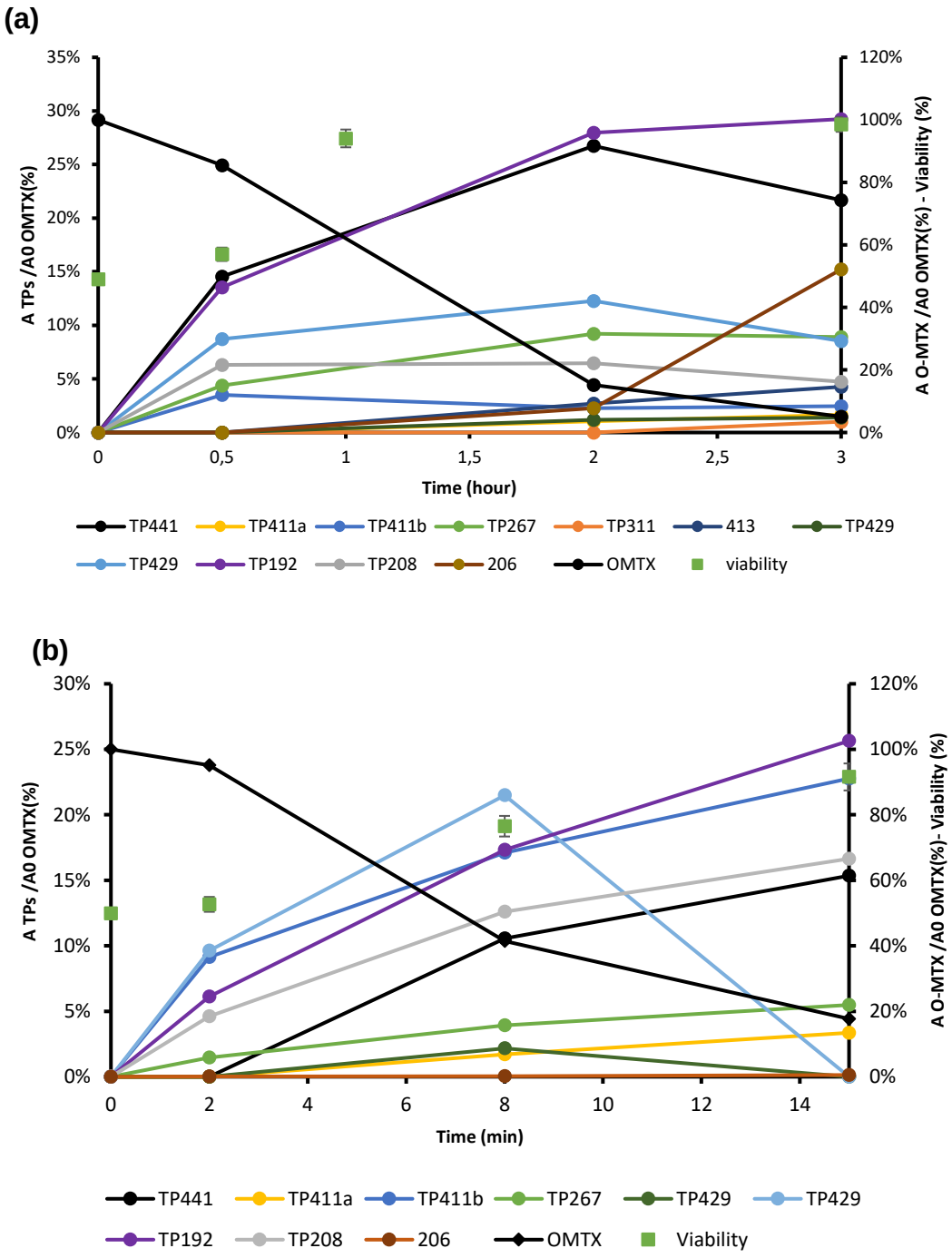


Figure 3.2- S9 Viability evolution (%) *(green square)* of cancer cells with OMTX *(black line)* and TPs *(colored lines*) evolution as function of the irradiation time: (a) 300-450 nm, pH7; (b) 254 nm, pH7.

3.2.1. References

Alouane, A., Labruère, R., Le Saux, T., Schmidt, F., Jullien, L., 2015. Self-Immolative Spacers: Kinetic Aspects, Structure-Property Relationships, and Applications. Angewandte Chemie International Edition 54, 7492–7509. https://doi.org/10.1002/anie.201500088

Benotti, M.J., Trenholm, R.A., Vanderford, B.J., Holady, J.C., Stanford, B.D., Snyder, S.A., 2009. Pharmaceuticals and Endocrine Disrupting Compounds in U.S. Drinking Water. Environmental Science & Technology 43, 597–603. https://doi.org/10.1021/es801845a

Besse, J.-P., Latour, J.-F., Garric, J., 2012. Anticancer drugs in surface waters. Environment International 39, 73–86. https://doi.org/10.1016/j.envint.2011.10.002

Booker, V., Halsall, C., Llewellyn, N., Johnson, A., Williams, R., 2014. Prioritising anticancer drugs for environmental monitoring and risk assessment purposes. Science of The Total Environment 473–474, 159–170. https://doi.org/10.1016/j.scitotenv.2013.11.145

Breithaupt, H., Küenzlen, E., 1982. Pharmacokinetics of methotrexate and 7-hydroxymethotrexate following infusions of high-dose methotrexate. Cancer Treatment Reports 66, 1733–1741.

Calza, P., Medana, C., Sarro, M., Rosato, V., Aigotti, R., Baiocchi, C., Minero, C., 2014a. Photocatalytic degradation of selected anticancer drugs and identification of their transformation products in water by liquid chromatography–high resolution mass spectrometry. Journal of Chromatography A 1362, 135–144. https://doi.org/10.1016/j.chroma.2014.08.035

Edwards, M., Topp, E., Metcalfe, C.D., Li, H., Gottschall, N., Bolton, P., Curnoe, W., Payne, M., Beck, A., Kleywegt, S., Lapen, D.R., 2009. Pharmaceutical and personal care products in tile drainage following surface spreading and injection of dewatered municipal biosolids to an agricultural field. Science of The Total Environment 407, 4220–4230. https://doi.org/10.1016/j.scitotenv.2009.02.028

Espinosa, A., Nélieu, S., Lieben, P., Skarbek, C., Labruère, R., Benoit, P., 2021. Photodegradation of methotrexate in aqueous solution: degradation kinetics and identification of transformation products. Environmental Science and Pollution Research https://doi.org/10.1007/s11356-021-15820-3

Fick, J., Söderström, H., Lindberg, R.H., Phan, C., Tysklind, M., Larsson, D.G.J., 2009. Contamination of surface, ground, and drinking water from pharmaceutical production. Environmental Toxicology and Chemistry 28, 2522. https://doi.org/10.1897/09-073.1

Fram, M.S., Belitz, K., 2011. Occurrence and concentrations of pharmaceutical compounds in groundwater used for public drinking-water supply in California. Science of The Total Environment 409, 3409–3417. https://doi.org/10.1016/j.scitotenv.2011.05.053

Gottschall, N., Topp, E., Metcalfe, C., Edwards, M., Payne, M., Kleywegt, S., Russell, P., Lapen, D.R., 2012. Pharmaceutical and personal care products in groundwater, subsurface drainage, soil, and wheat grain, following a high single application of municipal biosolids to a field. Chemosphere 87, 194–203. https://doi.org/10.1016/j.chemosphere.2011.12.018

Hughes, S.R., Kay, P., Brown, L.E., 2013. Global Synthesis and Critical Evaluation of Pharmaceutical Data Sets Collected from River Systems. Environmental Science & Technology 47, 661–677. https://doi.org/10.1021/es3030148

Johns, D.G., Iannotti, A.T., Sartorelli, A.C., Bertino, J.R., 1966. The relative toxicities of methotrexate and aminopterin. Biochemical Pharmacology 15, 555–561. https://doi.org/10.1016/0006-2952(66)90022-0

Johns, D.G., Loo, T.L., 1967. Metabolite of 4-Amino-4-deoxy-N10-methylpteroylglutamic Acid (Methotrexate). Journal of Pharmaceutical Sciences 56, 356–359. https://doi.org/10.1002/jps.2600560310

Kanjal, M.I., Muneer, M., Abdelhaleem, A., Chu, W., 2020. Degradation of methotrexate by UV/peroxymonosulfate: Kinetics, effect of operational parameters and mechanism. Chinese Journal of Chemical Engineering 28, 2658–2667. https://doi.org/10.1016/j.cjche.2020.05.033

Kim, I., Tanaka, H., 2009. Photodegradation characteristics of PPCPs in water with UV treatment. Environment International 35, 793–802. https://doi.org/10.1016/j.envint.2009.01.003

Kosjek, T., Negreira, N., de Alda, M.L., Barceló, D., 2015. Aerobic activated sludge transformation of methotrexate: Identification of biotransformation products. Chemosphere 119, S42–S50. https://doi.org/10.1016/j.chemosphere.2014.04.081

Kümmerer, K., 2019. From a problem to a business opportunity-design of pharmaceuticals for environmental biodegradability. Sustainable Chemistry and Pharmacy 12, 100136. https://doi.org/10.1016/j.scp.2019.100136

Kümmerer, K., 2017. Sustainable Chemistry: A Future Guiding Principle. Angewandte Chemie International Edition 56, 16420–16421. https://doi.org/10.1002/anie.201709949

Kümmerer, K., 2007. Sustainable from the very beginning: rational design of molecules by life cycle engineering as an important approach for green pharmacy and green chemistry. Green Chemistry 9, 899. https://doi.org/10.1039/b618298b

Kümmerer, K., Dionysiou, D.D., Olsson, O., Fatta-Kassinos, D., 2018. A path to clean water. Science 361, 222–224. https://doi.org/10.1126/science.aau2405

Lai, W.W.-P., Hsu, M.-H., Lin, A.Y.-C., 2017. The role of bicarbonate anions in methotrexate degradation via UV/TiO 2 : Mechanisms, reactivity and increased toxicity. Water Research 112, 157–166. https://doi.org/10.1016/j.watres.2017.01.040

Largeron, M., 2013. Protocols for the Catalytic Oxidation of Primary Amines to Imines: Protocols for the Catalytic Oxidation of Primary Amines to Imines. European Journal of Organic Chemistry 2013, 5225–5235. https://doi.org/10.1002/ejoc.201300315

Leder, C., Rastogi, T., Kümmerer, K., 2015. Putting benign by design into practice-novel concepts for green and sustainable pharmacy: Designing green drug derivatives by non-targeted synthesis and screening for biodegradability. Sustainable Chemistry and Pharmacy 2, 31–36. https://doi.org/10.1016/j.scp.2015.07.001

Lopez, B., Ollivier, P., Togola, A., Baran, N., Ghestem, J.-P., 2015. Screening of French groundwater for regulated and emerging contaminants. Science of The Total Environment 518–519, 562–573. https://doi.org/10.1016/j.scitotenv.2015.01.110

Lutterbeck, C.A., Baginska, E., Machado, Ê.L., Kümmerer, K., 2015. Removal of the anti-cancer drug methotrexate from water by advanced oxidation processes: Aerobic biodegradation and toxicity studies after treatment. Chemosphere 141, 290–296. https://doi.org/10.1016/j.chemosphere.2015.07.069

Mahro, M., Coelho, C., Trincão, J., Rodrigues, D., Terao, M., Garattini, E., Saggu, M., Lendzian, F., Hildebrandt, P., Romão, M.J., Leimkühler, S., 2011. Characterization and Crystallization of Mouse Aldehyde Oxidase 3: From Mouse Liver to *Escherichia coli* Heterologous Protein Expression. Drug Metabolism and Disposition 39, 1939–1945. https://doi.org/10.1124/dmd.111.040873

Montgomery, J.A., Piper, J.R., Elliott, R.D., Temple, C., Roberts, E.C., Shealy, Y.F., 1979. Analogs of methotrexate. Journal of Medicinal Chemistry 22, 862–868. https://doi.org/10.1021/jm00193a021

Moriyasu, A., Sugihara, K., Nakatani, K., Ohta, S., Kitamura, S., 2006. In vivo-In vitro Relationship of Methotrexate 7-Hydroxylation by Aldehyde Oxidase in Four Different Strain Rats. Drug Metabolism and Pharmacokinetics 21, 485–491. https://doi.org/10.2133/dmpk.21.485

Nair, M.G., Bridges, T.W., Henkel, T.J., Kisliuk, R.L., Gaumont, Y., Sirotnak, F.M., 1981. Folate analogs altered in the C9-N10 bridge region. 18. Synthesis and antitumor evaluation of 11-oxahomoaminopterin and related compounds. Journal of Medicinal Chemistry 24, 1068–1073. https://doi.org/10.1021/jm00141a010

Rastogi, T., Leder, C., Kümmerer, K., 2015a. A sustainable chemistry solution to the presence of pharmaceuticals and chemicals in the aquatic environment – the example of re-designing β-blocker Atenolol. RSC Advances 5, 27–32. https://doi.org/10.1039/C4RA10294K

Rastogi, T., Leder, C., Kümmerer, K., 2015b. Re-Designing of Existing Pharmaceuticals for Environmental Biodegradability: A Tiered Approach with β-Blocker Propranolol as an Example. Environmental Science & Technology 49, 11756–11763. https://doi.org/10.1021/acs.est.5b03051

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

Roth, M.E., Green, O., Gnaim, S., Shabat, D., 2016. Dendritic, Oligomeric, and Polymeric Self-Immolative Molecular Amplification. Chemical Reviews 116, 1309–1352. https://doi.org/10.1021/acs.chemrev.5b00372

Sastry, R.V.R.P., Venkatesan, C.S., Sastry, B.S., Mahesh, K., 2016. Identification and characterization of forced degradation products of pralatrexate injection by LC-PDA and LC–MS. Journal of Pharmaceutical and Biomedical Analysis 131, 400–409. https://doi.org/10.1016/j.jpba.2016.08.023

Schymanski, E.L., Jeon, J., Gulde, R., Fenner, K., Ruff, M., Singer, H.P., Hollender, J., 2014. Identifying Small Molecules via High Resolution Mass Spectrometry: Communicating Confidence. Environmental Science & Technology 48, 2097–2098. https://doi.org/10.1021/es5002105

Secrétan, P.-H., Karoui, M., Levi, Y., Sadou-Yayé, H., Tortolano, L., Solgadi, A., Yagoubi, N., Do, B., 2018. Pemetrexed degradation by photocatalytic process: Kinetics, identification of transformation products and estimation of toxicity. Science of The Total Environment 624, 1082–1094. https://doi.org/10.1016/j.scitotenv.2017.12.182

Sirotnak, F.M., Chello, P.L., Moccio, D.M., Piper, J.R., Montgomery, J.A., Parham, J.C., 1980. Analog specific aberrancies in antifolate inhibition of L1210 cell dihydrofolate reductase. Biochemical Pharmacology 29, 3293–3298. https://doi.org/10.1016/0006-2952(80)90306-8

ter Laak, T.L., van der Aa, M., Houtman, C.J., Stoks, P.G., van Wezel, A.P., 2010. Relating environmental concentrations of pharmaceuticals to consumption: A mass balance approach for the river Rhine. Environment International 36, 403–409. https://doi.org/10.1016/j.envint.2010.02.009

Vel Leitner, N.K., Berger, P., Legube, B., 2002. Oxidation of Amino Groups by Hydroxyl Radicals in Relation to the Oxidation Degree of the α-Carbon. Environmental Science & Technology 36, 3083–3089. https://doi.org/10.1021/es0101173

Vulliet, E., Cren-Olivé, C., Grenier-Loustalot, M.-F., 2011. Occurrence of pharmaceuticals and hormones in drinking water treated from surface waters. Environmental Chemistry Letters 9, 103–114. https://doi.org/10.1007/s10311-009-0253-7

Wu, C., Spongberg, A.L., Witter, J.D., Fang, M., Czajkowski, K.P., Ames, A., 2010. Dissipation and Leaching Potential of Selected Pharmaceutically Active Compounds in Soils Amended with Biosolids. Archives of Environmental Contamination and Toxicology 59, 343–351. https://doi.org/10.1007/s00244-010-9500-y

Zhao, S., Ma, H., Wang, M., Cao, C., Xiong, J., Xu, Y., Yao, S., 2010. Role of primary reaction initiated by 254 nm UV light in the degradation of p-nitrophenol attacked by hydroxyl radicals. Photochemical & Photobiological Sciences 9, 710. https://doi.org/10.1039/b9pp00187e

Chapitre 4. Etude de la biodégradation des deux analogues et de leurs impacts écotoxicologiques

Après avoir étudié la dégradation abiotique du MTX et du OMTX dans le chapitre précédent, ce troisième chapitre porte sur leur biodégradation. L'évolution de ces deux composés et de leurs TPs a été étudiée lors d'une incubation en présence de boue activée issue d'une station d'épuration. La cytotoxicité du mélange et les potentiels effets sur les communautés microbiennes de ces boues ont été évalués. Ce chapitre est découpé en deux parties. La partie **4.1** présente le développement analytique de la méthode UHPLC-MS/MS nécessaire au suivi des produits parents et des produits formés (TPs) dans différentes matrices : le surnageant de la boue activée ainsi que l'eau MilliQ et l'eau Volvic qui sont utilisées dans les tests d'écotoxicologie aquatique présentés au **Chapitre 5.** La partie **4.2** décrit la mise en place de l'incubation en présence de boue activée, l'évolution au cours du temps des composés parents et de leurs TPs respectifs, l'évolution de la cytotoxicité du mélange, ainsi que l'évaluation des potentiels effets sur les microorganismes de la boue activée au travers de différents indicateurs.

La première sous partie (**4.1**) est présentée sous format article car certains résultats serviront de « Supplementary Information » à un article portant sur la deuxième sous partie (**4.2**).

4.1. Développement analytique nécessaire au suivi des TPs

L'analyse par UHPLC-MS/MS du surnageant d'une boue dopée avec le MTX ou le OMTX vise à réaliser le suivi des composés parents et de leurs TPs. Le choix d'analyser uniquement le surnageant de la boue et non la boue elle-même, est appuyé par le fait que les composés étudiés sont majoritairement présents en phase aqueuse. Le développement de cette méthode analytique a bien pour objectif d'étudier les composés lors d'une incubation de boue, dopée avec une concentration de MTX et OMTX plus élevée que les concentrations environnementales. Il ne s'agit donc pas de développer une méthode analytique pour analyser des échantillons prélevés *in situ*. Contrairement à la méthode utilisée pour le suivi des composés lors de la photodégradation en eau MilliQ présentée au **Chapitre 3** (UHPLC-MS/MS en mode full scan), les expériences dans une matrice complexe telle que le surnageant de la boue activée ne permettrait pas de distinguer les pics chromatographiques pertinents directement depuis le courant d'ions total (Kosjek et al., 2015). C'est pourquoi une méthode plus spécifique et plus sensible telle que la détection en mode « multiple reaction monitoring » (MRM) est nécessaire, mais en contrepartie cette

méthode limite l'étude à l'observation de TPs ciblés. Il est probable que les mécanismes mis en jeu lors de la biodégradation soient différents de ceux de la photodégradation et mènent à la formation de TPs différents de ceux identifiés précédemment. Afin de développer une méthode complète et au vu du nombre limité d'étalons analytiques, des TPs suspectés dans le cas de la biodégradation du MTX, d'après Kosjek et al. (2015) et des structures équivalentes proposées pour OMTX ont été ajoutés à la liste des TPs identifiés lors des expériences de photodégradation. Contrairement aux structures des photo produits confirmés en haute résolution, ces nouvelles structures n'ont donc pas été identifiées formellement car l'étude a été réalisée en utilisant un spectromètre de masse de basse résolution. Finalement, une méthode simple, rapide, sans préparation spécifique d'échantillons et sur de faibles volumes a été développée pour permettre le suivi des TPs du MTX et du OMTX dans le surnageant de boues activées lors de l'incubation présentée en **Chapitre 4.2**. A des fins de comparaison et d'application potentielle dans d'autres contextes, le développement a été réalisé en parallèle dans l'eau MilliQ ainsi que dans une eau « de source » (eau Volvic) : ces compléments ont été utilisés pour les études écotoxicologiques présentées en **Chapitre 5**.

4.1.1. Introduction

Since their first detection in the aquatic environment in the 1970s (Hignite and Azarnoff, 1977), there is a growing interest in environmental contamination by pharmaceuticals. Anticancer drugs represent a low part of studies although they are of major interest due to their cytotoxicity, genotoxicity, mutagenicity and teratogenicity. Kosjek and Heath (2011) highlighted that one possible reason is their low environmental concentration levels in natural water (ng/L). Such a detection level became possible only in recent years thanks to the evolution of the analytical instrumentation and methods. Their occurrences in natural water are due to their partial removal of pharmaceuticals in WWTP which is strongly influenced by their physico-chemical properties. These properties include dissociation constant (pKa), octanol-water partition coefficient (Kow), organic carbon partition coefficient (Koc) among others (Kosjek and Heath, 2011). As most cytotoxic agents, MTX is highly polar (Rowney et al., 2009). At environmental pH of 7, it is likely to be dissociated thus increasing its aqueous mobility (Kosjek and Heath, 2011). With a log Kow < 1, it remains in the water phase. Therefore, MTX is unlikely to be eliminated by sorption onto sewage sludge (Kosjek et al., 2011) and is more susceptible to be biodegraded (Kiffmeyer et al., 1998; Kosjek et al., 2015). These physico-chemical properties have also to be considered when developing analytical procedures to detect and quantify the compounds in different

environmental matrices. Most published methods on the determination of pharmaceutical levels in aqueous solution are based on offline solid phase extraction (SPE) followed by LC-MS/MS (Castiglioni et al., 2005; Roberts and Thomas, 2006; Okuda et al., 2009; Langford and Thomas, 2009; Verlicchi et al., 2012; Galaon et al., 2017; Santos et al., 2018). In the case of MTX, limits of quantification (LOQs) from 0.3 to 6 ng/L were achievable thanks to SPE techniques (Castiglioni et al., 2005 ; Garcia-Ac et al., 2009; Martín et al., 2011; Ferrando-Climent et al., 2013). This pre-concentration technique is the most popular one for drug analysis and has become an essential tool in laboratories all over the world (Pavlovic et al., 2007). Offline SPE offers excellent performances, but its main disadvantage is the extensive labour required for sample preparation and the need for large sample volumes. For example, Martín et al. (2011) have obtained some of the lowest limit of detection (LOD), but they needed 250 mL of sample.

Online SPE is a robust technique for the rapid extraction and pre-concentration of aqueous samples. It also limits sample losses and contamination by human manipulation. It has already been used for MTX analysis in aqueous phase of WWTP (Garcia-Ac et al., 2009 ; Rabii et al., 2014) and would be suitable for the monitoring of a laboratory scale incubation, which only allowed small sample volumes.

The analytical method was developed based on previously identified MTX's and OMTX's TPs produced through an irradiation (Espinosa et al., 2021 and Espinosa et al., submitted) leading to a list of 11 TPs for MTX and 12 for OMTX, respectively. The analytical standards of these compounds were not available, except for (i) parent compounds (ii) three well-known metabolites or TPs of MTX, as the 2,4-diamino-N10-methyl-pteroic acid (DAMPA), the 7-hydroxymethotrexate (7-OH-MTX) and the 2,4-diamino-6-pteridinecarboxylic acid (AMPC) (Widemann et al., 2000) also known to be formed in activated sludge (Kiffmeyer et al., 1998 ; Kosjek et al., 2015) and (iii) one TP of OMTX, synthesised by ICMMO, the 4-(hydroxybenzoyl)-L-glutamic acid identified during photodegradation experiment named TP267 by Espinosa et al. (submitted). Moreover, other compounds could be produced in complex matrices as activated sludge (AS), especially by microorganism activities, so the initial TP list would be incomplete, especially in the case of OMTX because no literature is available for this molecule. Therefore, we proposed to enrich this list with potential TPs based on identified biodegradation products proposed by Kosjek et al. (2015) in the case of MTX and on their hypothetical equivalent structure for OMTX. For that purpose, the molecular ions of these potential TPs were included in the detection method, and then fragmentation studies were performed for these ions to obtain specific and sensitive MRM methods for the monitoring of these compounds.

The aim of this study was to develop a rapid and simple analytical method for the quantification of both MTX and OMTX and their respective TPs for a) their monitoring during the incubation of an

activated sludge at laboratory scale (working concentrations around 1 mg/L) by the analysis of the supernatant (**Chapter 4.2**), and b) their analyses in MilliQ and Volvic water for aquatic ecotoxicological tests (**Chapter 5**). The first part of this section presents the analytical optimizations and the strategy to enrich the method with suspected TPs, while the second part is about assessing the method's characteristics in terms of LOD, LOQ, linearity and matrix effect. The stability of molecules after sampling and the potential adsorption onto the sludge were also assessed to guarantee a pertinent exploitation of biodegradation experiment results.

4.1.2. Materials and methods

4.1.2.1. Chemicals and reagents

Methotrexate (MTX, CAS 59-05-2, > 98%) was obtained from Sigma Aldrich (St Quentin Fallavier, France), perchloric acid ($HClO_4$ 70%-72%) and NaOH 1 mol/L from Merk (Darmstadt, Germany). HCl 1 mol/L was prepared from a commercial 37% solution (Carlo Erba, Val de Reuil, France). Water was supplied by a Milli-Q water system (Millipore, Saint-Quentin-en-Yvelines, France). The analyses were performed using formic acid (98%-100%, Sigma Aldrich), acetonitrile (RS plus gradient, Carlo Erba) and methanol (RPE, Carlo Erba). OMTX and T267 were provided by Raphael Labruere, ICCMO and synthesised as described by Espinosa et al. (submitted). 2,4-diamino-N10-methyl-pteroic acid (DAMPA, CAS 19741-14-1, 100%); 7-Hydroxy Methotrexate Sodium Salt (7-OH-MTX,CAS 5939-37-7, >95%) and the 2,4-diaminopteridine-6-carboxylic acid (AMPC, CAS 716-74-5, ≥98%) were obtained from SCBT. The physico-chemical and biological properties of MTX and its dd analogue OMTX in Table 4.2- S1 (**Chapter 4.2**).

4.1.2.2. Aqueous samples

Volvic water was used to represent natural surface water in term of ionic composition and strength (MS at 180 °C of 130 mg/L and pH 7). Activated sludge (AS) was collected from a local municipal WWTP of Plaisir (Yvelines, France) with a capacity to process 7268 m^3 of water per day (data from 2019) from an aeration basin and its characterization are given in Table 4.2- S2.

4.1.2.3. Final analytical conditions for online-SPE-UHPLC-MS/MS

The analytical method was developed on an UHPLC-MS/MS triple quadrupole mass spectrometer (Acquity-TQD, Waters); detailed parameters are presented in the results. In addition, a two-position valve

was used to switch between the loading and elution modes of the online SPE cartridge (Oasis HLB, 2.1 x 20 mm, 25 µm particle size, Waters). The mobile phase was composed of water + 0.05% of formic acid (A) and acetonitrile + 0.05% of formic acid (B). The sample was loaded on the SPE cartridge with 100% A at 1 mL min^{-1} during 1.9 min. The equilibrium of the UHPLC column was kept with an isocratic pump (model 515, Waters) during the loading step of the SPE cartridge. Then, after switching the valve to the elution mode, the molecules retained on the SPE cartridge were eluted to the UHPLC column (C18 Gravity-SB Column, 100 x 2 mm, 1.8 µm particle size, Macherey-Nagel) in a back-flush mode with the same gradient profile described previously (Espinosa et al., 2021). MS/MS parameters were optimised for each compound are presented Tables 4.1- 1 and 4.1- 2.

4.1.3. Method development

4.1.3.1. SPE Optimisation

Because of the limited number of analytical standards (MTX, DAMPA, AMPC, 7-OH-MTX, OMTX and TP267), optimization was started on the MTX's photodegradation products. HLB cartridge type was chosen according to Ferrando-Climent et al. (2013) who showed that such material provided higher recoveries for all cytotoxic compounds including MTX compared with cationic exchange sorbent Oasis MCX and anionic exchange sorbent Oasis MAX.

The maximal injection volume for online-SPE was determined to avoid the loss of compounds through the SPE cartridge during the loading step by successive injections from 200 µL to 1000 µL, by 200 µL steps. Potential effects of pH were assessed by an adjustment of the samples at pH 2 or pH 7 with HCl or NaOH (0.05M).

The possible losses due to the online-SPE were evaluated and compared to offline SPE. Three ways of injections were done and for a direct comparison of peak areas. TPs were produced through a photo irradiation and an offline SPE extract obtained according to the method described in Espinosa et al. (2021) and were diluted in matrices to obtain the same injected amount (according to the concentration factor due to pre-concentration technique and injected volume) (Figure 4.1- 1):

1) Direct injection (6 µL) of a methanolic extract from offline SPE of a photo irradiated MTX juice as used for the identification of TPs Espinosa et al. (2021)

2) Online-SPE injection (1000 µL) of the same extract diluted with an appropriated factor

3) Online-SPE injection (1000 µL) of the photo irradiated MTX juice without manual pre-concentration and diluted with an appropriate factor.

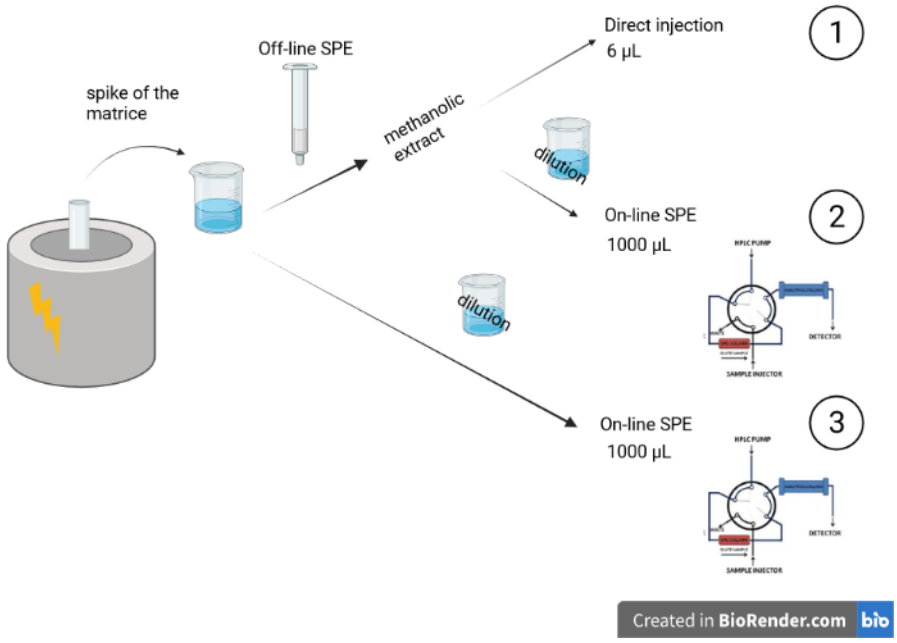


Figure 4.1- 1 Three ways of sample preparation and injection corresponding to 1), 2), and 3)

Created with BioRender.com

4.1.3.2. Identification of biodegradation products

Additionally to the standards and identified photoproducts (**Chapter 3**), the method was enriched with potential biodegradation products. To identify them, a simplified biodegradation experiment was performed with diluted activated sludge for both MTX and OMTX molecules following the protocol described in **Chapter 4.2**. Briefly, it consisted in spiking diluted activated sludge with MTX or OMTX and analysing the supernatant sampled at different times. A list of suspected biodegradation products was established according to Kosjek et al. (2015), and when their pseudo molecular ions $[M + H]^+$ were found in LC-MS. Then, the MS/MS parameters were optimised as described in 4.1.3.3ii. Because during photodegradation monitoring, some TPs, as pteridine derivatives, were only detected by ESI in positive mode, we chose to develop the analytical method only in this positive mode. As a matter of fact, we noticed a better sensitivity for all photoproducts, in accordance with Calza et al. (2014) and Negreira et al. (2013).

4.1.3.3. MS/MS parameters optimisation

i. For available standards :

For MTX, DAMPA, 7-OH-MTX, AMPC and TP267, direct infusion was carried out to: (1) select precursor ions in full-scan mode ions corresponding to the protonated molecules $[M + H]^+$ in the positive ionisation mode; (2) optimise sample cone and capillary voltage for each precursor ion; (3) select one or two daughter ions in daughter ion-scan mode, and (4) optimise collision energy for each daughter ion. The most abundant product ion (I) was selected for the quantification transition (precursor ion > product ion I) and the second most abundant product ion (II) was selected for the confirmation transition when possible (precursor ion > product ion II).

ii. For identified transformation products :

No standards were available for most of the TPs, identified for the first time or not. In order to optimise mass spectrometer parameters, those TPs were produced through photo irradiation and an offline SPE extract obtained according to the method described in Espinosa et al. (2021) was diluted in MilliQ water. The same procedure was done for aqueous sample experiment or for biodegradation experiment (**Chapter 4.2**). With the obtained spiked solutions, we proceeded to successive LC-MS/MS injections:

(1) In LC-MS full scan mode, varying the cone voltage by step of 5 V allowed to choose the best voltage based on the highest peak area for each compound after extracting the mass of interest from the chromatogram;

(2) In LC-MS/MS daughter scan mode at two collision energies (20 and 35 eV) for each precursor ion optimised cone voltage, in the way to produce daughter ions. The two most abundant ones were selected for the last step;

(3) In LC-MS/MS MRM mode, varying collision energy by steps of 2 eV for each transition chosen for each compound. The best collision energy based on the highest daughter ion peak area was selected for each transition.

4.1.3.4. Biodegradation and adsorption test

To ensure that the molecule disappearance observed in sludge supernatant during the incubation was due to biodegradation, some specific experiments were performed (method will be presented in 4.2.3.3) that consisted in incubating diluted activated sludge spiked with MTX or OMTX at 1 mg/L. Supernatants were analysed at different time steps. The amounts of remaining compounds (expressed in % of initial amounts) were compared with another incubation in presence of the biocide NaN_3 at 1

g/L, to stop (or at least to limit) the biodegradation process and possibly to evidence the occurrence of other processes such as adsorption. Experiments were done in in triplicate and in the dark to avoid photodegradation.

4.1.3.5. Stability of the parent compounds in sludge supernatant

To ensure that the molecule disappearance observed in sludge supernatant appeared during the incubation and was not due to instability, after sampling, we performed a centrifugation step that allowed to separate microorganisms from the supernatant and limit the biodegradation. Samples were stored at 10°C in the autosampler until their injection. Diluted activated sludge was spiked with 1 mg/L of MTX or OMTX (final concentration), then triplicate samples were immediately centrifuged at 12100g for 10 min in a MiniSpin centrifuge (Ependorf), and distributed in different vials for injection at different times (0, 10 and 48 h). Samples were stored at 10°C in the autosampler until their injection.

4.1.3.6. Characterization of the method

i. Limits of detection and quantification

Limits of detection (LODs) and limits of quantification (LOQs) were determined as the minimum detectable or quantifiable amount of each analyte available as standards with a signal-to-noise of 3 and 10, respectively. They were calculated from spiked samples obtained by successive dilutions with the available standards for the three studied aqueous matrices studied: MilliQ water, Volvic water and activated sludge supernatant.

ii. Calibration curve

Calibrations ranging from 25 ng/L to 1 mg/L were prepared by successive dilution except for the concentration of 1 µg/L to limit correlated error. Calibration curves, with at least 4 concentration levels, used a weighted 1/x quadratic regression function (equation 1). The correlation quadratic coefficient (r^2) was higher than 0.99 with residuals lower than 30%

$$Y = a_0 + a_1x + a_2x^2$$

Eq1.

iii. Matrix effects

Matrix effects in mass spectrometry were determined following equation 2:

$$\text{Matrix effect (\%)} = ((A_{extract} - A_{control}) - 1) / A_{water}) \times 100 \quad \text{Eq2.}$$

Where A extract is the area of the compound in the spiked extract; A control is the area of the compound in a negative control, which consists in a non-spiked extract (area supposed to be equal to 0); and A water is the area of the compound in MilliQ water spiked at the same concentration level and in which the matrix effect is not supposed to occur.

iv. Interday precision

Interday precision was assessed only for supernatant, at a concentration of 10 µg/L. Supernatants were spiked and injected each day along the biodegradation monitoring presented in **Chapter 4.2** because analyses lasted 3 days and the device response had to be controlled during all the analysis time.

4.1.4. Results and discussion

4.1.4.1. SPE optimisation

Effects of the injection volume were assessed from 200 to 1000 µL at acidic and neutral pH to determine the maximal injection volume avoiding the loss of compounds through the SPE cartridge during the loading step (breakthrough).

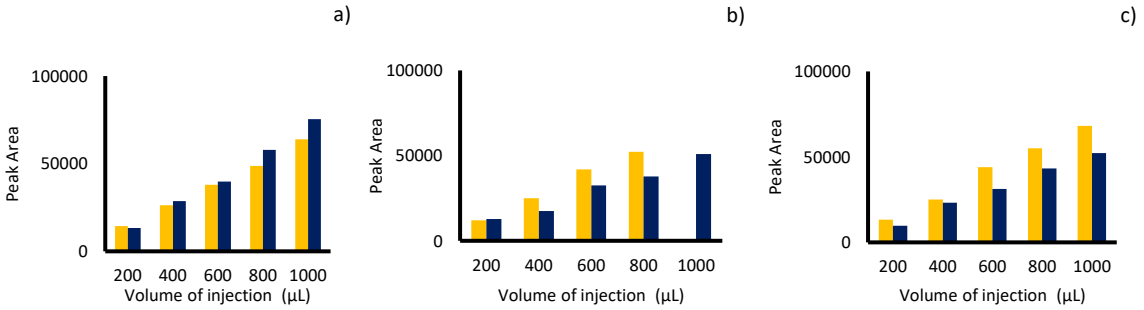


Figure 4.1- 2 Effects of the injection volume (online-SPE) on the MTX peak areas according to pH (pH 2 in yellow and pH 7 in blue) of aqueous sample.

a) MilliQ, b) Volvic (data missing for 1000 µL) and c) sludge supernatant

Considering the volume of injection, as shown for MTX in all matrices (Figure 4.1- 2), the highest peak areas were obtained with the highest volume of sample whatever the pH. This result was similar

for all detected photoproducts. It meant that the breakthrough did not occur for these compounds with 1 mL injection.

Considering pH effects, a pH of 7 (Figure 4.1- 2, blue bars) tended to generate higher areas in a MilliQ water sample (a) while pH 2 (Figure 4.1- 2, yellow bars) tended to better for Volvic (b) or sludge supernatant samples (c). Actually, the protonation on the MTX amino groups could make it suitable for cation-exchange SPE (Castiglioni et al., 2005 ; Negreira et al., 2013). But this pH effect depended on the compound. The following example is given for a 800 µL injection because data was missing for the 1000 µL Volvic one. TP266 (a) presented peak area slightly higher with pH 7 while for TP324 (b) peak areas were largely higher at pH 2 (Figure 4.1- 3). However, we did not find any unanimous trends and no significant differences were observed when the compounds were at high concentration (< 30% of variation of the area in case of MTX). Finally, the compromise was in favour of pH 7 because we have noticed a weak stability of OMTX in acidic water [8]. Additionally, this pH corresponded to the original pH of the studied environmental matrices. The choice to keep samples at their initial pH would be time saving with no additional step in sample preparation.

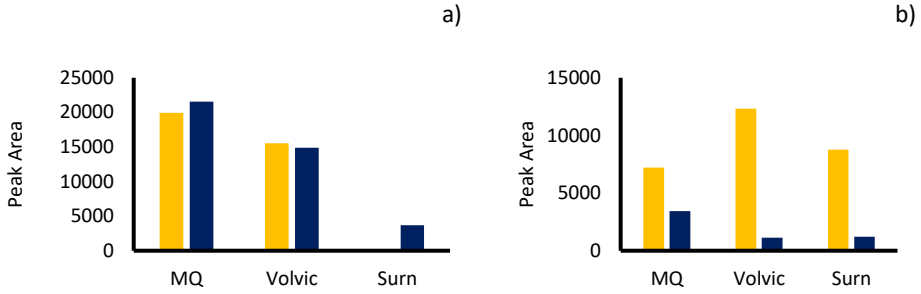


Figure 4.1- 3 Effects of the sample pH at pH 2 in yellow and pH 7 in blue (800 µL injection volume with online-SPE) for a) TP266 and b) TP324

Finally, the possible losses due to the online-SPE were evaluated and compared to offline SPE. The results for the MTX area for the 3 ways of injections are presented in Figure 4.1- 4. The online-SPE injection (2 and 3) did not reveal any losses compared to the direct injection (1). On contrary, the peak areas were higher than those for direct injection. The smallest areas obtained for direct injection could be due to the solvent of the sample that was 100% methanol. It generated large peaks and low precision for peak integration. The small difference (no replicates) between offline combined to online-SPE (2) and online-SPE (3) injections highlighted the good recovery of the offline SPE extraction. Finally, the online-

[8] Annexe 1 : Stabilité des composés MTX et OMTX à pH3 et pH7

SPE method (3) was suitable for developing our method. The results for almost all products showed these same trends.

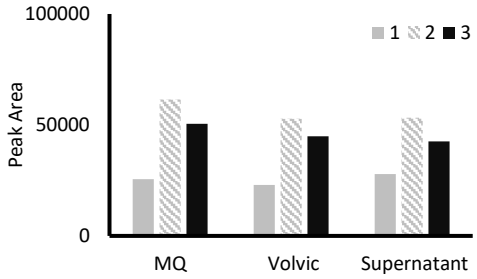


Figure 4.1- 4 MTX peak areas according to the sample preparation described in figure 4.1- 1 with (1) off line-SPE and direct injection (2) Offline combined to online-SPE injection and (3) Online-SPE injection

In conclusion, an online-SPE injection of a 1000 µL sample at pH 7 was chosen for allowing a rapid and simple sample preparation in agreement with the need of high frequency of sampling and the small available volumes to perform the monitoring of the incubation.

4.1.4.2. Identification of biodegradation products

As previously mentioned (see introduction 4.1.1), the list of TPs obtained from photodegradation was implemented with suspected biodegradation products from literature, which could be produced during the biodegradation experiments. For MTX, the three known biodegradation products according to Kosjek et al. (2015) for which standards were commercially available (7-OH-MTX, DAMPA and AMPC) were found in the sludge supernatant in the experiments described in 4.1.3.2.

Concerning the use of the standard AMPC, its mass and fragmentation lead to same results than the previously identified TP206 (Espinosa et al., 2021). Finally, the structure of TP206 we hypothesized, according to Lutterbeck et al. (2015) and Lai et al. (2017), could be the AMPC one, as proposed by Kosjek et al. (2015) and Kanjal et al. (2020). The lack of TP206 standard, and the weak information from fragmentation led to an hypothesis with a confidence level of 2b according to Schymanski et al. (2014).

For other suspected TPs, $[M+H]^+$ potentially corresponding to TP341 TP311k, TP190 and TP151 were also found (see the chemical structures proposed in Table 4.2- S3). In addition, in the case of TP341, two chromatographic peaks were present (retention time of 10.82 and 15.66 min) corresponding to two different compounds (Figure 4.1- 5)

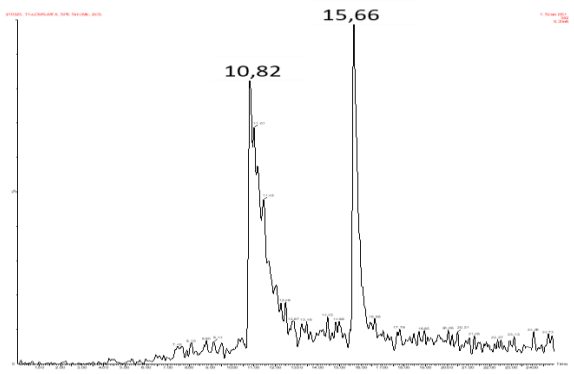


Figure 4.1- 5 Extracted ion chromatogram of $[M+H]^+$ = 342 in LC-MS full scan mode

After a new injection in CAD mode, the LC-MS/MS daughter mode revealed two different spectra (Figure 4.1- 6), corresponding to two different structures. However, without the use of high-resolution mass spectrometry, only hypotheses could be proposed, e.g. according to Kosjek et al. (2015) and Widemann et al. (2000), but another possibility was also considered (Figure 4.1- 7). Finally, the choice of major fragment for both compounds lead to two specific MRM transitions (TP341a and TP341b) for their detection and quantification (Figure 4.2- S1).

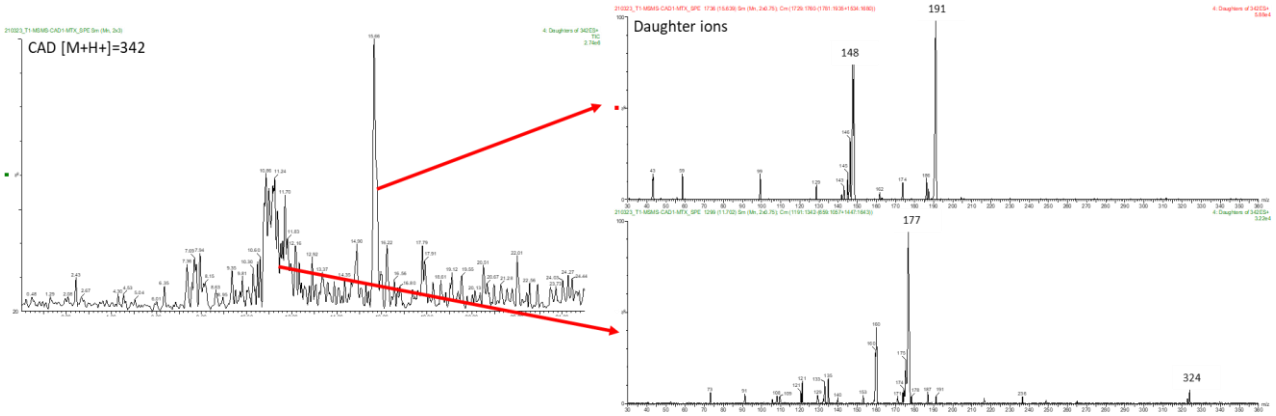
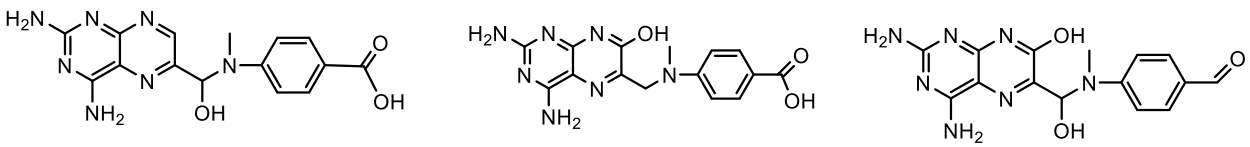


Figure 4.1- 6 LC-MS/MS daughter mode of [M+H]+ = 342



Kosjek et al., (2015) Widemann et al., (2000) ?

Figure 4.1- 7 Some hypothetical structures for TP341

Concerning OMTX, we assumed that equivalent biodegradation products of MTX could be formed, and their pseudo-molecular ions were searched in LC-MS. The hypothetical 7-OH-OMTX corresponding to the toxic 7-OH-MTX was found, as already detected during cytosol incubation (Espinosa et al., submitted), as well as the ODAMPA corresponding to the major DAMPA previously introduced, and finally TP328, a potential equivalent of TP341 previously presented Figure 4.1- 7. A summary of all structures (identified and hypothetical) is presented in Table 4.2- S3. Possible MS fragments of ODAMPA (Figure 4.1- 8) are presented in Figure 4.1- 9. Fragmentation of TP328 did not supply specific information about its structure (i.e. key fragment ions), and the 7-OH-OMTX one was already presented in Espinosa et al. (submitted).

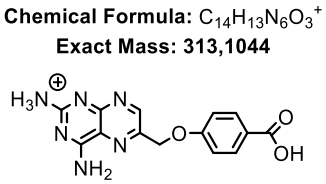


Figure 4.1- 8 ODAMPA structure in ESI positive mode

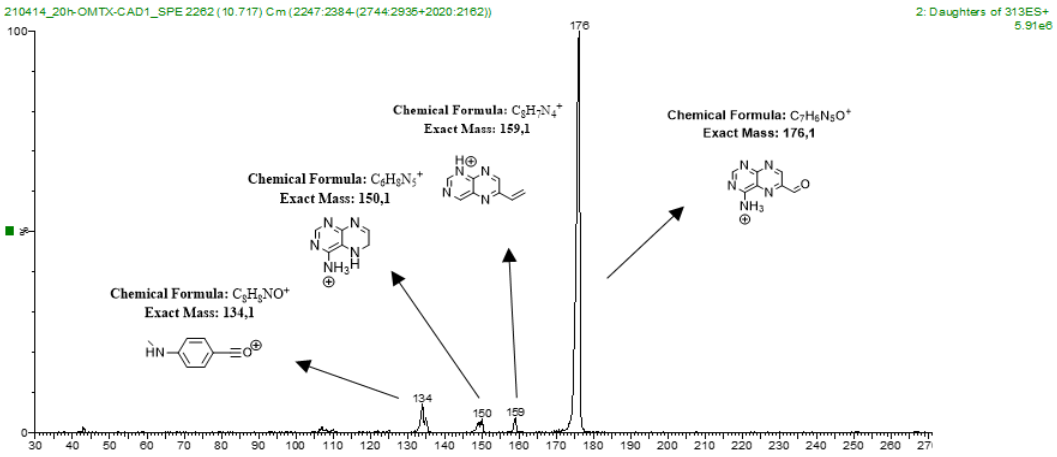


Figure 4.1- 9 Hypothetical fragments of suspected ODAMPA obtained in LC-MS/MS daughter mode of $[M+H]^+$ = 313

4.1.4.3. MSMS parameters optimisation

The cone voltage, the choice of 2 transitions and the collision energy are presented in Table 4.1- 1 for MTX, and Table 4.1- 2 for OMTX and their respective TPs. They were optimised by injection (see 4.1.3.3), but when it was not possible to optimize because of their minor production through

photodegradation, energies were fixed according to prior observation during identification (TP 424a or b, TP429a or b, TP411 a or b and TP281). The corresponding structures are presented in Table 4.2- S3.

Table 4.1- 1 MS–MS parameters (retention time, cone voltage; quantification and confirmation transitions; collision energy) for the selected compounds for the monitoring MTX method

(detected during P: photodegradation; B: Biodegradation)

			Quantification		Confirmation	
Molecule	**tr**	**Cone Voltage (V)**	**Transition**	**Coll energy (eV)**	**Transition**	**Coll energy (eV)**
7-OH-MTX (B)	14.9	22	471.1 > 191.1	20	471.1 > 324.1	20
MTX	10.9	40	455 > 308	20	455 > 134	34
TP 440 (P)	9.7	30	441 > 294	28	441 > 120	36
TP 424a or b (P)	13.5 or ?	32	425 > 308	22	425 > 134	38
TP 408a or b (P)	10	32	409 > 308	20	409 >134	36
TP 408a or b (P)	10.9	32	409 > 308	20	409 >134	36
TP 341a or b (B)	10.8	22	342 > 176.8	24	342 > 324	16
TP 341a or b (B)	15.6	22	342 > 191	26	342 > 147.6	40
DAMPA (B)	10.9	48	326.1 > 175.1	20	326.1 > 308.1	20
TP 324 (P+B)	8.9	45	325 > 308	16	325 >134	22
TP 311k (B)	9.4	40	312 > 175.2	18	312 > 120	22
TP 280 (P)	13	20	281 > 134	14	281 > 106	36
TP 266 (P+B)	9.9	22	267 > 120	28	267 > 92	38
TP 250 (P)	15.7	20	251 > 134	14	251 > 106	32
TP 208 (P)	4	35	209 > 191	18		
AMPC (B)	4.4	35	207> 163.1	16		
TP 190 (B)	15.86	40	191 > 147.6	18	191 > 105.2	26
TP 151 (B)	13.1	22	152 >137.14	22	152 > 120.13	22

c) **Table 4.1- 2** MS–MS parameters (retention time, cone voltage; quantification and confirmation transitions; collision energy) for the selected compounds for the monitoring OMTX method

(detected during P: photodegradation; B: Biodegradation)

			Quantification		Confirmation	
Molecule	**tr**	**Cone Voltage (V)**	**Transition**	**Coll energie (eV)**	**Transition**	**Coll energie (eV)**
7-OH-OMTX (B)	13.7	40	458.2 > 191	35	458.2 > 148.1	35
OMTX	10.7	52	442 > 175	28	442 > 313	20
TP 441 (P)	15.42	52	442 > 175	28	442 > 295	20
TP 429a or b (P)	10 or ?	40	430 > 396	15	430 > 340	22
TP 411a or b (P+B)	11.4 or ?	45	411.9 > 175	28	411.9 > 312.1	18
TP 328 (B)	14.6	22	329 > 190.7	22		
ODAMPA (B)	10.7	35	313.2 > 176	35	313.2 > 133.6	35
TP 311 (P)	10.75	40	312.2 > 175	36	312.2 > 295	28
TP 281 (P)	?	40	282 > 121	40		
TP 267 (P)	13.2	35	268 > 121.1	16	268 > 93.2	24
TP 208 (P)	4	35	209 > 191	18		
AMPC (B)	4.4	35	207 > 163.1	20		
TP 192 (P)	3.7	35	193 > 193	20		

4.1.4.4. Biodegradation and adsorption tests

The supernatants of the biodegradation and adsorption tests were analysed at 0, 7 and 14 hours, in presence or absence of NaN_3, in order to determine if adsorption occurred between the parent compounds and particulate phase of sludge. This process could lead to systematic underestimation of the concentration and could be considered as a displacement of the pollution from the aqueous to the solid phase (Seira et al., 2013). A decrease of MTX and OMTX peak areas was observed when incubation time increased (Figure 4.1- 10) and this decrease was more important in absence of NaN_3 than in presence of the biocide.

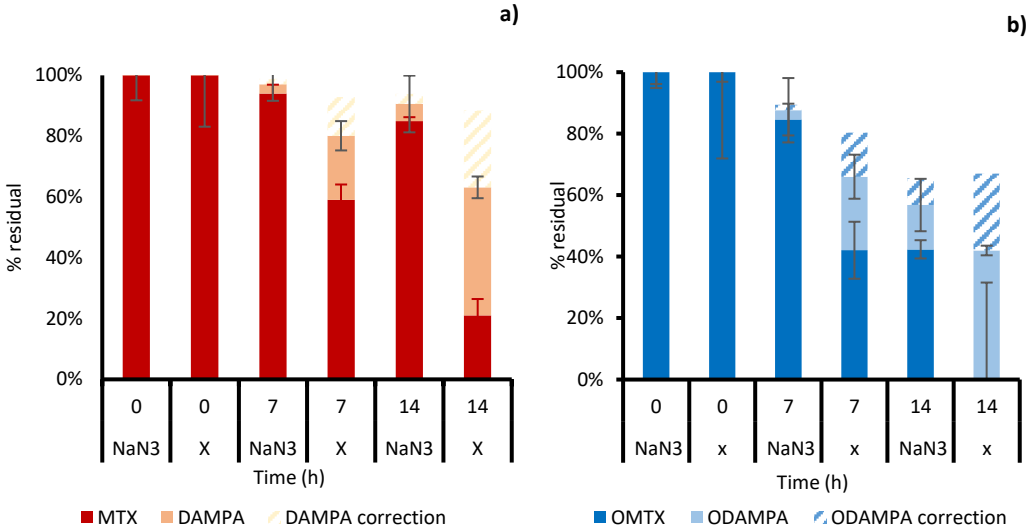


Figure 4.1- 10 Evolution with time of parent compounds and major TPs during diluted activated sludge incubation in presence or absence (X) of biocide NaN_3. Residual a) MTX and b) OMTX

The decrease of parent compounds in presence of the biocide NaN_3 could highlight adsorption process on solid phase, but the apparition of DAMPA, and ODAMPA indicated that NaN_3 was responsible for only a partial inhibition of microorganism activity, so did not totally curb the biodegradation process. These residual percentages were based on area ratio, with MTX and OMTX as references, but the ulterior use of DAMPA standard led us to use a correction factor of 1.6, because of underestimation due to a difference of MS response of DAMPA compared to MTX. Therefore, the same factor was used for ODAMPA even though no standard was available to confirm it.

Even without a total inhibition of microbial activity, it is possible to draw some conclusions at 14 h of contact between the molecules and the diluted activated sludge in presence of NaN_3. For MTX, 85% of residual compound and around 10% of DAMPA, so the part of adsorption were counting for less than

5%. For OMTX, 42% of residual compound, 14% of ODAMPA and a potentially 9% of underestimate ODAMPA. The 35% remaining represents further degradation and adsorption, the major process responsible for both MTX and OMTX dissipation were biodegradation.

4.1.4.5. Stability of the parent compounds in supernatant

Another point which could lead to underestimation of the compound concentrations, is their instability after sampling (Baker and Kasprzyk-Hordern, 2011). After sampling, the centrifugation step is supposed to separate microorganisms from the supernatant, stopping the biodegradation process, and samples were stored at 10°C in the autosampler until their injection. Therefore, the stability of MTX and OMTX was assessed during 48 hours at 10°C in the dark (Figure 4.1- 11). Up to 10 h, the variations of peak areas were below 5% for both molecules, while after 48 h these variations were higher for OMTX. Therefore, the analyses should be performed within 10 hours following the sampling for OMTX and 48 h for MTX. If it will not be possible, then a decrease in OMTX areas could occur.

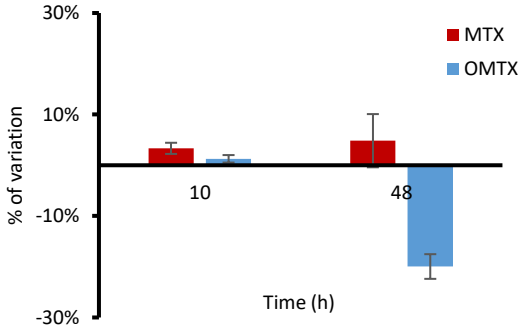


Figure 4.1- 11 Stability of MTX and OMTX in supernatant (at 10°C)

4.1.4.6. Characterization of the method

Table 4.1- 3 gathers performances of the method and will be commented in the next sections. Only a few standards were available, AMPC and TP267 presented bad calibration curves and high LOQs, and were not used for quantification.

Table 4.1- 3 Performances of the analytical method

	MilliQ				Volvic			
Compound	LOD (ng/L)	LOQ (ng/L)	calibration range	R^2	LOD (ng/L)	LOQ (ng/L)	calibration range	R^2
MTX	23	77	500 ng/L - 1mg/L	0.9999	5	16	100 ng/L - 1 mg/L	0.9999
DAMPA	5	16	500 ng/L - 1mg/L	0.9999	5	16	250 ng/L - 1 mg/L	0.9998
7-OH-MTX	21	71	1µg/L - 100 µg/L	0.9992	5	15	500 ng/L - 100 µg/L	0.9996
AMPC	>224	>746	1µg/L - 100 µg/L	-	>250	>746	-	-
OMTX	1	5	500 ng/L - 1mg/L	0.9999	4	15	50 ng/L - 1mg/L	0.9999
TP267	>54	>179	1µg/L - 1mg/L	0.9996	>188	>625	-	-

	Supernatant				
Compound	LOD (ng/L)	LOQ (ng/L)	calibration range	R^2	interday 10µg/L
MTX	5	17	1µg/L - 1 mg/L	0.9999	13%
DAMPA	2	5	1 µg/L - 1 mg/L	0.9997	20%
7-OH-MTX	6	19	100 ng/L - 1 mg/L	0.9999	26%
AMPC	>92	>307	-	-	-
OMTX	4	13	500 ng/L - 1mg/L	0.9999	3%
TP267	>3000	>10000	-	-	-

i. Limits of detection and quantification

The obtained LOQs were low (5 to 19 ng/L) for DAMPA and 7-OH-MTX as well as parent compounds, which correspond to 0.002% of residual compound for the monitoring of an incubation spiked at 1 mg/L, but we noticed weak sensibility for AMPC and TP267. This difference is not surprising because of their different structure compared to other TPs and parent compounds (4-hydroxybenzoyl-L-glutamic acid and pteridine derivatives). It could be presumed the same for other pteridine structures, like TP208. For TP267 especially, its high LOQ in supernatant (10000 ng/L, corresponding to 1% residual) is not surprising because of a large peak shape.

ii. Calibration curve

A wide range of concentration was explored for the calibration curves, up to 1 mg/L. They included 4 to 6 levels of concentrations and R^2 were always above 0.999, except for AMPC and TP267 for which no calibration curves were exploitable.

iii. Matrix effects

Matrix effects determined from Eq. 2 are known to vary depending on the compounds and their concentrations. They were evaluated at different levels ranging from 250 ng/L to 1 mg/L, which is the concentration used for spiking in biodegradation experiments. MTX and DAMPA presented lower matrix effects than other compounds (Figure 4.1- 12a). For MTX, absolute matrix effects were <50% at low concentrations and fall at <15% above 10 µg/L. For DAMPA, matrix effects were < 20 % above 500 ng/L in supernatant and <10 % above 1000 ng/L in Volvic.

For more impacted compounds (Figure 4.1- 12b) TP267 presented low matrix effects above 10 µg/L and moderate (50%) to high (>100%) matrix effects below that concentration (not presented because <LOQ). AMPC present moderate matrix effet above 10µg/L. OMTX ion enhancement was highlighted in Volvic water even at high concentrations and was less marked in supernatant, but still > 50 % below 0.1 mg/L. While for 7-OH-MTX the enhancements in supernatant and Volvic led to better LODs and LOQs than in MilliQ as seen in Table 4.1- 3. Globally the positive effects could lead to overestimation of molecules at low concentrations when areas are compared together. This matrix impacts could be avoided by a calibration in the matrix.

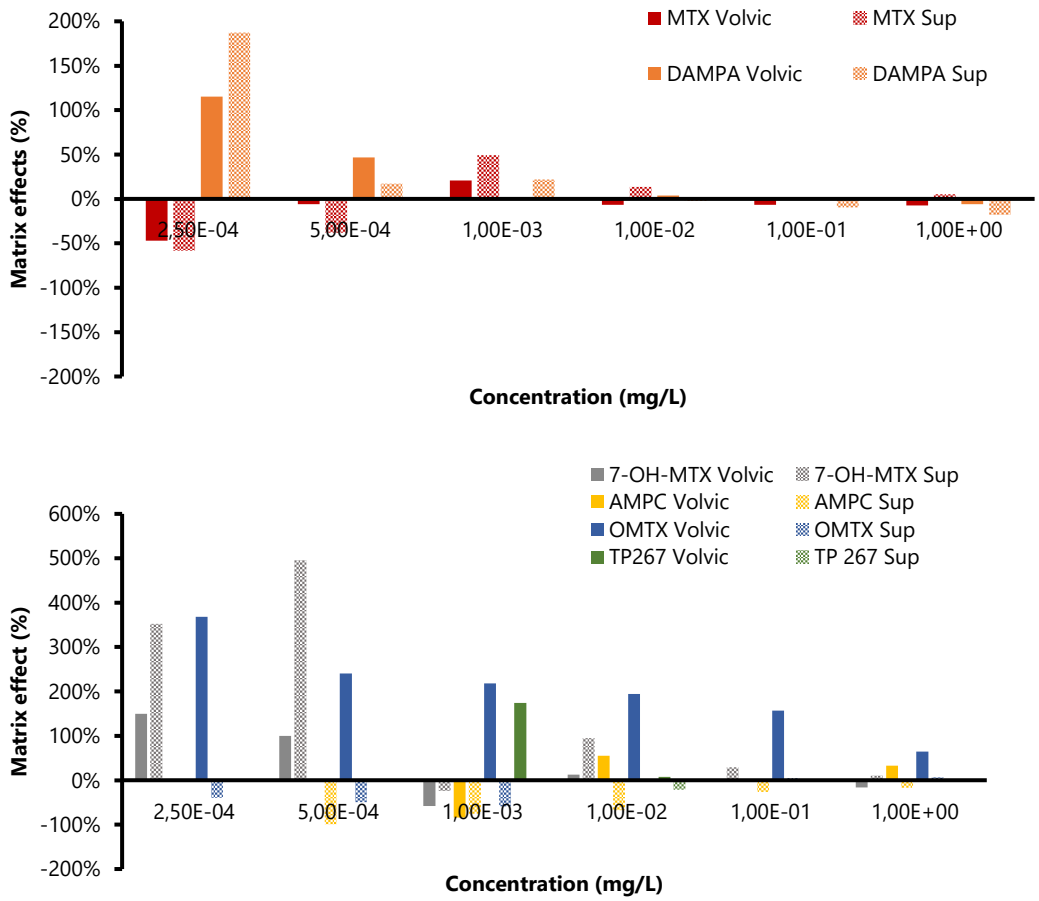


Figure 4.1- 12 Matrix effects (in %) depending on concentration levels in Volvic water and sludge supernatant a) low effects and b) high effects

iv. Interday precision

For parent compounds, the variation was <15 % for a concentration of 10 µg/L (corresponding to 0.1 % of residual parent compound), this precision was higher for TPs but did not exceed 26 %. It guaranteed a low response deviation along the 3 days of analysis needed to monitor the biodegradation presented in **Chapter 4.2.**

4.1.5. Conclusions

A simple and rapid online-SPE-UHPLC-MS/MS method for the simultaneous monitoring of a wide variety of MTX and OMTX transformation products was developed. The quantification of both parent compounds, DAMPA and 7-OH-MTX with LOQ from 5 to 19 ng/L allow performing a biodegradation

study with activated sludge and tracking residual concentration around 0.002 % of initial concentration at 1 mg/L MTX or OMTX. The proposed method should thus be efficient for the monitoring of parent compounds and TPs during the foreseen biodegradation experiment. Only 1 mL of sample is necessary, meeting the constraints of laboratory-scale. The suspected TP approach to overcome the lack of standards allowed the enrichment of the method with the addition of several potential biodegradation products of MTX and OMTX. The matrix effects that were generally high or very high at low concentrations highlighted the need to prepare standards directly in the matrices to perform suitable calibration.

The preliminary tests also showed that (i) samples could be analysed at their initial pH (around pH 7) without any risks of breakthrough during the loading of online-SPE, (ii) biodegradation of parent compounds was the main process during experiments with sewage sludge, and (iii) parent compounds were very stable in vials after centrifugation and prior to their injection, at least 48 h for MTX and 10 hours for OMTX. For OMTX, a loss of 20% of the initial concentration was shown at 48 h.

4.1.6. References

Baker, D.R., Kasprzyk-Hordern, B., 2011. Critical evaluation of methodology commonly used in sample collection, storage and preparation for the analysis of pharmaceuticals and illicit drugs in surface water and wastewater by solid phase extraction and liquid chromatography–mass spectrometry. Journal of Chromatography A 1218, 8036–8059. https://doi.org/10.1016/j.chroma.2011.09.012

Calza, P., Medana, C., Sarro, M., Rosato, V., Aigotti, R., Baiocchi, C., Minero, C., 2014. Photocatalytic degradation of selected anticancer drugs and identification of their transformation products in water by liquid chromatography–high resolution mass spectrometry. Journal of Chromatography A 1362, 135–144. https://doi.org/10.1016/j.chroma.2014.08.035

Castiglioni, S., Bagnati, R., Calamari, D., Fanelli, R., Zuccato, E., 2005. A multiresidue analytical method using solid-phase extraction and high-pressure liquid chromatography tandem mass spectrometry to measure pharmaceuticals of different therapeutic classes in urban wastewaters. Journal of Chromatography A 1092, 206–215. https://doi.org/10.1016/j.chroma.2005.07.012

Espinosa, A., Nélieu, S., Lieben, P., Skarbek, C., Labruère, R., Benoit, P., 2021. Photodegradation of methotrexate in aqueous solution: degradation kinetics and identification of transformation products. Environmental Science and Pollution Research https://doi.org/10.1007/s11356-021-15820-3

Espinosa, A., Rascol, E., Abellàn Flos, M., Skarbek, C., Lieben, P., Bannerman, E., Diez Marinez, A., Pethe, S., Benoit, P., Nélieu, S., Labruère, R., Submitted in Angewandte Chemie International Edition

Ferrando-Climent, L., Rodriguez-Mozaz, S., Barceló, D., 2013. Development of a UPLC-MS/MS method for the determination of ten anticancer drugs in hospital and urban wastewaters, and its application for the screening of human metabolites assisted by information-dependent acquisition tool (IDA) in sewage samples. Analytical and Bioanalytical Chemistry 405, 5937–5952. https://doi.org/10.1007/s00216-013-6794-4

Galaon, T., Petre, J., Iancu, V.I., Cruceru, L., Niculescu, M., Chiriac, F.L., Puiu, D.M., Mihalache, M., 2017. detection of cytostatic drugs in municipal waste water and their transfer to surface water, in: SIMI 2017. Presented at the International Symposium "The Environment and the Industry," National Research and Development Institute for Industrial Ecology, pp. 229–236. https://doi.org/10.21698/simi.2017.0029

Garcia-Ac, A., Segura, P.A., Gagnon, C., Sauvé, S., 2009. Determination of bezafibrate, methotrexate, cyclophosphamide, orlistat and enalapril in waste and surface waters using on-line solid-phase extraction liquid chromatography coupled to polarity-switching electrospray tandem mass spectrometry. Journal of Environmental Monitoring 11, 830. https://doi.org/10.1039/b817570e

Hignite, C., Azarnoff, D.L., 1977. Drugs and drug metabolites as environmental contaminants: chlorophenoxyisobutyrate and salicylic acid in sewage water effluent. Life Sciences 20, 337–341. https://doi.org/10.1016/0024-3205(77)90329-0

Kanjal, M.I., Muneer, M., Abdelhaleem, A., Chu, W., 2020. Degradation of methotrexate by UV/peroxymonosulfate: Kinetics, effect of operational parameters and mechanism. Chinese Journal of Chemical Engineering 28, 2658–2667. https://doi.org/10.1016/j.cjche.2020.05.033

Kiffmeyer, T., Götze, H.-J., Jursch, M., Lüders, U., 1998. Trace enrichment, chromatographic separation and biodegradation of cytostatic compounds in surface water. Fresenius' Journal of Analytical Chemistry 361, 185–191. https://doi.org/10.1007/s002160050859

Kosjek, T., Heath, E., 2011. Occurrence, fate and determination of cytostatic pharmaceuticals in the environment. TrAC Trends in Analytical Chemistry 30, 1065–1087. https://doi.org/10.1016/j.trac.2011.04.007

Kosjek, T., Negreira, N., de Alda, M.L., Barceló, D., 2015. Aerobic activated sludge transformation of methotrexate: Identification of biotransformation products. Chemosphere 119, S42–S50. https://doi.org/10.1016/j.chemosphere.2014.04.081

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

Lutterbeck, C.A., Baginska, E., Machado, Ê.L., Kümmerer, K., 2015. Removal of the anti-cancer drug methotrexate from water by advanced oxidation processes: Aerobic biodegradation and toxicity studies after treatment. Chemosphere 141, 290–296. https://doi.org/10.1016/j.chemosphere.2015.07.069

Martín, J., Camacho-Muñoz, D., Santos, J.L., Aparicio, I., Alonso, E., 2011. Simultaneous determination of a selected group of cytostatic drugs in water using high-performance liquid chromatography-triple-quadrupole mass spectrometry. Journal of Separation Science 34, 3166–3177. https://doi.org/10.1002/jssc.201100461

Negreira, N., López de Alda, M., Barceló, D., 2013. On-line solid phase extraction–liquid chromatography–tandem mass spectrometry for the determination of 17 cytostatics and metabolites in waste, surface and ground water samples. Journal of Chromatography A 1280, 64–74. https://doi.org/10.1016/j.chroma.2013.01.031

Okuda, T., Yamashita, N., Tanaka, H., Matsukawa, H., Tanabe, K., 2009. Development of extraction method of pharmaceuticals and their occurrences found in Japanese wastewater treatment plants. Environment International 35, 815–820. https://doi.org/10.1016/j.envint.2009.01.006

Pavlović, D.M., Babić, S., Horvat, A.J.M., Kaštelan-Macan, M., 2007. Sample preparation in analysis of pharmaceuticals. TrAC Trends in Analytical Chemistry 26, 1062–1075. https://doi.org/10.1016/j.trac.2007.09.010

Rabii, F.W., Segura, P.A., Fayad, P.B., Sauvé, S., 2014. Determination of six chemotherapeutic agents in municipal wastewater using online solid-phase extraction coupled to liquid chromatography-tandem mass spectrometry. Science of The Total Environment 487, 792–800. https://doi.org/10.1016/j.scitotenv.2013.12.050

Roberts, P., Thomas, K., 2006. The occurrence of selected pharmaceuticals in wastewater effluent and surface waters of the lower Tyne catchment. Science of The Total Environment 356, 143–153. https://doi.org/10.1016/j.scitotenv.2005.04.031

Rowney, N.C., Johnson, A.C., Williams, R.J., 2009. Cytotoxic drugs in drinking water: a prediction and risk assessment exercise for the Thames catchment in the United-Kingdom. Environmental Toxicology and Chemistry 28, 2733. https://doi.org/10.1897/09-067.1

Santos, M.S.F., Franquet-Griell, H., Alves, A., Lacorte, S., 2018. Development of an analytical methodology for the analysis of priority cytostatics in water. Science of The Total Environment 645, 1264–1272. https://doi.org/10.1016/j.scitotenv.2018.07.232

Schymanski, E.L., Jeon, J., Gulde, R., Fenner, K., Ruff, M., Singer, H.P., Hollender, J., 2014. Identifying Small Molecules via High Resolution Mass Spectrometry: Communicating Confidence. Environmental Science & Technology 48, 2097–2098. https://doi.org/10.1021/es5002105

Seira, J., Claparols, C., Joannis-Cassan, C., Albasi, C., Montréjaud-Vignoles, M., Sablayrolles, C., 2013. Optimization of pressurized liquid extraction using a multivariate chemometric approach for the determination of anticancer drugs in sludge by ultra-high performance liquid chromatography–tandem mass spectrometry. Journal of Chromatography A 1283, 27–38. https://doi.org/10.1016/j.chroma.2013.01.114

Verlicchi, P., Al Aukidy, M., Galletti, A., Petrovic, M., Barceló, D., 2012. Hospital effluent: Investigation of the concentrations and distribution of pharmaceuticals and environmental risk assessment. Science of The Total Environment 430, 109–118. https://doi.org/10.1016/j.scitotenv.2012.04.055

Widemann, B.C., Sung, E., Anderson, L., Salzer, W.L., Balis, F.M., Monitjo, K.S., McCully, C., Hawkins, M., Adamson, P.C., 2000. Pharmacokinetics and metabolism of the methotrexate metabolite 2, 4-diamino-N(10)-methylpteroic acid. The Journal of Pharmacology and Experimental Therapeutics 294, 894–901.

4.2. Biodegradation by activated sludge of methotrexate and its eco-designed analogue and feedback impact on the microbiological community

4.2.1. Abstract

Pharmaceuticals and their degradation products contaminate wastewater at a range of ng/L to mg/L and may be discharged into aquatic environment from municipal sewage effluents because of their partial removal. This study focuses on the biodegradation in activated sludge of methotrexate (MTX), an anticancer drug of high environmental concern due to its cytotoxic mode of action and its ability to produce a controversy and potentially toxic metabolite, the 7-OH-MTX. This evolution was compared with that of an eco-compatible analogue (OMTX) which was purposefully synthetized with the assumption of higher biodegradation and lower environmental impact of its transformation products (TPs). The OMTX degradation kinetic was faster than the MTX one but in the same order of magnitude. The TPs monitoring highlighted similar mechanisms of degradation for both compounds, the DAMPA and the identification of its equivalent ODAMPA, are resulting from an hydrolysis of the glutamate moiety and reach level of 90% of parent molecule, and appeared as stable against biodegradation. In accordance with cytotoxicity assessment, the initial action mode of the parent compound was lost at the end of the 48 h incubation, indicating DAMPA and ODAMPA did not exert cytotoxic action. The harmful 7-OH-MTX was found in minor concentration (<2%) and was transient as its equivalent, the 7-OH-OMTX. No remarkable and persistent impacts on the microbiological community of the sludge were evidenced through indicators of the carbon and nitrogen cycles and enzyme activities after a 48-hours incubation for both compounds.

4.2.2. Introduction

Water contamination by pharmaceuticals has several origins. Verlicchi et al. (2012) pointed out hospital effluents to be one of the main sources. Moreover, considering specific wastewater treatment for such effluents would represent a high cost (Verlicchi et al., 2015). Pharmaceutical residues may also be present in domestic wastewaters representing an equivalent or higher release than hospital effluents because of large household treatment uses (Mahnik et al., 2007; Kosjek and Heath, 2011; Ferrando-Besse et al., 2012; Climent et al., 2013). In addition, pharmaceutical residues can also be released from pharmaceutical factories (Kessler, 2010). Wastewater treatment plants (WWTP) have been designed to remove chemical oxygen demand (COD), nutrients and pathogens. The most popular process is

biological wastewater treatment by activated sludge, where microorganisms play a key role in the removal of organic contaminants even if it could remain partial or inefficient for some of them (Kraigher et al., 2008; Delgado et al., 2010; Amariei et al., 2017; Amariei et al., 2020; Çeçen and Gül, 2021). Polar pharmaceuticals are mostly removed by microbial degradation (Quintana et al., 2005) while other compounds can be removed from water by a transfer by adsorption onto the sludge (Çeçen and Gül, 2021), or in a lesser extent undergo hydrolysis or volatilization depending on their properties (Booker et al., 2014; Amariei et al., 2020). Furthermore, these contaminants could disturb wastewater treatment processes because of their toxicity on the microbial community of sludge, and lead to poor biological oxygen demand removal, disrupted nitrification, deflocculation (Love and Bott, 2002) and even cause operation failure (Ren, 2004; Yao et al., 2012). For example, Yao et al. (2012) reported that the activity of enzyme such catalase, dehydrogenase, urease, phosphatase and protease were impacted in the presence of 10 mM of tetrahydrofuran after a period of 85 days. Alvarino et al. (2014) found that acetaminophen and doxycycline at level of hundreds of mg/L would perturbing the nitrification process by affecting community structure. Kraigher et al. (2008) observed shift in bacterial community structure exposed to few µg/L of pharmaceutical during months. Concerning acute toxicity, Amarei et al. (2017) reported effect on oxygen uptake and esterase activity from few mg/L of triclosan while ibuprofen effects were observed at more than 700 mg/L within the day after the exposition.

The following indicators linked to carbon and nitrogen cycles could give indications of process efficiency: oxygen consumption and enzyme activities can be good indicators of the activities of the mixed species sludge community (Dalzell et al., 2002; Molina-Muñoz et al., 2010; Yao et al., 2012; Amariei et al., 2017).

Because cytotoxic agents are designed to disrupt cell replication and act non-selectively on all growing cells with a very potent mechanism of action (cytotoxicity, genotoxicity, mutagenicity and teratogenicity), they could cause adverse effects even at low concentrations as ng/L (Johnson et al., 2008; Besse et al., 2012; Parrella et al., 2014a and b; Galaon et al., 2017). Methotrexate (MTX), one of the most widely prescribed worldwide antineoplastic agents (Kosjek and Heath, 2011) is an analogue of folic acid. As a competitive inhibitor of dihydrofolate-reductase (DHFR), an enzyme involved in the metabolism of folic acid, MTX therefore impacts cell division. It is also used for meningeal leukemia, psoriasis and autoimmune disease as rheumatoid arthritis (Sabry et al., 2003). It is one of the three antimetabolites the most frequently detected in wastewater (Li et al., 2021). Found in hospital effluents up to 4.8 µg/L (Olalla et al., 2018), it can also outcome from domestic water due to at home treatments (Besse et al., 2012). In wastewater effluent methotrexate concentrations can range from 1.8 to 53 ng/L (Castiglioni et

al., 2005; Rabii et al., 2014). Rabii et al. (2014) found between 13 and 60 ng/L in influent and effluent of WWTP with no significant decrease in concentration after a short residence time (<3 h). However, the MTX fate in WWTP is unclear, as Wang et al. (2018) classed MTX as "easily degradable cytostatic drugs" and Kiffmeyer et al. (1998) reported its total biodegradation within seven days, while other authors reported a weak biodegradation (Henschel et al., 1997; Lutterbeck et al., 2015). Data about MTX in surface water are scarce except Ferrando-Climent et al. (2014) who detected few ng/L in surface water directly to the output of a WWTP. In addition to the parent compound, the formation of potentially toxic TPs has to be addressed. Data are needed on their ecotoxicological effects on aquatic organisms in surface waters and sediments (Besse et al., 2012).

Kiffmeyer et al. (1998) revealed that 7-hydroxymethotrexate (7-OH-MTX) was a major and persistent TP of MTX during aerobic degradation by activated sludge, while it appeared only as a transient species in the study of Kosjek et al. (2015). The 7-OH-MTX pharmacological activity is controversial: it was described as acting via the same cytotoxic mechanism as methotrexate with a lower potency (Fabre and Goldman, 1985), and could be found in the aquatic environment at higher levels than the parent compound. For this reason, Besse et al. (2012) included it in the priority list for environmental assessment and recommended conducting occurrence studies.

Limiting contamination could go through innovative and anticipative approaches by developing compounds with a better biodegradability leading to innocuous and non-persistent transformation products called "benign by design" concept by Kümmerer (2007). Espinosa et al. (submitted) proposed an eco-designed analog for MTX, the oxygenated analog OMTX, and studied its photodegradation pathways compared with MTX (Espinosa et al., 2021). The OMTX structure was promising in terms of degradation kinetics at low wavelength (related to specific treatment as drinking water one) but, neither 7-OH-MTX for MTX nor the equivalent compound for OMTX were formed under photolysis in pure water (Espinosa et al., 2021; Espinosa et al., submitted).

This study aimed to go one step further and investigate the biodegradation of MTX and its eco-compatible analogue OMTX in the supernatant of an activated sludge by a) identifying and monitoring TPs formed during the biodegradation of both molecules and comparing biodegradation kinetics; b) comparing the evolution of cytotoxicity; and c) assessing potential impact of molecules on the microbiological community, through indicators of the carbon and nitrogen cycles and enzyme activities.

4.2.3. Material and methods

4.2.3.1. Chemicals and reagents

See **Chapter 4,** part 4.1.2.1

4.2.3.2. Analyses and measurements

i. Analytical conditions for online-SPE-UHPLC-MS/MS

The previously developed method for analyses of parent compounds MTX and OMTX and their respective TPs by online-SPE-UHPLC-MS/MS was used. Details of this analytical development and characterization of the method were presented in **Chapter 4.1**

ii. Cytotoxicity

See **Chapter3,** part 3.2.3.1

iii. Enzymatic activities

Phosphatase (PHOS), β-glucosidase (βGLU), urease (URE), arylamidase (ARN), dehydrogenase (DHA), nitrate reductase (NITRED) as well as potential nitrification (NIT), were measured with colorimetric methods using ISO 20130:2018 (PHOS, βGLU, URE and ARN), ISO 23753-1:2019 (DHA), ISO15685:2004 (NIT) and Abdelmagid and Tabatabai (1987) (NITRED), protocols.

A volume of 50 mL of Activated sludge (AS) was taken in triplicate, under agitation for each sample, and centrifuged at 4000 rpm for 15 min. For PHOS, β GLU, URE and ARN and NIT, 25 mL of water were adding to the pellet before depositing in microplates. For DHA and NITRED, pellets were directly used for reactions. A control, without incubation with substrates, was performed for each sample. The substrates used were 4-nitrophenylphosphate, 4-nitrophenyl-β-D-glucopyranoside, urea, L-leucine β-naphthylamide hydrochloride, triphenyltetrazolium chloride, potassium nitrate and ammonium sulphate, respectively for PHOS, βGLU, URE, ARN, DHA, NITRED and NIT.

The concentration of released product was related either to a range of: *para*-nitrophenol (λ=405 nm) for the PHOS and βGLU activities; NH_4Cl (λ=650 nm) for urease; and β-naphthylamide for RNA (λ=540 nm); triphenyl formazan (TPF) for DHA (λ=485 nm); sodium nitrite for NITRED λ=520 nm) and sodium nitrate for NIT (λ=540 nm), with Spectrostar nano (BMG labtech). Enzymatic activities were calculated and expressed in mU (nanomole equivalent of product released per min) per g of dry sludge.

iv. Oxygen consumption

The OxiTop® system (WTW Weilheim, Germany) was used to study the effect of MTX and OMTX on microbial respiration during a 48 h-incubation of suspended sludge spiked with each compound and compared with unspiked controls incubation. This manometric measurement is based on a pressure difference due to the oxygen consumption measured at the beginning (T0+2 h because of the time needed for preparation) and at the end of the 48 h-incubation (Tf+2 h). The oxygen is used by microorganisms, and the released carbon dioxide is then captured by the KOH pellets, placed in the rubber hood in the bottle above the water phase. According to Baginska et al. (2015) Equation.1 has been used for calculations of the biological oxygen demand (BOD) values.

$$BOD\ (mg.L^{-1}) = \frac{M(O_2)}{RT_m}\left[\frac{(V_{tot} - V_i)}{V_i} + \frac{\alpha T_m}{T_0}\right]\Delta p(O_2)$$

Equation.1

With BOD: biological oxygen demand (mg L^{-1}); $M(O_2)$: molecular mass of O_2 (32 g mol^{-1}); R: gas constant (83.144 L mbar mol^{-1} K^{-1}); T_0: reference temperature (273.15 K); T_m: measuring temperature (°K); V_t: bottle volume (mL); V_l: sample volume (mL); α: Bunsen absorption coefficient (0.03103); Δp (O_2): difference of the oxygen partial pressure (mbar)

v. Total C and N contents

Total C and N contents were measured on homogenised and freeze dried samples by an elementary analyser, Vario isotope select Elementar®. The contents are related to the mean of dry mass content of the activated sludge. The mineral N concentration was analysed in the supernatant by colorimetry on a continuous flow analyser (Skalar, The Netherlands) ad described by Doublet et al. (2010). Finally, total and organic carbon in supernatant were analysed by TOC® 5050A Shimadzu® Detector.

vi. pH and conductivity

The pH and conductivity were measured on homogenised samples on SevenGo® Duo pH/conductivity meter SG23 for conductivity and 780 pH Meter Metrohm® for pH.

4.2.3.3. Incubation experiment

i. Experimental setup

The feasibility of monitoring the MTX and OMTX degradation and the concomitant formation of TPs depends both on the initial concentration of parent molecules and on the amount of activated sludge. They had to be set according to kinetics allowing frequent and practical sampling for not missing the evolution of some TPs. The amount of activated sludge was set at a determined concentration by diluting a raw sludge sample in its own supernatant obtained after its centrifugation at 11000 *g* during 10 min.

After a serie of preliminary tests (see below 4.2.4.1), the amount of suspended solid concentration was set at 0.96 ± 0.10 g dry matter/L by a dilution of 1/20 in sludge supernatant and was spiked at 1 mg/L of MTX or OMTX. Incubations were conducted in 1 L glass bottles with a total volume of 0.55 L of diluted activated sludge (AS) and aerated with a magnetic agitator. The 48 h-experiments were performed at 20 °C and protected from light to avoid photodegradation. Control samples without MTX or OMTX were also incubated. Figure 4.2- 1 presents the setup of this experiment. The oxygen consumption was followed with Oxitop systems in a dedicated set of test bottles because this manometric measure does not allow the opening of the bottles. This setup was also used to evaluate the enzymatic activities at the end of the experiment (Figure 4.2- 1A). A second set was used for these evaluations at initial time due to the large sample volume required for the analyses (Figure 4.2- 1B). A third set of test bottles was incubated in the same conditions for regular samplings to monitor TPs and cytotoxicity and for carbon and nitrogen analyses at initial and final time (Figure 4.2- 1C). Finally, evaluation of the possible abiotic degradation was evaluated in MilliQ water control (Figure 4.2- 1D). Each condition was run in triplicate.

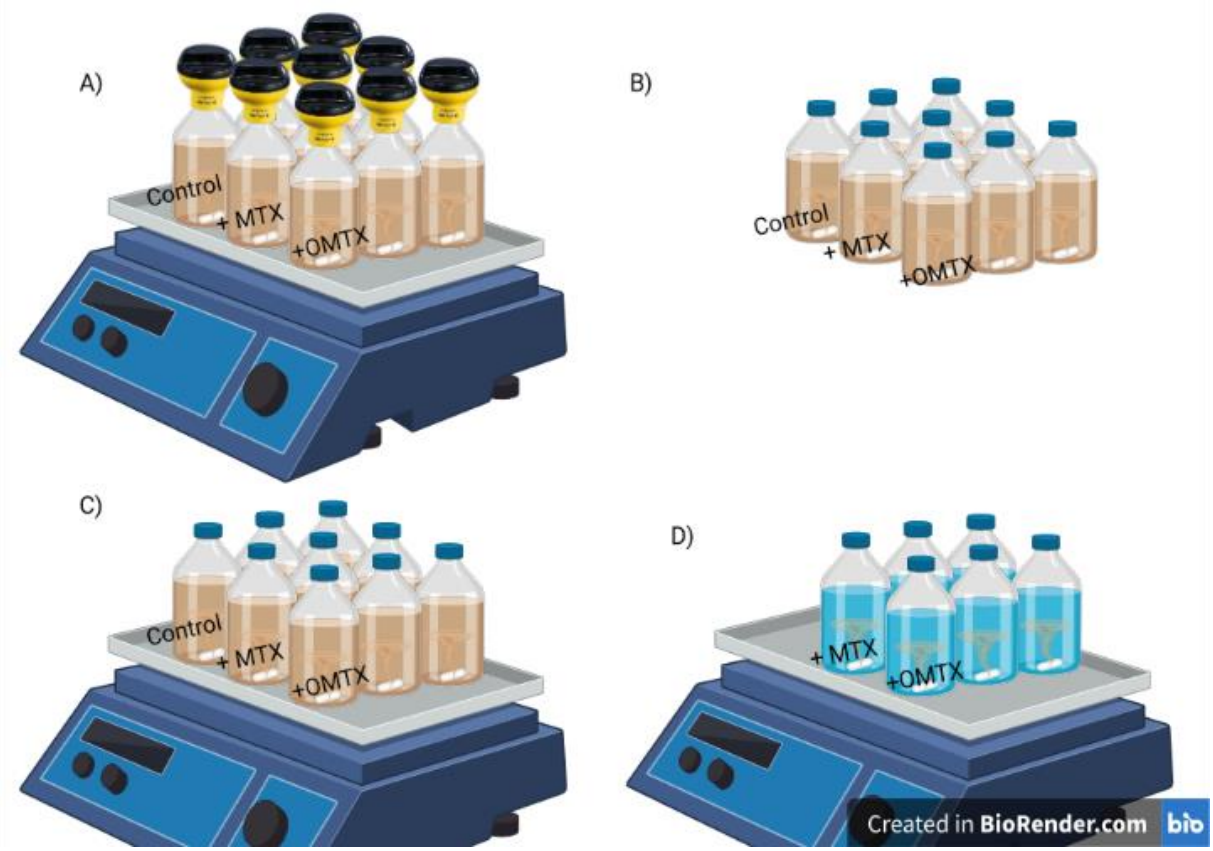


Figure 4.2- 1 Set up of the experiment A, C, D for 48 h-incubation and B) for immediate enzymatic assessment

ii. Sampling procedure for the different measurements

For contaminant monitoring, 2 mL of incubated AS were sampled at different times and were centrifuged at 12100 *g* for 10 min in a MiniSpin centrifuge (Ependorf) for the online-SPE-UHPLC-MS/MS analysis of the supernatant (carried out within the day). For cytotoxicity, the same procedure than for TP monitoring but the supernatants were conserved at -40°C until assessment. Cytotoxic assessment of the supernatant was done only for initial and final time of the incubation. For C and N analyses, 60 mL of samples were freeze dried for total analyses, or 8 mL were kept at -20°C with or without centrifugation (11000 *g* during 10 min) depending on analyses (on supernatant or homogenised sample, respectively). For enzymatic activity assessment, 50 mL of homogenised samples were used at the beginning of the incubation (+2 h) and at the end of the 48 h-incubation (+50 h).

4.2.3.4. Statistical analyses

The effects of MTX and OMTX on physico-chemical characteristics of the diluted activated sludge, respiration and enzymatic activities were determined with an analysis of variance (ANOVA) after the Shapiro–Wilk and Bartlett tests for normality of the residuals and data homoscedasticity checking. Then, Tukey's HSD (honestly significant difference) post-hoc test allowed pairwise comparisons. When the previous conditions were not met, the test of Kruskal–Wallis followed by the Dunn test with a Bonferroni correction for pairwise comparisons was used to compare the different treatments. All statistical analyses were performed at the significance level $p < 0.05$ with the R Studio software version 4.1.0. Results were provided as averages and standard deviations.

4.2.4. Results and Discussion

4.2.4.1. Preliminary tests

The feasibility of monitoring parent compounds and TPs were checked regarding the frequency of sampling and time required for analysis preparation between two samplings. We looked at the impact of sludge concentration on biodegradation kinetics of parent compounds, as well as the evolution profile of TPs. These preliminary tests were done with MTX (because OMTX was available only in limited amounts). The comparison of two spiking levels and different sludge preparations by dilution as previously detailed are presented in Figure 4.2- 2a and b, respectively. As expected, the biodegradation rate was related to the sludge concentration. The total disappearance of MTX was achieved in less than 2 h when incubated in raw sludge, that would not be suitable for several samplings, while biodegradation in supernatant did not occur within 3 h.

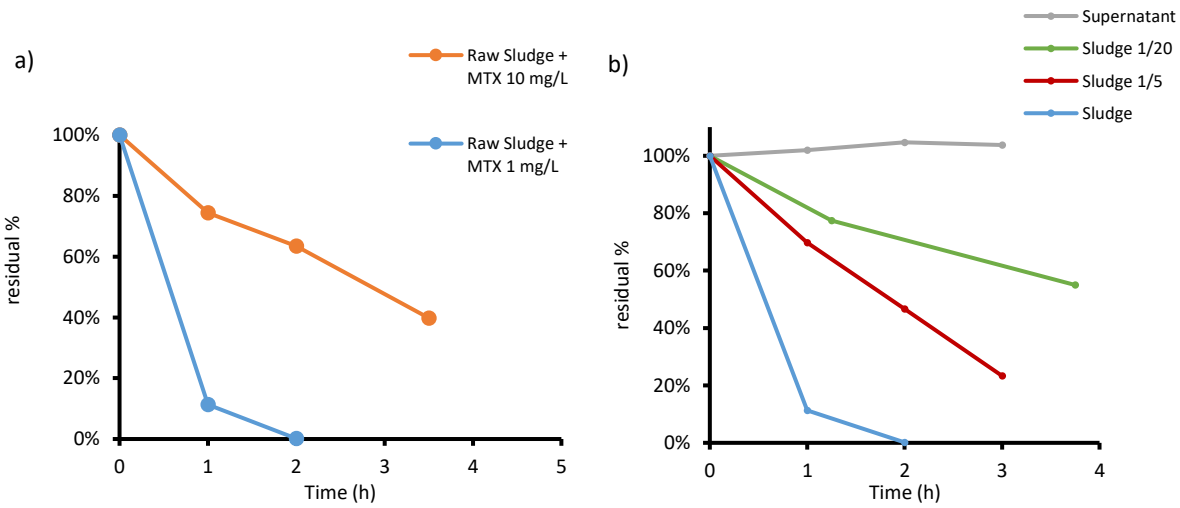


Figure 4.2- 2 Effect of MTX concentration (a) and sludge dilution with spike of MTX at 1mg/L (b) on the MTX dissipation expressed in % of the initial amount

Concerning the formation of TP Kiffmeyer et al. (1998) found the previously presented harmful 7-OH-MTX to be major and persistent while Kosjek et al. (2015) found it as minor and transient highlighting that evolution of TPs could vary according to the composition of the wastewater, activated sludge and the concentration of the parent compound. Its evolution, as the evolution of the other known major biodegradation products of MTX, the 2,4-diamino-N(10)-methylpteroic acid (DAMPA) (Kosjek et al., 2015), were monitored for the different concentrations of MTX or sludge. As presented in Figure 4.2- 3, these factors did not affect the profile of DAMPA and 7-OH-MTX when considering their concentration relative to the residual MTX *(not presented as a function of time incubation because the same residual % was obtained at different incubation time, according to sludge concentration).*

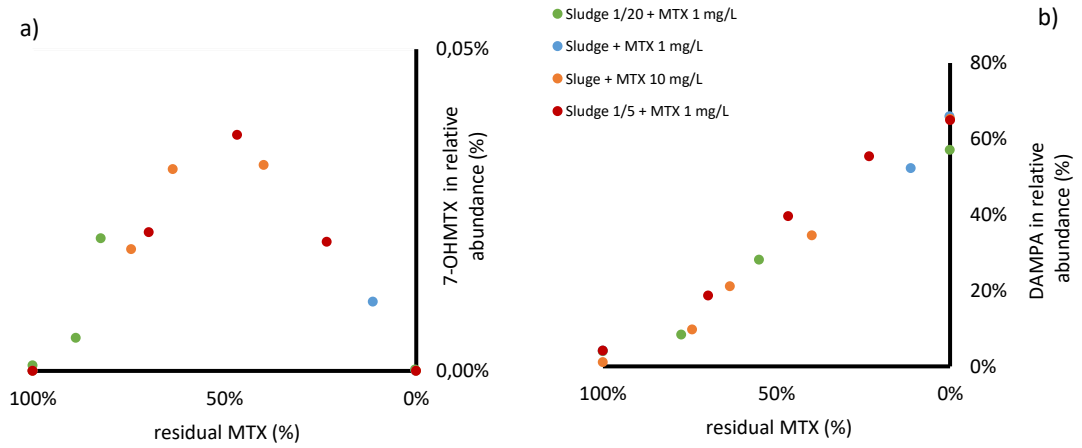


Figure 4.2- 3 Effect of sludge dilution on 7-OH-MTX (a) and DAMPA (b) formation (! different ordinate scales) expressed in % of the initial amount

The ageing of sludge had a negative impact on the degradation of both compounds. Indeed, the degradation rate was decreased by a factor of 4.5 to 5.4 for OMTX and MTX, respectively when comparing a sludge suspension prepared 48 h after sampling in WWTP, and after a 3 week storage at 4°C (Figure 4.2- 4). For this reason, it was decided that the biodegradation experiments should be run as soon as the sludge was collected.

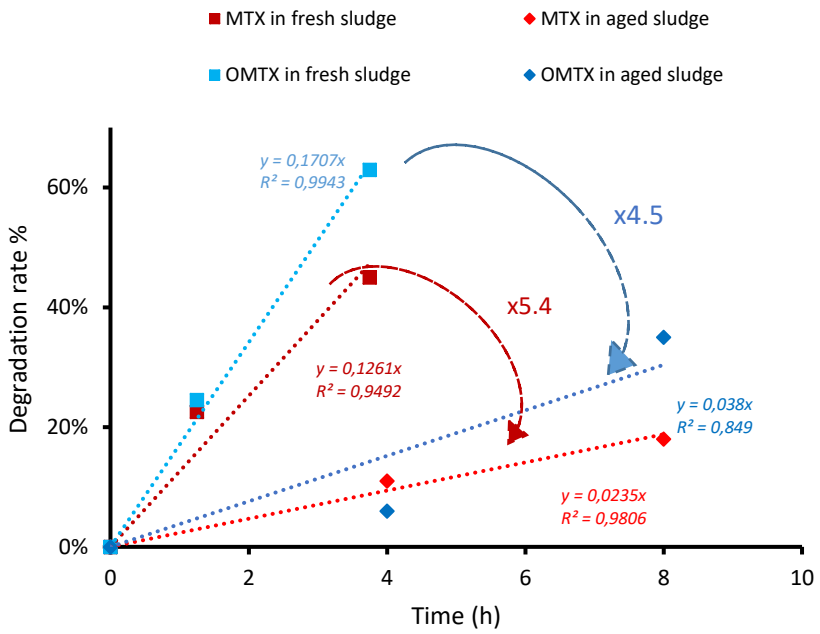


Figure 4.2- 4 Comparison of MTX and OMTX degradation by fresh and 3-weeks storage sludge at 4°C

Finally, the dilution of activated sludge was set at a dilution factor of 1/20, corresponding to a concentration of suspended solid of 0.96 ± 0.10 g/L. The spike of parent compound at 1 mg/L, allowing the use of a limited amount of molecules, regular samplings for the monitoring of TPs and in accordance with analytical performances, LOQ reaching few ng/L as previously presented in **Chapter 4.1**.

4.2.4.2. Degradation kinetics of parents compounds

MTX and OMTX were stable in MilliQ water (control) over the 48 h of incubation, highlighting the absence of abiotic degradation. In the activated sludge suspensions, OMTX was faster dissipated than MTX, we could notice that this observation is contrary to predicated biodegradation half life (Tableau 4.2- S1). Its concentration reached less than 8% of the initial concentration after 8h of incubation, while it took more than 12 h for MTX (Figure 4.2- 5) to reach such a percentage. This dissipation was attributed to biodegradation as previously demonstrated in **Chapter 4.1** (Figure 4.1- 10). The degradation of parent compounds seems to follow a zero-order kinetics with values of 0.07 h^{-1} and 0.12 h^{-1} for MTX and OMTX, respectively. Biodegradation rates could be expected to follow a first-order model (Çeçen and Gül, 2021) but zero-order kinetics could give a good representation of the removal capacity of the sludge at high contaminant concentrations, as already observed in literature for Malathion (Rodríguez-Espinosa et al., 2018) or Naproxen (Falås et al., 2012).

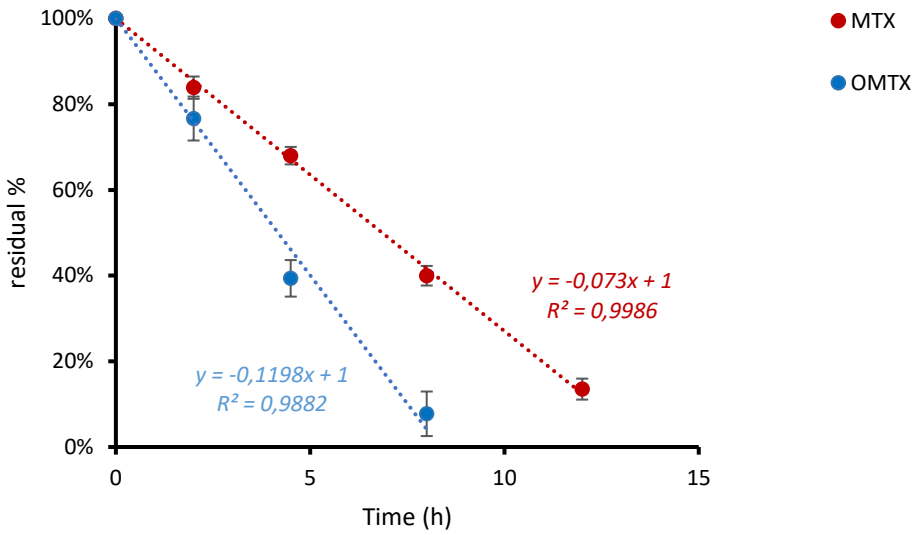


Figure 4.2- 5 Biodegradation kinetics : Residual amounts of MTX and OMTX expressed in % of the initial amount

4.2.4.3. Monitoring of TPs

DAMPA and its analogue ODAMPA were found at the highest concentrations whereas 8 and 4 other TPs were found at low concentrations for MTX and OMTX, respectively. The major TPs of MTX and OMTX, respectively the DAMPA and the ODAMPA identified in **Chapter 4.1** are presented in Figure 4.2-6**.**

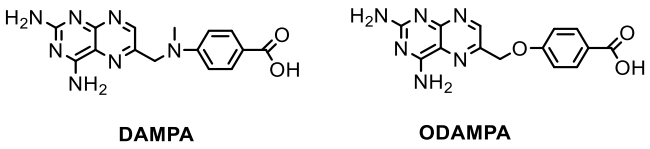


Figure 4.2- 6 DAMPA and its oxygenated equivalent ODAMPA

In MilliQ water (control), their detection at T0 was negligible (< 0.5%) and stable along the 48 h incubation (data not shown). Furthermore, it is worth noting that these two TPs were not detected during photodegradation process (Espinosa et al., 2021; Espinosa et al., submitted), indicating that DAMPA and ODAMPA resulted from a biodegradation process. Evolution profiles of these two were respectively accumulated with time, reaching 80-90 % of the initial concentration of parent compound after 48 h of incubation (Figure 4.2- 7a et b). According to Kosjek et al. (2015) "the transformation of primary amide

to carboxylate is a well-known microbiological transformation that occurs under aerobic conditions". These authors also demonstrated the stability of DAMPA to further biodegradation.

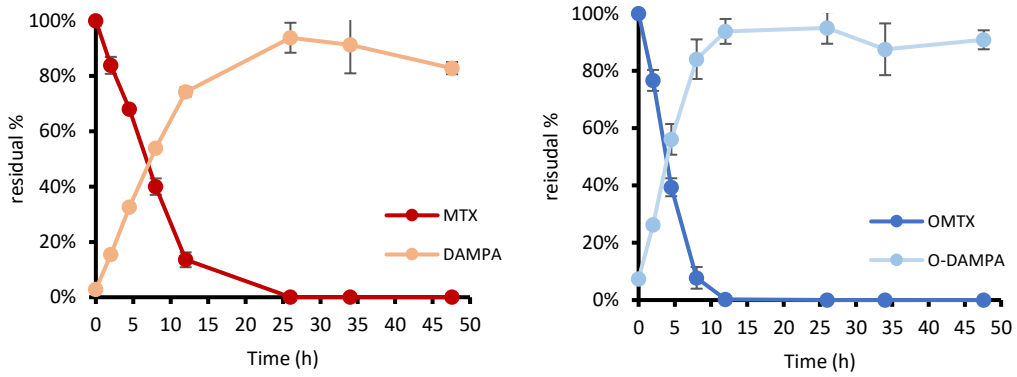


Figure 4.2- 7 Evolution of MTX and DAMPA (a) and OMTX and ODAMPA (b) during the 48 h incubation - Results expressed in % of the initial amount

Among minor TPs of MTX (< 2% of initial amount) presented in Figure 4.2- 8, TP266 and TP324 were also detected during photodegradation by Espinosa et al. (2021). In addition, they were detected in other photodegradation studies (Calza et al., 2014; Lai et al., 2017) and biodegradation ones (Kosjek et al., 2015; Lutterbeck et al., 2015). Surprisingly, TP266 was also formed in MilliQ controls at maximum residual percentage of 0.10 vs 0.02% in sludge. This could be due to: (i) overestimation of concentration due to high matrix effects at low concentrations, as they were observed in **Chapter 4.1** for some TPs; (ii) the result from an hydrolysis also occurring in abiotic condition; or (iii) the fact that the areas and therefore the concentrations were very low leading to probably high uncertainties.

Other minor TPs, such as TP341a and b, TP311k and TP150, identified during biodegradation experiments by Kosjek et al. (2015) were also detected in our study (Figure 4.2- 8). TP190 was not detected in our study, but according to Kosjek et al. (2015) it could result from an oxidative cleavage of DAMPA leading also to TP150, and this TP190 could further be transformed into AMPC. The late apparition of this latter one confirmed that it is not a primary breakdown product. AMPC was also detected as a secondary product for OMTX (Figure 4.2- 9b).

The harmful 7-OH-MTX was detected only as a transient species in accordance with Kosjek et al. (2015), reaching a maximum level of 0.03%, equivalent to 1.3 µg/L after 4h of incubation and finally decreasing rapidly (Figure 4.2- 8b), contrary to Kiffmeyer et al. (1998) who qualified it as major and persistent. It could result from a difference in the composition of activated sludge, or in the concentration

of the parent compound. The 7-OH-OMTX presented the same kinetic profile as its analogue (Figure 4.2- 9b).

TP 341a or b (Figure 4.2- 8a) reached respectively 1.96% and 1.13%. The metabolite proposed by Widemann et al. (2000) seemed to be the most suitable hypothesis for this compound. Indeed, hydroxylation (among other oxidation reactions), was commonly reported during organic micro-pollutants biotransformation under aerobic heterotrophic conditions, and could be carried out by multiple microorganisms (Kennes-Veiga et al., 2021). Furthermore, hydrolysis carried out by hydrolases, was already observed in activated sludge (Achermann et al., 2018). So, one of this TP341a or b could result from either the hydroxylation of DAMPA or from the hydrolysis of 7-OH-MTX and this last route is in accordance with the 7-OH-MTX profile (Figure 4.2- 8a). The equivalent pathway could be suggested for the OMTX equivalent TP, TP328 (Figure 4.2- 9a). Finally, TP411 a or b reached a maximum level of 0.007% after 2 h (Figure 4.2- 9b).

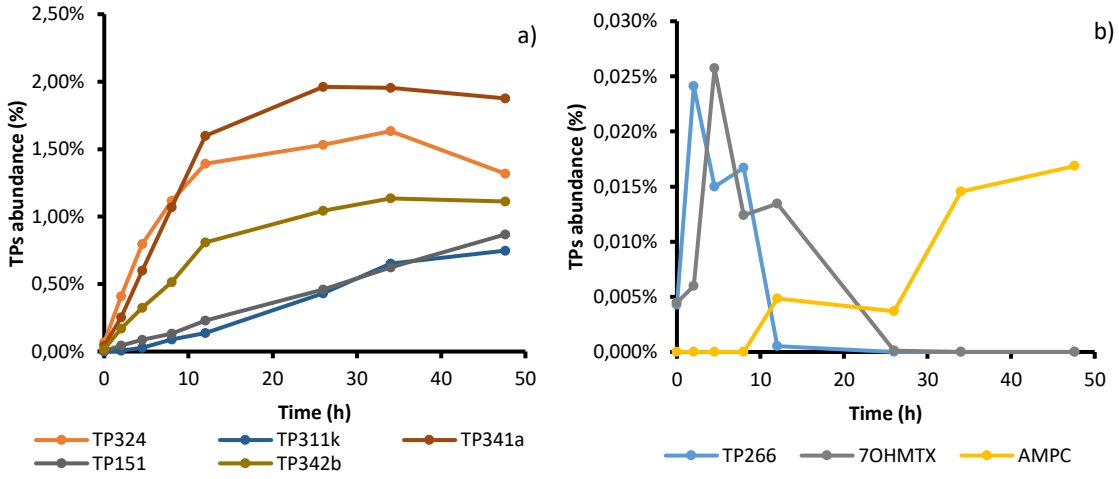


Figure 4.2- 8 Minor biodegradation products of MTX: between 0.5 and 2% a) and below 0.5% b) - Results expressed in % of the initial amount

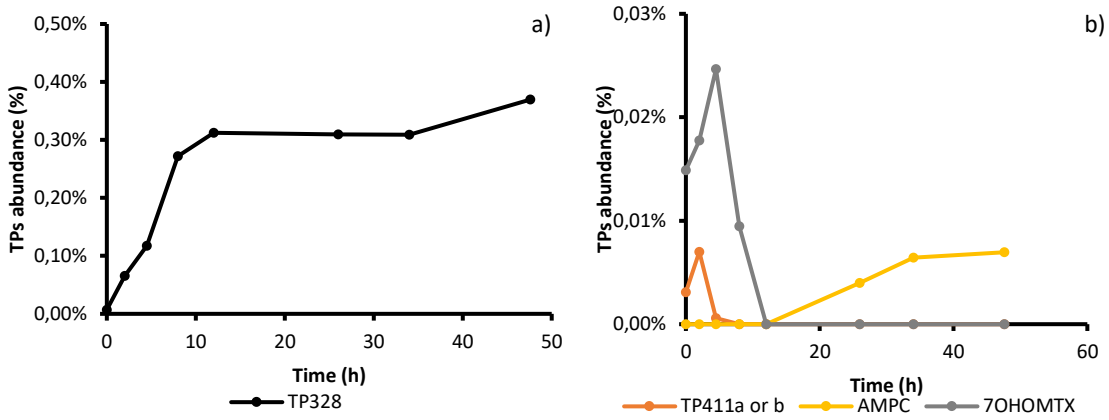


Figure 4.2- 9 Minor biodegradation products of OMTX above 0.3% a) and below 0.03% b) - Results expressed in % of the initial amount

4.2.4.4. Cytotoxicity assessment

The supernatants of diluted activated sludge spiked with MTX or OMTX were used for cytotoxicity assessment at the beginning and at the end of incubation to allow comparison of cytotoxic effect of parent compounds and TPs presents at final time.

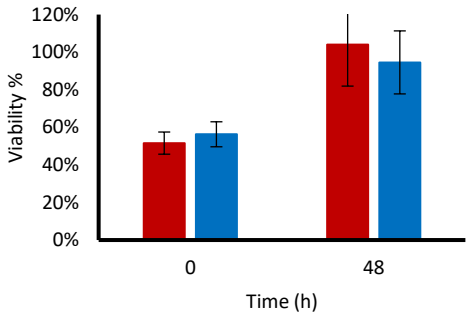


Figure 4.2- 10 Evolution of viability of A549 cells exposed to supernatant of diluted activated sludge samples spiked with MTX (red) or OMTX (blue) before incubation and at the end (48 h)

Sample dilutions for setting a ~50% inhibition of cell growth for the initial time of incubation (T0) samples allowed to observe the evolution of cytotoxicity. At T0, only parent compounds were present and results were similar for MTX and OMTX because of their similarIC_{50} (Espinosa et al., submitted). Figure 4.2- 10 shows an increase of viability between T0 and T48, indicating that the cytotoxic effect was diminished at the end of the incubation, when parent compounds were totally degraded (Figure 4.2-7) and mostly converted into DAMPA for MTX, and ODAMPA for OMTX. This loss of cytotoxic action of DAMPA compared with that of MTX is in accordance with literature (Widemann et al., 2000).

4.2.4.5. Assessment of the potential impact on the microbial community present in the diluted activated sludge

The potential impact of molecules on the microbiological community was assessed through indicators of the general metabolism and the carbon and nitrogen cycles. No significant impact between MTX and OMTX were evidenced and there were almost no significant differences comparing MTX or OMTX treatments with control without pharmaceutical after 2 h or 48 h of incubation (Table 4.2- 1).

Table 4.2- 1 Responses of the monitored indicators compared with control for impact assessment (significance level $p < 0.05$)

	Indicator	Significant impact in presence of MTX	Significant impact in presence of OMTX
General	Oxygen consumption	No	↗
	Dehydrogenase (DHA)	No	No
	C/N Ratio	No	No
	Dry mass	No	No
Carbon cycle	B-Glucosidase (B-GLU)	No	No
	Total Carbon (C_{TOT})	No	No
	Inorganic Carbon (C_{IN})	No	No
	Organic Carbon (C_{ORGa})	No	No
Nitrogen cycle	Urease (URE)	↗	No
	Arylamidase (ARN)	No	No
	Potential Nitrification (NIT)	No	No
	Nitrate Reductase (NITRED)	No	No
	Total Nitrogen (N_{TOT})	No	No
	Inorganic Nitrogen (N_{IN})	No	No

For the majority of the followed indicators, no impact of molecules MTX or OMTX could be observed when comparing to the control, except for the oxygen consumption and the urease activity.

The oxygen consuming reactions correspond to oxidation of organic substances, synthesis of organic biomass, and auto-oxidation of organic biomass (Delgado et al., 2010) and according to Amariei et al. (2017), the potential impact on microbial activity evaluated through respiration measurement could be more impaired than enzymatic one. As presented in Figure 4.2- 11, the addition of MTX or OMTX seemed to increase the oxygen consumption linked to the respiration, and compared to the control, it was significant only for OMTX (while it was not different between MTX and OMTX). It could be an indicator of a stress response of the sludge. However, this statistical increase represented a low variation (<15 %) and it was finally not considered as an indication of ecotoxicity regarding the sensitivity of the measurement. The Dehydrogenase (DHA), an enzyme of the respiratory chain (Yao et al., 2012)

characteristics of overall microbial activity (Vargas-García et al., 2010), seemed to increase after 2 h when spiked with MTX or OMTX, but because of the high uncertainty caused by levels near to the detection limit of DHA, non-significant differences were observed (data not shown).

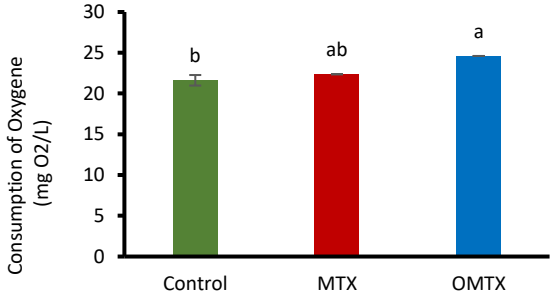


Figure 4.2- 11 Oxygen consumption in (mg O_2/L) after the 48 h of incubation

for control, spiked MTX and spiked OMTX diluted activated sludge

Concerning nitrogen cycle, urease (URE), an extracellular enzyme which catalyses the hydrolysis of nitrogen-containing organic compounds and transforms urea into ammonium, is a key enzyme in activated sludge (Yao et al., 2012). Urease activity was affected in presence of MTX after 2 hours of incubation (Figure 4.2- 12a) while it was not significant for OMTX. At the end of the incubation, the activity was back to the same level as the control (Figure 4.2- 12b). Another enzyme linked to the nitrogen cycle, the arylamidase (ARN) showed a tendency to increase in presence of MTX, but this trend was not significant (data not shown). Furthermore, no difference was observed in the nitrate, nitrites and total nitrogen contents of the sludge supernatant.

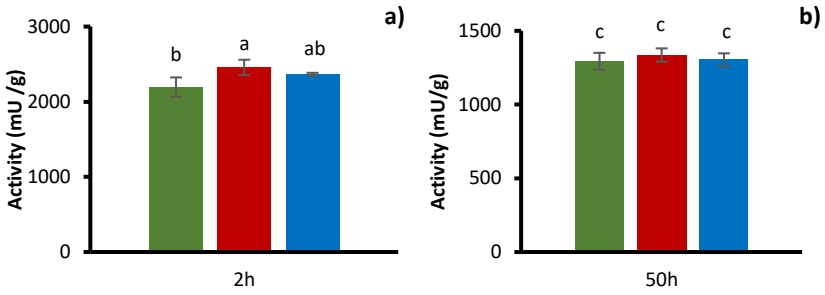


Figure 4.2- 12 Urease activity after 2 h (a) and 50 h (b) of incubation

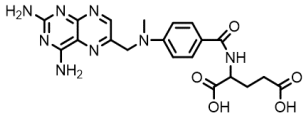
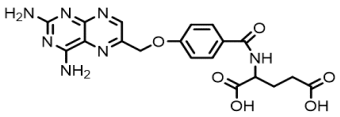
for the Control (green), the spiked MTX (red) and the spiked OMTX (blue) diluted activated sludge and comparing to control at 2 h

3.1.1. Conclusions

Biodegradation of MTX and OMTX were in the same order of magnitude in terms of kinetics, even if OMTX biodegraded slightly faster. Both compounds exhibited a similar behaviour during the biodegradation in sludge supernatant. The harmful 7-hydroxylated compounds (7-OH-MTX and its equivalent 7-OH-OMTX), whose toxicity was suspected for 7-OH-MTX (Besse et al., 2012) and justified to study an eco-designed analog, were finally found as minor and transient. Major TPs of both compounds resulted in hydrolysis of the glutamic acid part with the formation of one major and persistent TP, the DAMPA for MTX and the equivalent ODAMPA for OMTX with the loss of the cytotoxic action for both. Even if DAMPA loosed the cytotoxic action and is known as a weak inhibitor of DHFR (Widemann et al., 2000), it may potentially serve as the precursors of antifolates, in particular the synthesis of MTX starting from DAMPA was observed inside parasites (Nduati et al., 2005; Muregi et al., 2008) and such enzymatic synthesis could happen in different micro-organisms leading to only temporary degradation of MTX. ODAMPA could present the same effect and lead to environmental negative impact. Contrary to photodegradation, the advantages of the eco-designed OMTX were not highlighted under biodegradation by activated sludge. The formation of persistent TPs would need further investigations, even if no remarkable and persistent effects on microbial activity through indicators assessed were noticed for this short term and punctual exposition. However, we should be aware that even if the level of cytostatics would be lower in environmental conditions than in the present experiment, the continuous influx and the "cocktail effects" could lead to more contrasted results in the long term.

3.1.2. Supplementary information

Table 4.2- S1 Physico-chemical and biological properties of MTX and its eco-designed analog OMTX

	MTX	**OMTX**
Name	*acide (2S)-2-[(4-{[(2,4-diamino-7,8-dihydroptéridin-6-yl)méthyl](méthyl)amino}phényl)formamido] pentanedioïque*	*N-[a-(2-amino-4-hydroxy-6- pteridinyl)-p-anisoyl]-L-glutamic acid*
CAS	59-05-2	57963-55-0
Formula	$C_{20}H_{22}N_8O_5$	$C_{19}H_{19}N_7O_6$
Structure		
Exact Mass	454.1713	441.1397
pKa [b]	4.15 3.56	4.15 3.5
pKa [c]	3.36 4.70 5.71	X
pKa [d]	X	4.55
LogKow: Octanol-Water [b]	0.94	0.53
LogKow: Octanol-Water [a]	-1.85	-0.177
Bioconcentration Factor [a]	7.78	5.77
Biodeg. Half-Life (days) [a]	5.03	7.41
Fish Biotrans. Half-Life (days) [a]	0.198	0.205
Henry's Law (atm-m3/mole) [a]	1.45e-11	1.46e-11
Koc Soil Adsorp. Coeff. (L/kg) [a]	54.2	44.5
Vapor Pressure (mmHg) [a]	7.17e-9	7.21-9
Water Solubility (mol/L) [a]	1.89e-4	1.33e-4

[a] *https://comptox.epa.gov/dashboard/chemical/properties/DTXSID80330337: OPERA is a free and open source/open data suite of QSAR Models providing predictions and additional information ;* [b] *Chemdraw;* [c] *Rubino et al. (2001);* [d] *Experimental*

Table 4.2- S2 Raw activated sludge properties

	Raw Activated sludge
Dry mass (g/L)	4.69 ± 0.15
Suspended matter (g/L)	4.19 ± 0.13
Dry volatile matter (g/L)	3.16 ± 0.07
pH	7.1 ± 0.1
Total Carbon in particulate phase (mgC/L)	1569.5 ± 18.1
Total Carbon in aqueous phase (mgC/L)	44,12 ± 6.69
Inorganic Carbon in aqueous phase (mgC/L)	38,16 ± 5.52
Organic Carbon in aqueous phase (mgC/L)	5,96 ± 1.22
Total Nitrogen in particulate phase (mgN/L)	284.4 ± 2.4
Inroganic Nitrogen in aqueous phase (mgN/L)	0.895 ± 0.007

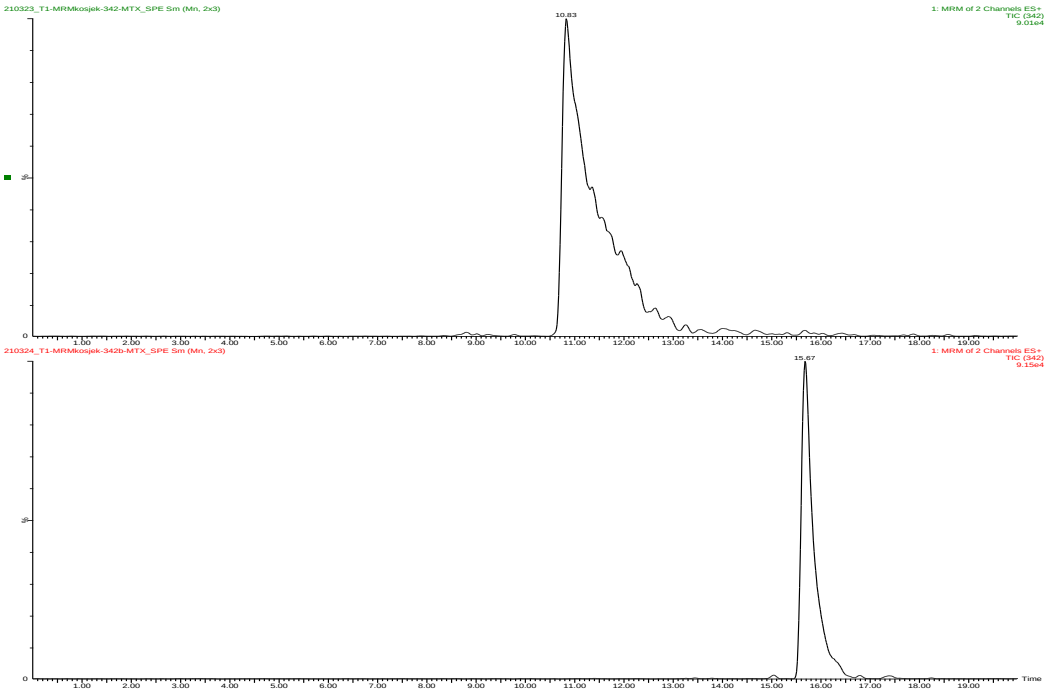
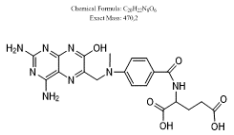
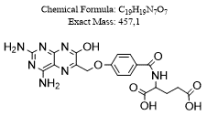
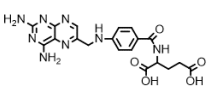
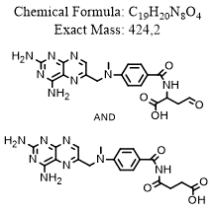
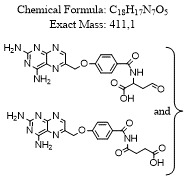
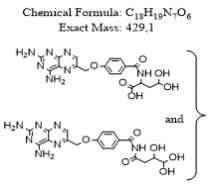
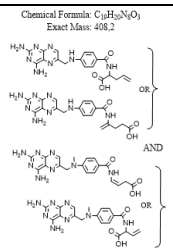
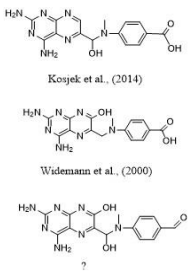
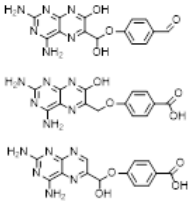
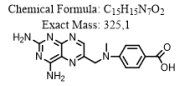
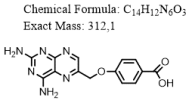
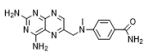
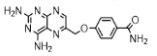
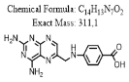
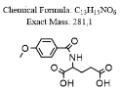
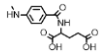
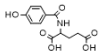
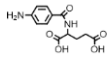
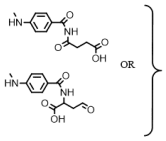
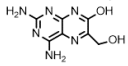
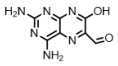
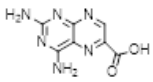
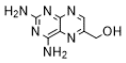


Figure 4.2- S1 MRM of TP 341a and TP 341b

Table 4.2- S3 Final list of structures of MTX and corresponding OMTX TPs (* when same structure is common to both molecules)

MTX TPs	Structures	OMTX TPs	Structures
7-OH-MTX		7-OH-OMTX	
TP 440	Chemical Formula: $C_{19}H_{20}N_8O_5$ Exact Mass: 440,2 		
TP 424a and b	Chemical Formula: $C_{19}H_{20}N_8O_4$ Exact Mass: 424,2 	TP 411a and b	Chemical Formula: $C_{18}H_{17}N_7O_5$ Exact Mass: 411,1 
		TP 441	Unidentified
		TP 429b and a	Chemical Formula: $C_{18}H_{18}N_7O_6$ Exact Mass: 429,1 
TP 408 b and a	Chemical Formula: $C_{18}H_{20}N_8O_3$ Exact Mass: 408,2 		
TP 341a and b Hypothesis		TP 328 Hypothesis	Chemical Formula: $C_{14}H_{12}N_6O_4$ Exact Mass: 328,1 
DAMPA	Chemical Formula: $C_{15}H_{15}N_7O_2$ Exact Mass: 325,1 	ODAMPA	Chemical Formula: $C_{14}H_{12}N_6O_3$ Exact Mass: 312,1 

TP 324	Chemical Formula: $C_{15}H_{16}N_8O$ Exact Mass: 324,1 	TP 311	Chemical Formula: $C_{14}H_{13}N_7O_2$ Exact Mass: 311,1 
TP 311k *(name TP311 is already given for an OMTX TP, and "k" correspond to "Kosjek")*	Chemical Formula: $C_{14}H_{13}N_7O_2$ Exact Mass: 311,1 		
		TP 281	Chemical Formula: $C_{12}H_{13}NO_6$ Exact Mass: 281,1 
TP 280	Chemical Formula: $C_{13}H_{16}N_2O_5$ Exact Mass: 280,1 	TP 267	Chemical Formula: $C_{12}H_{13}NO_6$ Exact Mass: 267,1 
TP 266	Chemical Formula: $C_{12}H_{14}N_2O_5$ Exact Mass: 266,1 		
TP 250	Chemical Formula: $C_{12}H_{14}N_2O_4$ Exact Mass: 250,1 		
TP 208*	Chemical Formula: $C_7H_8N_6O_2$ Exact Mass: 208,1 		
TP 206 *	Chemical Formula: $C_7H_6N_6O_2$ Exact Mass: 206,1 		
AMPC*	Chemical Formula: $C_7H_6N_6O_2$ Exact Mass: 206,0552 		
		TP 192	Chemical Formula: $C_7H_8N_6O$ Exact Mass: 192,1 
TP 190	Chemical Formula: $C_7H_6N_6O$ Exact Mass: 190,1 		
TP 151	Chemical Formula: $C_8H_9NO_2$ Exact Mass: 151,1 		

3.1.3. References

Abdelmagid, H.M., Tabatabai, M.A., 1987. Nitrate reductase activity of soils. Soil Biology and Biochemistry 19, 421–427. https://doi.org/10.1016/0038-0717(87)90033-2

Achermann, S., Falås, P., Joss, A., Mansfeldt, C.B., Men, Y., Vogler, B., Fenner, K., 2018. Trends in Micropollutant Biotransformation along a Solids Retention Time Gradient. Environmental Science & Technology 52, 11601–11611. https://doi.org/10.1021/acs.est.8b02763

Alvarino, T., Katsou, E., Malamis, S., Suarez, S., Omil, F., Fatone, F., 2014. Inhibition of biomass activity in the via nitrite nitrogen removal processes by veterinary pharmaceuticals. Bioresource Technology 152, 477–483. https://doi.org/10.1016/j.biortech.2013.10.107

Amariei, G., Boltes, K., Rosal, R., Leton, P., 2020. Enzyme response of activated sludge to a mixture of emerging contaminants in continuous exposure. PLoS ONE 15, e0227267. https://doi.org/10.1371/journal.pone.0227267

Amariei, G., Boltes, K., Rosal, R., León, P., 2017. Toxicological interactions of ibuprofen and triclosan on biological activity of activated sludge. Journal of Hazardous Materials 334, 193–200. https://doi.org/10.1016/j.jhazmat.2017.04.018

Baginska, E., Haiß, A., Kümmerer, K., 2015. Biodegradation screening of chemicals in an artificial matrix simulating the water–sediment interface. Chemosphere 119, 1240–1246. https://doi.org/10.1016/j.chemosphere.2014.09.103

Besse, J.-P., Latour, J.-F., Garric, J., 2012. Anticancer drugs in surface waters. Environment International 39, 73–86. https://doi.org/10.1016/j.envint.2011.10.002

Booker, V., Halsall, C., Llewellyn, N., Johnson, A., Williams, R., 2014. Prioritising anticancer drugs for environmental monitoring and risk assessment purposes. Science of The Total Environment 473–474, 159–170. https://doi.org/10.1016/j.scitotenv.2013.11.145

Calza, P., Medana, C., Sarro, M., Rosato, V., Aigotti, R., Baiocchi, C., Minero, C., 2014. Photocatalytic degradation of selected anticancer drugs and identification of their transformation products in water by liquid chromatography–high resolution mass spectrometry. Journal of Chromatography A 1362, 135–144. https://doi.org/10.1016/j.chroma.2014.08.035

Castiglioni, S., Bagnati, R., Calamari, D., Fanelli, R., Zuccato, E., 2005. A multiresidue analytical method using solid-phase extraction and high-pressure liquid chromatography tandem mass spectrometry to measure pharmaceuticals of different therapeutic classes in urban wastewaters. Journal of Chromatography A 1092, 206–215. https://doi.org/10.1016/j.chroma.2005.07.012

Çeçen, F., Gül, G., 2021. Biodegradation of five pharmaceuticals: estimation by predictive models and comparison with activated sludge data. Int. International Journal of Environmental Science and Technology 18, 327–340. https://doi.org/10.1007/s13762-020-02820-y

Dalzell, D.J.B., Alte, S., Aspichueta, E., de la Sota, A., Etxebarria, J., Gutierrez, M., Hoffmann, C.C., Sales, D., Obst, U., Christofi, N., 2002. A comparison of five rapid direct toxicity assessment methods to determine toxicity of pollutants to activated sludge. Chemosphere 47, 535–545. https://doi.org/10.1016/S0045-6535(01)00331-9

Delgado, L.F., Schetrite, S., Gonzalez, C., Albasi, C., 2010. Effect of cytostatic drugs on microbial behaviour in membrane bioreactor system. Bioresource Technology 101, 527–536. https://doi.org/10.1016/j.biortech.2009.08.051

Doublet, J., Francou, C., Poitrenaud, M., Houot, S., 2010. Sewage sludge composting: Influence of initial mixtures on organic matter evolution and N availability in the final composts. Waste Management 30, 1922–1930. https://doi.org/10.1016/j.wasman.2010.04.032

Espinosa, A., Nélieu, S., Lieben, P., Skarbek, C., Labruère, R., Benoit, P., 2021. Photodegradation of methotrexate in aqueous solution: degradation kinetics and identification of transformation products. Environmental Science and Pollution Research https://doi.org/10.1007/s11356-021-15820-3

Espinosa, A., Rascol, E., Abellàn Flos, M., Skarbek, C., Lieben, P., Bannerman, E., Diez Marinez, A., Pethe, S., Benoit, P., Nélieu, S., Labruère, R., Submitted in Angewandte Chemie International Edition

Fabre, G., Goldman, I.D., 1985. Formation of 7-hydroxymethotrexate polyglutamyl derivatives and their cytotoxicity in human chronic myelogenous leukemia cells, in vitro. Cancer Research 45, 80–85.

Falås, P., Andersen, H.R., Ledin, A., la Cour Jansen, J., 2012. Impact of solid retention time and nitrification capacity on the ability of activated sludge to remove pharmaceuticals. Environmental Technology 33, 865–872. https://doi.org/10.1080/09593330.2011.601764

Ferrando-Climent, L., Rodriguez-Mozaz, S., Barceló, D., 2014. Incidence of anticancer drugs in an aquatic urban system: From hospital effluents through urban wastewater to natural environment. Environmental Pollution 193, 216–223. https://doi.org/10.1016/j.envpol.2014.07.002

Ferrando-Climent, L., Rodriguez-Mozaz, S., Barceló, D., 2013. Development of a UPLC-MS/MS method for the determination of ten anticancer drugs in hospital and urban wastewaters, and its application for the screening of human metabolites assisted by information-dependent acquisition tool (IDA) in sewage samples. Analytical and Bioanalytical Chemistry 405, 5937–5952. https://doi.org/10.1007/s00216-013-6794-4

Galaon, T., Petre, J., Iancu, V.I., Cruceru, L., Niculescu, M., Chiriac, F.L., Puiu, D.M., Mihalache, M., 2017. detection of cytostatic drugs in municipal waste water and their transfer to surface water, in: simi 2017. presented at the International Symposium "The

Environment and the Industry," National Research and Development Institute for Industrial Ecology, pp. 229–236. https://doi.org/10.21698/simi.2017.0029

Henschel, K.-P., Wenzel, A., Diedrich, M., Fliedner, A., 1997. Environmental Hazard Assessment of Pharmaceuticals. Regulatory Toxicology and Pharmacology 25, 220–225. https://doi.org/10.1006/rtph.1997.1102

Johnson, A.C., Jürgens, M.D., Williams, R.J., Kümmerer, K., Kortenkamp, A., Sumpter, J.P., 2008. Do cytotoxic chemotherapy drugs discharged into rivers pose a risk to the environment and human health? An overview and UK case study. Journal of Hydrology 348, 167–175. https://doi.org/10.1016/j.jhydrol.2007.09.054

Kennes-Veiga, D.M., Vogler, B., Fenner, K., Carballa, M., Lema, J.M., 2021. Heterotrophic enzymatic biotransformations of organic micropollutants in activated sludge. Science of The Total Environment 780, 146564. https://doi.org/10.1016/j.scitotenv.2021.146564

Kessler, R., 2010. INDUSTRY ISSUES: Pharmaceutical Factories as a Source of Drugs in Water. Environmental Health Perspectives 118. https://doi.org/10.1289/ehp.118-a383

Kiffmeyer, T., Götze, H.-J., Jursch, M., Lüders, U., 1998. Trace enrichment, chromatographic separation and biodegradation of cytostatic compounds in surface water. Fresenius' Journal of Analytical Chemistry 361, 185–191. https://doi.org/10.1007/s002160050859

Kosjek, T., Heath, E., 2011. Occurrence, fate and determination of cytostatic pharmaceuticals in the environment. TrAC Trends in Analytical Chemistry 30, 1065–1087. https://doi.org/10.1016/j.trac.2011.04.007

Kosjek, T., Negreira, N., de Alda, M.L., Barceló, D., 2015. Aerobic activated sludge transformation of methotrexate: Identification of biotransformation products. Chemosphere 119, S42–S50. https://doi.org/10.1016/j.chemosphere.2014.04.081

Kraigher, B., Kosjek, T., Heath, E., Kompare, B., Mandic-Mulec, I., 2008. Influence of pharmaceutical residues on the structure of activated sludge bacterial communities in wastewater treatment bioreactors. Water Research 42, 4578–4588. https://doi.org/10.1016/j.watres.2008.08.006

Kümmerer, K., 2007. Sustainable from the very beginning: rational design of molecules by life cycle engineering as an important approach for green pharmacy and green chemistry. Green Chemistry 9, 899. https://doi.org/10.1039/b618298b

Lai, W.W.-P., Hsu, M.-H., Lin, A.Y.-C., 2017. The role of bicarbonate anions in methotrexate degradation via UV/TiO 2 : Mechanisms, reactivity and increased toxicity. Water Research 112, 157–166. https://doi.org/10.1016/j.watres.2017.01.040

Li, D., Chen, H., Liu, H., Schlenk, D., Mu, J., Lacorte, S., Ying, G.-G., Xie, L., 2021. Anticancer drugs in the aquatic ecosystem: Environmental occurrence, ecotoxicological effect and risk assessment. Environment International 153, 106543. https://doi.org/10.1016/j.envint.2021.106543

Love, N.G., Bott, C.B., 2002. Evaluating the role of microbial stress response mechanisms in causing biological treatment system upset. Water Science and Technology 46, 11–18. https://doi.org/10.2166/wst.2002.0449

Lutterbeck, C.A., Baginska, E., Machado, Ê.L., Kümmerer, K., 2015. Removal of the anti-cancer drug methotrexate from water by advanced oxidation processes: Aerobic biodegradation and toxicity studies after treatment. Chemosphere 141, 290–296. https://doi.org/10.1016/j.chemosphere.2015.07.069

Mahnik, S.N., Lenz, K., Weissenbacher, N., Mader, R.M., Fuerhacker, M., 2007. Fate of 5-fluorouracil, doxorubicin, epirubicin, and daunorubicin in hospital wastewater and their elimination by activated sludge and treatment in a membrane-bio-reactor system. Chemosphere 66, 30–37. https://doi.org/10.1016/j.chemosphere.2006.05.051

Molina-Muñoz, M., Poyatos, J.M., Rodelas, B., Pozo, C., Manzanera, M., Hontoria, E., Gonzalez-Lopez, J., 2010. Microbial enzymatic activities in a pilot-scale MBR experimental plant under different working conditions. Bioresource Technology 101, 696–704. https://doi.org/10.1016/j.biortech.2009.08.071

Muregi, F.W., Kino, H., Ishih, A., 2008. Plasmodium berghei: Lack of antimalarial activity of an analogue of folate precursor, 2,4-diamino-6-hydroxymethylpteridine in a mouse model. Experimental Parasitology 120, 286–289. https://doi.org/10.1016/j.exppara.2008.08.006

Nduati, E., Hunt, S., Kamau, E.M., Nzila, A., 2005. 2,4-Diaminopteridine-Based Compounds as Precursors for De Novo Synthesis of Antifolates: a Novel Class of Antimalarials. Antimicrobial Agents and Chemotherapy 49, 3652–3657. https://doi.org/10.1128/AAC.49.9.3652-3657.2005

Olalla, A., Negreira, N., López de Alda, M., Barceló, D., Valcárcel, Y., 2018. A case study to identify priority cytostatic contaminants in hospital effluents. Chemosphere 190, 417–430. https://doi.org/10.1016/j.chemosphere.2017.09.129

Parrella, A., Kundi, M., Lavorgna, M., Criscuolo, E., Russo, C., Isidori, M., 2014a. Toxicity of exposure to binary mixtures of four anti-neoplastic drugs in Daphnia magna and Ceriodaphnia dubia. Aquatic Toxicology 157, 41–46. https://doi.org/10.1016/j.aquatox.2014.09.012

Parrella, A., Lavorgna, M., Criscuolo, E., Russo, C., Fiumano, V., Isidori, M., 2014b. Acute and chronic toxicity of six anticancer drugs on rotifers and crustaceans. Chemosphere 115, 59–66. https://doi.org/10.1016/j.chemosphere.2014.01.013

Quintana, J., Weiss, S., Reemtsma, T., 2005. Pathways and metabolites of microbial degradation of selected acidic pharmaceutical and their occurrence in municipal wastewater treated by a membrane bioreactor. Water Research 39, 2654–2664. https://doi.org/10.1016/j.watres.2005.04.068

Rabii, F.W., Segura, P.A., Fayad, P.B., Sauvé, S., 2014. Determination of six chemotherapeutic agents in municipal wastewater using online solid-phase extraction coupled to liquid chromatography-tandem mass spectrometry. Science of The Total Environment 487, 792–800. https://doi.org/10.1016/j.scitotenv.2013.12.050

Ren, S., 2004. Assessing wastewater toxicity to activated sludge: recent research and developments. Environment International 30, 1151–1164. https://doi.org/10.1016/j.envint.2004.06.003

Rodríguez-Espinosa, P.F., Mendoza-Pérez, J.A., Tabla-Hernandez, J., Martínez-Tavera, E., Monroy-Mendieta, M.M., 2018. Biodegradation and kinetics of organic compounds and heavy metals in an artificial wetland system (AWS) by using water hyacinths as a biological filter. International Journal of Phytoremediation 20, 35–43. https://doi.org/10.1080/15226514.2017.1328397

Rubino, F.M., 2001. Separation methods for methotrexate, its structural analogues and metabolites. Journal of Chromatography B: Biomedical Sciences and Applications 764, 217–254. https://doi.org/10.1016/S0378-4347(01)00402-9

Sabry, S.M., Abdel-Hady, M., Elsayed, M., Fahmy, O.T., Maher, H.M., 2003. Study of stability of methotrexate in acidic solution Spectrofluorimetric determination of methotrexate in pharmaceutical preparations through acid-catalyzed degradation reaction. Journal of Pharmaceutical and Biomedical Analysis 32, 409–423. https://doi.org/10.1016/S0731-7085(03)00239-5

Vargas-García, M.C., Suárez-Estrella, F., López, M.J., Moreno, J., 2010. Microbial population dynamics and enzyme activities in composting processes with different starting materials. Waste Management 30, 771–778. https://doi.org/10.1016/j.wasman.2009.12.019

Verlicchi, P., Al Aukidy, M., Galletti, A., Petrovic, M., Barceló, D., 2012. Hospital effluent: Investigation of the concentrations and distribution of pharmaceuticals and environmental risk assessment. Science of The Total Environment 430, 109–118. https://doi.org/10.1016/j.scitotenv.2012.04.055

Verlicchi, P., Al Aukidy, M., Zambello, E., 2015. What have we learned from worldwide experiences on the management and treatment of hospital effluent? — An overview and a discussion on perspectives. Science of The Total Environment 514, 467–491. https://doi.org/10.1016/j.scitotenv.2015.02.020

Wang, X., Zhang, J., Chang, V.W.C., She, Q., Tang, C.Y., 2018. Removal of cytostatic drugs from wastewater by an anaerobic osmotic membrane bioreactor. Chemical Engineering Journal 339, 153–161. https://doi.org/10.1016/j.cej.2018.01.125

Widemann, B.C., Sung, E., Anderson, L., Salzer, W.L., Balis, F.M., Monitjo, K.S., McCully, C., Hawkins, M., Adamson, P.C., 2000. Pharmacokinetics and metabolism of the methotrexate metabolite 2, 4-diamino-N(10)-methylpteroic acid. The Journal of Pharmacology and Experimental Therapeutics 294, 894–901.

Yao, Y., Lu, Z., Min, H., Gao, H., Zhu, F., 2012. The effect of tetrahydrofuran on the enzymatic activity and microbial community in activated sludge from a sequencing batch reactor. Ecotoxicology 21, 56–65. https://doi.org/10.1007/s10646-011-0765-3

Chapitre 5. Comparaison de la toxicité aigüe du methotrexate avec celle de son analogue éco-conçu

Ce chapitre présente sous la forme d'un manuscrit, les résultats des tests d'écotoxicité aquatique sur les deux molécules mères, MTX et OMTX, réalisés à l'INERIS. Ce manuscrit sera prochainement soumis à une revue telle que Ecotoxicology and Environmental Safety. Les éléments du **Chapitre 4**, indiqués par des notes en bas de page seront positionnés en Supplementary Materials.

Il est nécessaire de sélectionner des organismes sensibles afin de permettre la comparaison entre les deux molécules. En accord avec la littérature présentée en **Chapitre 1**, l'algue *R. subcapitata* a donc été retenue, cependant l'exposition lumineuse nécessaire à la croissance des algues provoque une dégradation des molécules, le support analytique selon la méthode développée au **Chapitre 4.1** a permis de suivre la stabilité des molécules mais aussi d'observer les produits de transformations afin de critiquer les résultats obtenus.

La bactérie *A. fischeri* a aussi été sélectionnée en raison de la rapidité et de la simplicité de ce test et afin d'éviter la dégradation des molécules. Cependant la littérature est hétérogène quant à sa sensibilité. Un tel test permettrait aussi de suivre l'évolution de la toxicité au cours de la photodégradation ou de la biodégradation comme proposé par différents auteurs (Calza et al., 2014 ; Lutterbeck et al., 2015a; Lai et al., 2017). La bactérie s'est cependant révélée non sensible pour les deux molécules et n'a pas permis de poursuivre l'évaluation des effets ecotoxicologiques des produits de transformation.

Nous avions aussi sélectionné une espèce d'enchytréide, *Enchytraeus albidus,* pour travailler sur un organisme présent dans les milieux aquatiques et terrestres. Il nous paraissait intéressant de considérer cet organisme encore non étudié sur des anticancéreux. Les essais préliminaires, uniquement réalisés sur la molécule MTX n'ont pas rapporté d'effets létaux ou sublétaux (concentration maximale testée: 100 mg/L), cette expérimentation n'a pas été poursuivie et les premiers résultats ne sont pas présentés.

Comparison of the acute aquatic toxicity of the methotrexate and its eco designed analog

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5.1. Abstract

Through the introduction within the molecular structure of a sensitive chemical group, the eco-design of drugs is meant to enhance their breakdown after their excretion by the human body, in particular during water treatment. Such a strategy may represent a promising route for decreasing the impacts of pharmaceutical residues on ecosystems and their adverse effects on living organisms. The aquatic toxicity of cytotoxic methotrexate (MTX) and its eco-designed analog OMTX was assessed by using two standardized tests: the luminescent marine bacterial test on *Aliivibrio fischer*i and the algal growth inhibition test on *Raphidocelis subcapitata*. *A. fischeri* was not sensitive to these molecules contrary to the algae *R. subcapitata*. OMTX was non-toxic to algae while MTX was revealed to be highly toxic with an EC_{50} 47 times lower than the OMTX one. As both molecules are sensitive to light, the aqueous solution was analysed by online-SPE-UHPLC-MS/MS after a 72 h exposition. The fate of both parent molecules supports the toxicity of MTX but also revealed that its transformation products could be involved. The lack of aquatic toxicity for OMTX confirms that eco-conception is a promising approach, and this analog deserves further investigation.

5.2. Introduction

Pharmaceuticals reach the environment because of their poor metabolization in the human body (Turci et al., 2003), resulting in their excretion by urine and faeces and then their presence in hospital and domestic wastewaters, and their partial removal in wastewater treatment plants (WWTP). Among the large variety of pharmaceuticals, anticancer drugs are detected in WWTP influents and effluents and are suspected to represent risk for aquatic species because of their heavy potent mode of action (Buerge et al., 2006; Besse et al., 2012; Booker et al., 2014; Kümmerer et al., 2016). As cytotoxics, they are reported to exert genotoxic, mutagenic and carcinogenic effects (Zounková et al., 2007; Booker et al., 2014; Česen et al., 2016) even at very low concentrations (Parrella et al., 2014). As a matter of fact, they are designed to disrupt cell replication and act non-selectively on all growing cells (Johnson et al., 2008; Besse et al., 2012; Galaon et al., 2017). Beyond their own impact, they could have synergic effect in mixtures (Parrella et al., 2014; Isidori et al., 2016) and their transformation products (TPs) could be even more toxic (Negreira et al., 2014; Borgatta et al., 2015; Česen et al., 2016).

Methotrexate (MTX) is a cytotoxic compound from antimetabolite class L01BA according to the Anatomical Therapeutic Classification, which inhibits folate-dependent metabolic processes, by structural analogy with folic acid and finally compromising cell proliferation. MTX is also used for rheumatoid arthritis, psoriasis and autoimmune disease (Sabry et al., 2003). Largely excreted unchanged in urine (81%) (Benet et al., 2011), it is one of the three antimetabolites the most frequently detected in wastewater (Li et al., 2021). MTX was found at level of µg/L in hospital effluents and at ng/L in WWTP influents (Garcia-Ac et al., 2009; Catastini et al., 2008; Yin et al., 2010; Negreira et al., 2013; Martín et al., 2014; Olalla et al., 2018). Even if Kiffmeyer et al. (1998), Kosjek et al. (2015) and ([9]) reported its complete biodegradation in a few hours to few days during activated sludge experiments MTX is nevertheless found in WWTP effluent at ng/L level (Catastini et al., 2008; Ferrando-Climent et al., 2013; Negreira et al., 2014). However, there is a lack of data on its occurrence in surface and drinking waters. Rabii et al. (2014) revealed that MTX was not significantly eliminated from water by physico-chemical treatment in municipal WWTPs with low residence time, and detected it at levels up to 53 ng/L in the effluent discharge. Ferrando-Climent et al. (2014) also detected it at 5 ng/L in a river after a WWTP discharge although MTX is known to be photosensitive (Calza et al., 2014; Espinosa et al., 2021). Due to its potential presence in the environment, its ecotoxicological effects have been assessed in recent years, Table (5-1) summarises the available data for aquatic toxicity assessment. Data obtained are heterogeneous, even on the same species, making it difficult to draw robust conclusions from this data set. For example, Calza et al. (2014) observed a 100% luminescence inhibition on *A. fisheri* at level of 15 mg/L and Barışçı et al. (2018) reported an EC_{50} of 3 mg/L, while Henschel et al. (1997) and Białk-Bielińska et al. (2017) did not notice significant effect at 100 mg/L.

[9] cf résultats du Chapitre 4.2

Table 5- 1 Ecotoxicological data (EC_{50} in mg/L) for MTX towards selected organisms

	Latin name	Organism	EC_{50} (mg/L)	Endpoint	Références
Vegetal	*Latuca sativa*	Letttuce	2,2	Root growth of the seeds (120 h)	Lutterberck et al., 2015b
	Scenedesmus subspicatus	Algae	260	Growth inhibition (72 h)	Henschel et al., 1997
	Raphidocelis subcapitata	Algae	192*	Growth inhibition (ECOSAR)	Sanderson et al., 2003
	Raphidocelis subcapitata	-	9,51	Growth inhibition (72 h)	Bialk-Bielińska et al., 2017
	Isochrysis galbana	Algae	84	Growth inhibition (96 h)	G. V. Aguirre-Martínez et al., 2016
	Lemna minor	Duckweed	0,08**	Growth inhibition (7 d)	Bialk et al., 2017
	Lemna minor	-	0.02	Growth inhibition (7 d)	Markovic et al., 2021
Animal	*Daphnia magna*	Crustacean	*651**	Growth inhibition (ECOSAR)	Sanderson et al., 2003
	Daphnia magna	-	> 1000	Immobilization (48 h)	Henschel et al., 1997
	Daphnia magna	-	> 100	Immobilization (48 h)	Bialk-Bielińska et al., 2017
	Ceriodaphnia dubia	Crustacean	1.15	Reproduction (7 d)	Lutterbeck et al., 2014
	Artemia salina L. eggs	Shrimp eggs	< 15	Mortality (96 h)	Muneer et al., 2020
	Paracentrotus lividus	Urchins sea	1000	Fertilization process	Aguirre-Martínez et al., 2016
	Paracentrotus lividus	-	1.5	Larval development	Aguirre-Martínez et al., 2016
	Brachydaniorerio embryos	Fish embryos	85	Mortality (96 h)	Henschel et al., 1997
	Pimephales promelas	Fish	3.83, 10^{5}*	(ECOSAR)	Sanderson et al., 2003
Protoz-oans	*Tetrahymena pyriformis*		45	Growth inhibition 48 h	Henschel et al., 1997
Bacteria	*Aliivibrio fischeri*	Marine bacteria	1220	Luminescence inhibition (30 min)	Henschel et al., 1997
	Aliivibrio fischeri	-	900	Luminescence inhibition (30 min)	Aguirre-Martínez et al., 2016
	Aliivibrio fischeri	-	> 2.5	Luminescence inhibition (15 min)	Lai et al., 2017
	Aliivibrio fischeri	-	> 100	Luminescence inhibition (30 min)	Bialk-Bielińska et al., 2017
	Aliivibrio fischeri	-	> 4	Luminescence inhibition (30 min)	Lutterbeck et al., 2014
	Aliivibrio fischeri	–	< 15	Luminescence inhibition (30 min)	Calza et al., 2014
	Aliivibrio fischeri	-	3	Luminescence inhibition (15 min)	Barışçı et al., 2018

**ECOSAR prediction : ecotoxicological (Quantitative) Structure Activity Relationship ((Q)SAR) screening according to Sanderson et al. (2003)*

*** Semi-static conditions*

No toxic effects at the maximal concentration tested

Furthermore, sub-lethal effects can occur at lower concentrations. Lutterbeck et al. (2015b) and Muneer et al. (2020) revealed cytogenetic modification on onion *Allium cepa* at few mg/L. MTX was the most phytotoxic among the cytotoxic compounds tested (Lutterbeck et al., 2015b). At a concentration close to environmental ones (4 µg/L), Kleinert et al. (2021) observed an increase in dihydrofolate-reductase (DHFR) activity on the mussel *Elliptio complanata* gonad, consistently with MTX mode of action.

Looking closer to the fate of MTX, its two well-known metabolites are 7-hydroxy-MTX (7-OH-MTX) and 4-amino-4-deoxy-N10-methylpteroic acid (DAMPA) (Widemann et al., 2000). While the

DAMPA was found to lose the cytotoxic action (Widemann et al., 2000), the 7-OH-MTX was described as acting via the same cytotoxic mechanism as methotrexate with a lower potency (Fabre and Goldman, 1985). Furthermore, 7-OH-MTX is also formed in activated sludge (Kiffmeyer et al., 1998; Kosjek et al., 2015; and [10]) and present a risk for the aquatic environment (Besse et al., 2012). Concerning its ecotoxicological effect, it seems to be less toxic than MTX : under semi static conditions the EC_{50} on *Lemna minor* was 0.16 mg/L for MTX against 8.3 mg/L for 7-OH-MTX. However, the susceptibility of MTX and 7-OH-MTX to be photodegraded conducted to a difficult comparison because of their instability during the tests (Białk-Bielińska et al., 2017). The toxicity of MTX mixture with its TPs after Advanced Oxidation Processes (AOPs, based on radical reactions) treatment was also studied on *A. fischeri*, Calza et al. (2014) and Lutterbeck et al. (2015a) observed a decrease in toxicity after UV catalysis. However, Calza et al. (2014) mentioned a slight persistence of effects indicating some TPs are not totally innocuous. On the opposite, Lai et al. (2017) observed an increase in toxicity after UV catalysis treatment in presence of bicarbonate anions and related this increase to a secondary TP corresponding to the 4-aminobenzamide moiety of the molecule. Barışçı et al. (2018) also mentioned an increase after electrooxidation process but attributed it to 2,4 diamine-pteridine derivatives structures.

To overcome contamination by persistent and toxic compounds, innovative and anticipative approaches are emerging as the "benign by design" concept, which imply the design of chemicals that will, after use, fragment in the environment into innocuous and non-persistent transformation products (Kümmerer, 2007). In this eco-conception approach, an already known bioisoster of methotrexate where an oxygen atom replaces the N-methyl moiety was synthesized as described in Espinosa et al. (submitted). This compound displayed a comparable *in vivo* antitumor activity against L-1210 lymphoid leukemia in mice (Nair et al., 1981) and similar cytotoxicity against A549 cancer cells (Espinosa et al., submitted). Its photodegration and its biodegradation were previously studied in comparison with the methotrexate one (Espinosa et al., submitted ; and [10]).

To go one-step further in the study of environmental MTX impact compared to its eco-designed analog (OMTX), this study aims to assess their toxicity on the bacteria *A. fischeri* and on the algae *R. subcapitata*. Algae tests are performed under continuous illumination and analyses of parent compounds and TPs were combined to the toxic assessment because both MTX and OMTX are high susceptible to photodegradation (Booker et al., 2014; Calza et al., 2014; Espinosa et al., 2021; Espinosa et al., submitted).

[10] cf résultats du Chapitre 4.2

Białk-Bielińska et al. (2017) have demonstrated the total degradation of MTX at the end of the algae test, highlighting the importance of combining chemical analyses to the ecotoxicity tests to understand the nature of the observed effects and obtain reliable data. To support stability analysis, we used a previously developed analytical method for the quantification of both parent compounds and TPs issuing from photodegradation and biodegradation of MTX and OMTX ([11])

5.3. Material and methods

5.3.1. Chemical

MTX (MTX, CAS 59-05-2, > 98%) was obtained from Sigma Aldrich and NaOH 1 mol/L from Merk. The analyses were performed using formic acid (98–100%, Sigma Aldrich), acetonitrile (RS plus gradient, Carlo Erba), and methanol (RPE, Carlo Erba). OMTX was synthesised as described by Espinosa et al. (submitted) (see physico-chemical and biological properties of MTX and its eco designed analogue OMTX - Supplementary Information[12]). MTX and OMTX stock solutions were prepared at 1239 and 1246 mg/L in Volvic water + 2% NaCl for the *A. fischeri* tests and in MilliQ water for the algae *R. subcapitata* test.

5.3.2. Ecotoxicological tests

5.3.2.1. Marine bacteria *Aliivibrio fischeri*

The luminescence inhibition of the marine bacterium *Aliivibrio fischeri* (formerly *Vibrio fischeri)* test was realised according to NF EN ISO 11348-3 on a Microtox Model 500 Toxicity Analyzer (Modern Water). This acute toxicity test allows determining the inhibition of the luminescence emitted by the marine bacteria after a contact time of 15 and 30 min by using a correction factor which is a measure of intensity changes of control samples during the exposure time. Freeze-dried bacteria, reconstitution solution, and diluent (2% NaCl) were obtained from Modern Water. Successive dilutions of the stock solutions were prepared in MilliQ Water (MTX : 16, 31, 62, 124, 248, 496, 991 mg/L and OMTX : 16, 31, 62, 125, 249, 498, 997 mg/L). The test design considered two replicates per concentration. The sensitivity of the bacteria batch was checked with the following reference substances (zinc sulfate heptahydrate, 3,5 dichlorophenol, potassium dichromate). Results are expressed in terms of Effective Concentration EC_{50} (i.e. effective concentration of the substance at which at which luminescence of *A. fischeri* is halved).

[11] cf résultats du Chapitre 4.1
[12] Cf Chapitre 4, Tableau 4.1- S2

5.3.2.2. Micro algae *Raphidocelis subcapitata*

Algae growth inhibition test was carried out in 24-well plates according to OECD 201 using *Raphidocelis subcapitata* (formerly *Pseudokirchneriella subcapitata*) (CCAP 278/4, SAMS Limited Culture Collection of Algae from Scottish Marine Institute, UK). Range of concentrations of the test substance : MTX (2, 4, 9, 19, 43, 94, 207, 455 and 974 mg/L) and OMTX (2, 4, 9, 19, 43, 94, 207, 455, 958 mg/L) were prepared by mixing appropriate quantities of growth medium, test sample and an inoculum of exponentially growing algal cells for a period of 72 hours. The initial cell density was 8.10^3 cellules/ml. Tests were conducted in a controlled temperature room (21.4°C-22.4°C) under continuous illumination (5513 ± 365 Lux). The test was carried out without pH adjustment, pH range were between 7 and 8 and pH between initial and final time were + 0.3. The study design consisted in 3 replicates per concentration (9 concentration levels), 6 controls and 2 blanks (without algae) one each plate of 2.5 mL wells. The cell density was determined after 72 h via measurements of algae cell counts (BeckmanCoulter Coulter® ZII) and chlorophyll-fluorescence (Victor-X3, PerkinElmer). Inhibition is measured as a reduction in specific growth rate, relative to control cultures grown under identical conditions. EC_{10} and EC_{50} values were calculated using a logistic Hill model with bootstrap estimation of confidence intervals. Calculations were performed with REGTOX software.

5.3.3. Analytical monitoring during the algae test

Photostability of parent compounds was checked toward analysis of parents and TPs at initial and final time (0 and 72 h). The previously developed analytical methods by online-SPE-UHPLC-MS/MS[13] were used. For the T0 analysis, 1 mL aliquots were withdrawn from an extra plate without algae and for Tf in another plate with algae exposed in the same condition as the ecotoxicological tests. Sampled were stocked at -20°C and protected from the light before analysis[14].

5.4. Results and discussion

5.4.1. Ecotoxicological tests

[13] Cf résultats Chapitre 4.1

[14] Des analyses complémentaires sur des témoins sans algues, conservés à -20°C, pourront être analysés ultérieurement afin de les comparer à ceux exposés en présence d'algues.

According to the results presented in Table (5- 2), the effect of both MTX and OMTX on the luminescence of *A. fischeri* was low (EC_{50} > 1000 mg/L) revealing they were non-toxic to the marine bacteria. This result is in accordance with Henschel et al. (1997); Białk-Bielińska et al. (2017) and Lutterbeck et al. (2014) as presented in Table 5- 1. We could notice that at the highest concentration tested, OMTX induced more effect than MTX (33% for both replicates against 22% and 24% of inhibition) but these concentrations are unlikely to occur in the environment.

MTX can be considered as toxic to algae based on measurements of algae cell counts with EC_{50} of 14.0 mg/L (fluorescence gave similar results). These results are close to those of Białk-Bielińska et al. (2017) who determined EC_{50} of 9.51 mg/L. The EC_{50} of OMTX appears to be 48-times higher than that of MTX, thus allowing to classify OMTX as less toxic. This difference will be discussed in regard to the photostability of compounds in the next section.

Table 5- 2 ECx values for aquatic organisms determined for MTX and its ecodesigned analog OMTX in mg/L

Compound	*A. fischeri* (EC_{50}) *(% of inhibition at 1000 mg/L for the 2 replicate)*	*R. subcapitata* (EC_{50}) (Confidence Interval, ±2.5%)	*R. subcapitata* (EC_{10}) (Confidence Interval, ±2.5%)
	30 min	72 h	
MTX	> 1000 *(22 and 24% of inhibition)*	14.0 (12.8-15.4)	4.4 (3.5-5.5)
OMTX	> 1000 *(33 and 33% of inhibition)*	664.3 (609.4-719.0)	167.0 (134.0-203.6)

5.4.2. Chemical analysis during the algae test

5.4.2.1. Evolution of the parent compound concentrations

The comparison of MTX concentration at initial and final time of the 72 h-light exposition during algae tests highlighted a weak stability (Table 5- 3), in accordance with its known photosensitivity (Calza et al., 2014 ; Espinosa et al., 2021). At concentrations close to the EC_{50}, the MTX was almost totally degraded with only 0.2 % of the parent compound remaining.

In contrast, OMTX appeared more stable than MTX with residual percentages of 77 % and 86 % respectively in each assay (Table 5- 3). Thus, the comparison of MTX and OMTX EC_{50} has to be considered

in function of those contrasted stabilities. Furthermore, the high toxic effect on the algal growth observed for MTX can result from a combined exposure of algae to MTX and its TPs.

Table 5- 3 Comparison of the initial and final (72 h) parent concentrations (in mg/L) during the algae test, at nominal concentration levels framing EC_{50} values

Compound	Experimental concentrations framing EC_{50} value (mg/L)	
	Initial	Final (72 h)
MTX	9	0.02
	19	0.04
OMTX	455	350
	985	824

In general, biodegradation rate increases as analyte concentrations decrease (Białk-Bielińska et al., 2017). Thanks to analyses of all concentration levels, we confirm this behaviour for OMTX, as shown in Figure (5- 1a) lower is the initial concentration, lower is the residual percentage and higher is the degradation level. However, MTX presented an opposite behaviour (Figure 5- 1b): higher was the concentration of MTX, higher was the degradation. This behaviour was already seen by Hsu et al. (2019), who demonstrated in their study of MTX photodegradation a self-sensitized mechanism due to the generation of triplet excited-state species issuing from MTX itself or from TPs containing a pteridine structure.

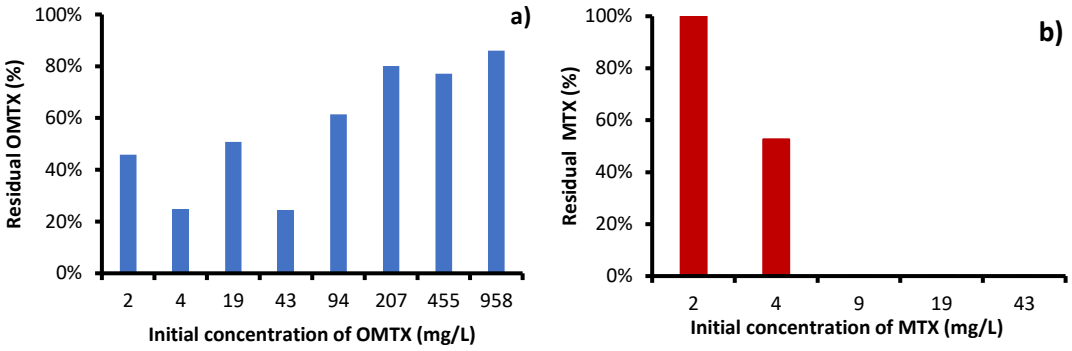


Figure 5- 1 Residual amounts expressed in % initial amounts after 72 h of exposition to light in function of the initial concentrations of OMTX (a) and MTX (b)

5.4.2.2. Formation of transformation products (TPs)

After a 72 h-light exposition, TPs observed could result from photodegradation, or from metabolization by the cultures (algae and associated bacteria) [15]. For MTX, TP 208 and TP 206 were the major TPs and were present at higher amounts when the MTX degradation was more advanced (Figure 5- 2a). These pteridine derivatives support the Hsu et al. (2019) hypothesis of a self-sensitized mechanism. These TPs were also detected in previous photodegradation studies (Calza et al., 2014; Lutterbeck et al., 2015a; Lai et al., 201; Kanjal et al., 2020). In our previous study, we have demonstrated that these TPs were non-cytotoxic against A549 cancer cells (Espinosa et al., 2021). Their contribution in toxicity on algae is not documented but Calza et al. (2014) and Barışçı et al. (2018) suspected diamino-pteridine derivatives structures to have a lower toxicity against *A. fischeri* compared to MTX but they could act as potent inhibitors of pteridine-dependent enzymes (Dhondt et al., 1991). TP 280, previously detected as minor by Espinosa et al. (2021) and Calza et al. (2014), and a new product corresponding to an isomer of MTX were also detected. This isomer could contribute in the toxicity because of its major presence and its probable structure similar to the MTX one, as supported by the detection of the same fragment ions than MTX in MS/MS daughter ion spectra acquired in positive mode, i.e. the m/z 308 ion issuing from the cleavage of the amide bond between the glutamic and p-methylaminobenzoic acid residues as well as by the m/z 134 ion formed by cleavage of the C–N bond between the p-methylaminobenzoyl and diaminopteridinemethylene groups (Espinosa et al. 2021). As a complement, the minor TPs issued from MTX as presented in Figure (5- 3a) were TP 266, TP 324, TP 440, TP 250, also previously observed during photodegradation (Espinosa et al., 2021) and the 7-OH-MTX. Two of those minor compounds, TP 440 and 7-OH-MTX, are known to act as MTX itself (Fabre and Goldman, 1985; Nduati et al., 2005) but their presence < 1% would not have a significant role in the toxicity observed. Attributing the contribution in toxicity to each compound would require further investigation, the monitoring of these TPs during the 72 h and the identification of the structure of MTX isomer furthermore regarding stability of compound, in environmental condition they would be find in mixture with tis TPs and other pollutants.

Concerning the TPs of OMTX, in our previous study the major photoproducts were TP 208 and TP 192 (Espinosa et al., submitted). But in the present study, these two TPs were only minor after the 72 h of exposition (Figure 5- 3b). This difference could be due to the difference in light exposition considering wavelength and energy, or to algae metabolism, which would have caused a lower degradation rate

[15] Les témoins exposés sans algues pourront être analysés ultérieurement, les résultats pourront être intégrés au Supplementary information

and/or difference in photodegradation mechanism. Here, the major TPs were TP 267, TP 411and TP 429 (Figure 5- 2b) and have never been shown to act as photosensitizers.

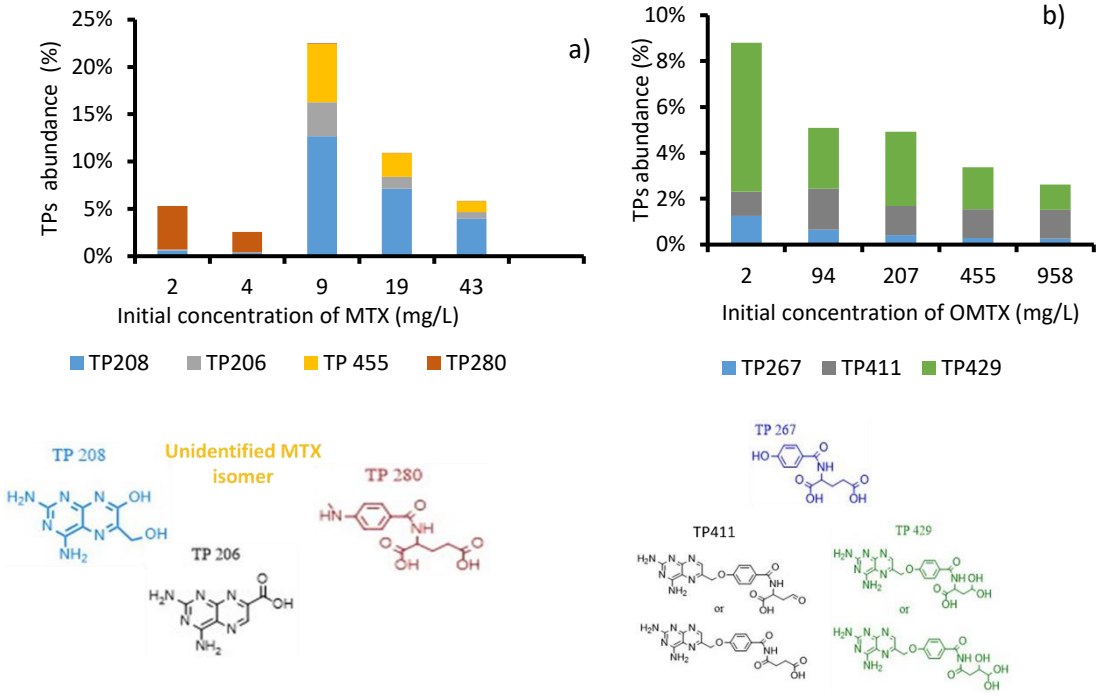


Figure 5- 2 Abundance of major TPs (expressed in % of initial amount of parent compounds) after a 72 h-light exposition as a function of initial concentration of parent compounds, MTX (a) and OMTX (b)

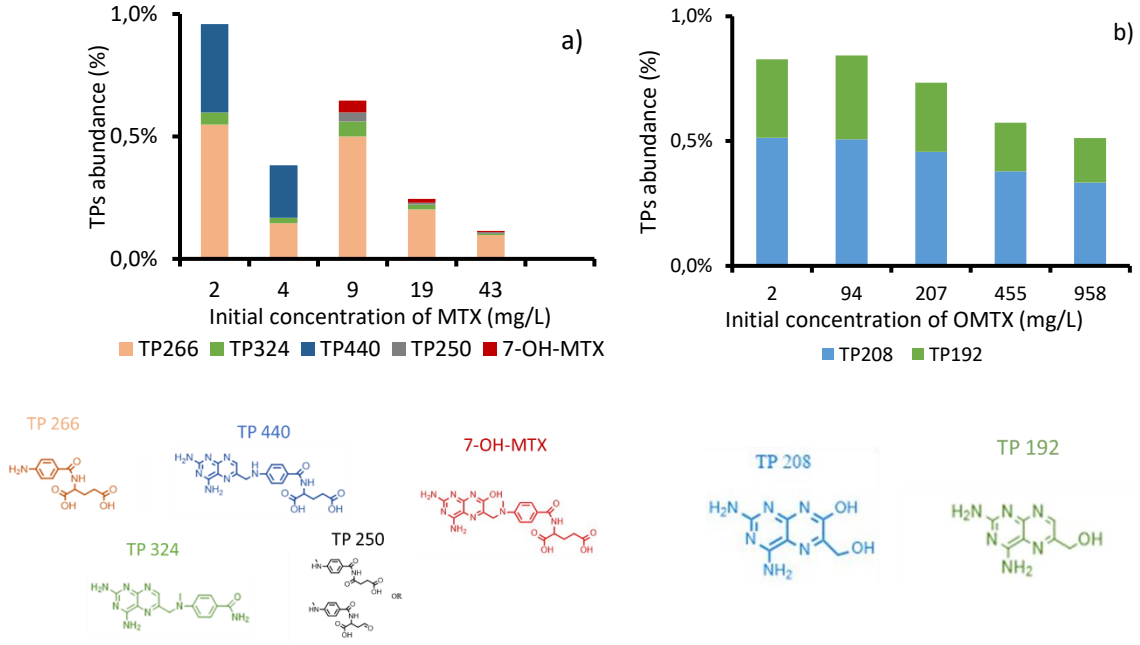


Figure 5- 3 Abundance of minor TPs (expressed in % of initial amount of parent compounds) after a 72 h-light exposition as a function of initial concentration of parent compounds, MTX (a) and OMTX (b)

5.5. Conclusion

The ecotoxicological assessment towards two aquatic organisms of the anticancer drug MTX in comparison with its ecodesigned analog OMTX was performed in combination with chemical analysis. The marine bacteria *A. fischeri* was not sensitive to both compounds in contrast with the algae *R. subcapitata*. While MTX was very toxic to the algae *R. subcapitata* in accordance with previous studies, we did not observe a toxic impact of OMTX (EC_{50} 14 mg/L against 664 mg/L). The chemical analysis revealed the weak stability of MTX under algae test and the identification of TPs resulting of bio and photodegradagtion gave some clues on chemical structure involvement in the observed toxicity. The mixture of pteridine derivatives and the unidentified isomer of MTX as well as the MTX itself before its degradation could be involved. Furthermore, this hypothesis is supported when regarding OMTX' TPs, the abundance of pteridines derivatives were negligeable. However, to elucidate the role of each MTX TPs, experiments under semi static condition and a regular monitoring of parents and TPs compounds, as well as complementary TPs identification would be required.

This first simple and rapid aquatic toxicity assessment revealed that OMTX presents lower eco toxicological impact. This result confirms that eco-conception is a promising approach. This eco-designed analog would deserve complementary investigations for acute and chronic toxicity assessment on a larger variety of organisms likely to be sensitive to allow the comparison of these two molecules as *C. dubia* or *L. minor*.

5.6. References

Aguirre-Martínez, G.V., Okello, C., Salamanca, M.J., Garrido, C., Del Valls, T.A., Martín-Díaz, M.L., 2016. Is the step-wise tiered approach for ERA of pharmaceuticals useful for the assessment of cancer therapeutic drugs present in marine environment? Environmental Research 144, 43–59. https://doi.org/10.1016/j.envres.2015.10.028

Barışçı, S., Turkay, O., Ulusoy, E., Şeker, M.G., Yüksel, E., Dimoglo, A., 2018. Electro-oxidation of cytostatic drugs: Experimental and theoretical identification of by-products and evaluation of ecotoxicological effects. Chemical Engineering Journal 334, 1820–1827. https://doi.org/10.1016/j.cej.2017.11.105

Benet, L.Z., Broccatelli, F., Oprea, T.I., 2011. BDDCS Applied to Over 900 Drugs. The AAPS Journal 13, 519–547. https://doi.org/10.1208/s12248-011-9290-9

Besse, J.-P., Latour, J.-F., Garric, J., 2012. Anticancer drugs in surface waters. Environment International 39, 73–86. https://doi.org/10.1016/j.envint.2011.10.002

Białk-Bielińska, A., Mulkiewicz, E., Stokowski, M., Stolte, S., Stepnowski, P., 2017. Acute aquatic toxicity assessment of six anti-cancer drugs and one metabolite using biotest battery – Biological effects and stability under test conditions. Chemosphere 189, 689–698. https://doi.org/10.1016/j.chemosphere.2017.08.174

Booker, V., Halsall, C., Llewellyn, N., Johnson, A., Williams, R., 2014. Prioritising anticancer drugs for environmental monitoring and risk assessment purposes. Science of The Total Environment 473–474, 159–170. https://doi.org/10.1016/j.scitotenv.2013.11.145

Borgatta, M., Decosterd, L.-A., Waridel, P., Buclin, T., Chèvre, N., 2015. The anticancer drug metabolites endoxifen and 4-hydroxy-tamoxifen induce toxic effects on Daphnia pulex in a two-generation study. Science of The Total Environment 520, 232–240. https://doi.org/10.1016/j.scitotenv.2015.03.040

Buerge, I.J., Buser, H.-R., Poiger, T., Müller, M.D., 2006. Occurrence and Fate of the Cytostatic Drugs Cyclophosphamide and Ifosfamide in Wastewater and Surface Waters †. Environmental Science & Technology, 40, 7242–7250. https://doi.org/10.1021/es0609405

Calza, P., Medana, C., Sarro, M., Rosato, V., Aigotti, R., Baiocchi, C., Minero, C., 2014. Photocatalytic degradation of selected anticancer drugs and identification of their transformation products in water by liquid chromatography–high resolution mass spectrometry. Journal of Chromatography A 1362, 135–144. https://doi.org/10.1016/j.chroma.2014.08.035

Catastini, C., Mullot, J.-U., Boukari, S., Mazellier, P., Levi, Y., Cervantes, P., Ormsby, J.-N., 2008. Identification de molécules anticancéreuses dans les effluents hospitaliers. European journal of water quality 39, 171–180. https://doi.org/10.1051/water/2008004

Česen, M., Eleršek, T., Novak, M., Žegura, B., Kosjek, T., Filipič, M., Heath, E., 2016. Ecotoxicity and genotoxicity of cyclophosphamide, ifosfamide, their metabolites/transformation products and their mixtures. Environmental Pollution 210, 192–201. https://doi.org/10.1016/j.envpol.2015.12.017

Dhondt, J.-L., Hayte, J.-M., Millot, F., Klein, R., Blais, J.-C., Pfleiderer, W., 1991. 2,4-Diamino-7-hydroxy-pteridines, a new class of catabolites of methotrexate. European Journal of Biochemistry, 200, 237–244. https://doi.org/10.1111/j.1432-1033.1991.tb21072.x

Espinosa, A., Nélieu, S., Lieben, P., Skarbek, C., Labruère, R., Benoit, P., 2021. Photodegradation of methotrexate in aqueous solution: degradation kinetics and identification of transformation products. Environmental Science and Pollution Research, https://doi.org/10.1007/s11356-021-15820-3

Espinosa, A., Rascol, E., Abellàn Flos, M., Skarbek, C., Lieben, P., Bannerman, E., Diez Marinez, A., Pethe, S., Benoit, P., Nélieu, S., Labruère, R., Submitted in Angewandte Chemie International Edition

Fabre, G., Goldman, I.D., 1985. Formation of 7-hydroxymethotrexate polyglutamyl derivatives and their cytotoxicity in human chronic myelogenous leukemia cells, in vitro. Cancer Research, 45, 80–85.

Ferrando-Climent, L., Rodriguez-Mozaz, S., Barceló, D., 2014. Incidence of anticancer drugs in an aquatic urban system: From hospital effluents through urban wastewater to natural environment. Environmental Pollution 193, 216–223. https://doi.org/10.1016/j.envpol.2014.07.002

Ferrando-Climent, L., Rodriguez-Mozaz, S., Barceló, D., 2013. Development of a UPLC-MS/MS method for the determination of ten anticancer drugs in hospital and urban wastewaters, and its application for the screening of human metabolites assisted by information-dependent acquisition tool (IDA) in sewage samples. Analytical and Bioanalytical Chemistry 405, 5937–5952. https://doi.org/10.1007/s00216-013-6794-4

Galaon, T., Petre, J., Iancu, V.I., Cruceru, L., Niculescu, M., Chiriac, F.L., Puiu, D.M., Mihalache, M., 2017. Detection of cytostatic drugs in municipal waste water and their transfer to surface water, in: SIMI 2017. Presented at the International Symposium "The Environment and the Industry," National Research and Development Institute for Industrial Ecology, pp. 229–236. https://doi.org/10.21698/simi.2017.0029

Garcia-Ac, A., Segura, P.A., Gagnon, C., Sauvé, S., 2009. Determination of bezafibrate, methotrexate, cyclophosphamide, orlistat and enalapril in waste and surface waters using on-line solid-phase extraction liquid chromatography coupled to polarity-

switching electrospray tandem mass spectrometry. Journal of Environmental Monitoring 11, 830. https://doi.org/10.1039/b817570e

Henschel, K.-P., Wenzel, A., Diedrich, M., Fliedner, A., 1997. Environmental Hazard Assessment of Pharmaceuticals. Regulatory Toxicology and Pharmacology 25, 220–225. https://doi.org/10.1006/rtph.1997.1102

Hsu, M.-H., Tsai, C.-J., Lin, A.Y.-C., 2019. Mechanism and pathways underlying the self-sensitized photodegradation of methotrexate under simulated solar irradiation. Journal of Hazardous Materials 373, 468–475. https://doi.org/10.1016/j.jhazmat.2019.03.095

Isidori, M., Lavorgna, M., Russo, C., Kundi, M., Žegura, B., Novak, M., Filipič, M., Mišík, M., Knasmueller, S., de Alda, M.L., Barceló, D., Žonja, B., Česen, M., Ščančar, J., Kosjek, T., Heath, E., 2016. Chemical and toxicological characterisation of anticancer drugs in hospital and municipal wastewaters from Slovenia and Spain. Environmental Pollution 219, 275–287. https://doi.org/10.1016/j.envpol.2016.10.039

Johnson, A.C., Jürgens, M.D., Williams, R.J., Kümmerer, K., Kortenkamp, A., Sumpter, J.P., 2008. Do cytotoxic chemotherapy drugs discharged into rivers pose a risk to the environment and human health? An overview and UK case study. Journal of Hydrology 348, 167–175. https://doi.org/10.1016/j.jhydrol.2007.09.054

Kanjal, M.I., Muneer, M., Abdelhaleem, A., Chu, W., 2020. Degradation of methotrexate by UV/peroxymonosulfate: Kinetics, effect of operational parameters and mechanism. Chinese Journal of Chemical Engineering 28, 2658–2667. https://doi.org/10.1016/j.cjche.2020.05.033

Kiffmeyer, T., Götze, H.-J., Jursch, M., Lüders, U., 1998. Trace enrichment, chromatographic separation and biodegradation of cytostatic compounds in surface water. Fresenius' Journal of Analytical Chemistry 361, 185–191. https://doi.org/10.1007/s002160050859

Kleinert, C., Poirier-Larabie, S., Gagnon, C., André, C., Gagné, F., 2021. Occurrence and ecotoxicity of cytostatic drugs 5-fluorouracil and methotrexate in the freshwater unionid Elliptio complanata. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 244, 109027. https://doi.org/10.1016/j.cbpc.2021.109027

Kosjek, T., Negreira, N., de Alda, M.L., Barceló, D., 2015. Aerobic activated sludge transformation of methotrexate: Identification of biotransformation products. Chemosphere 119, S42–S50. https://doi.org/10.1016/j.chemosphere.2014.04.081

Kümmerer, K., 2007. Sustainable from the very beginning: rational design of molecules by life cycle engineering as an important approach for green pharmacy and green chemistry. Green Chemistry 9, 899. https://doi.org/10.1039/b618298b

Kümmerer, K., Haiß, A., Schuster, A., Hein, A., Ebert, I., 2016. Antineoplastic compounds in the environment—substances of special concern. Environmental Science and Pollution Research, 23, 14791–14804. https://doi.org/10.1007/s11356-014-3902-8

Lai, W.W.-P., Hsu, M.-H., Lin, A.Y.-C., 2017. The role of bicarbonate anions in methotrexate degradation via UV/TiO 2 : Mechanisms, reactivity and increased toxicity. Water Research 112, 157–166. https://doi.org/10.1016/j.watres.2017.01.040

Li, D., Chen, H., Liu, H., Schlenk, D., Mu, J., Lacorte, S., Ying, G.-G., Xie, L., 2021. Anticancer drugs in the aquatic ecosystem: Environmental occurrence, ecotoxicological effect and risk assessment. Environment International 153, 106543. https://doi.org/10.1016/j.envint.2021.106543

Lutterbeck, C.A., Baginska, E., Machado, Ê.L., Kümmerer, K., 2015a. Removal of the anti-cancer drug methotrexate from water by advanced oxidation processes: Aerobic biodegradation and toxicity studies after treatment. Chemosphere 141, 290–296. https://doi.org/10.1016/j.chemosphere.2015.07.069

Lutterbeck, C.A., Kern, D.I., Machado, Ê.L., Kümmerer, K., 2015b. Evaluation of the toxic effects of four anti-cancer drugs in plant bioassays and its potency for screening in the context of waste water reuse for irrigation. Chemosphere 135, 403–410. https://doi.org/10.1016/j.chemosphere.2015.05.019

Lutterbeck, C.A, Kern, D.I, Machado, Ê.L., Kümmerer, K., 2014. Ecotoxicological evaluation of selected anticancer drugs. Toxicology Letters 229, S71–S72. https://doi.org/10.1016/j.toxlet.2014.06.280

Markovic, M., Neale, P.A., Nidumolu, B., Kumar, A., 2021. Combined toxicity of therapeutic pharmaceuticals to duckweed, Lemna minor. Ecotoxicology and Environmental Safety 208, 111428. https://doi.org/10.1016/j.ecoenv.2020.111428

Martín, J., Camacho-Muñoz, D., Santos, J.L., Aparicio, I., Alonso, E., 2014. Occurrence and ecotoxicological risk assessment of 14 cytostatic drugs in wastewater. Water, Air, and Soil Pollution 225. https://doi.org/10.1007/s11270-014-1896-y

Muneer, M., Kanjal, M.I., Iqbal, M., Saeed, M., Khosa, M.K., Ud Den, N.Z., Ali, S., Nazir, A., 2020. Gamma and UV radiations induced treatment of anti-cancer methotrexate drug in aqueous medium: Effect of process variables on radiation efficiency evaluated using bioassays. Applied Radiation and Isotopes 166, 109371. https://doi.org/10.1016/j.apradiso.2020.109371

Nair, M.G., Bridges, T.W., Henkel, T.J., Kisliuk, R.L., Gaumont, Y., Sirotnak, F.M., 1981. Folate analogs altered in the C9-N10 bridge region. 18. Synthesis and antitumor evaluation of 11-oxahomoaminopterin and related compounds. Journal of Medicinal Chemistry, 24, 1068–1073. https://doi.org/10.1021/jm00141a010

Nduati, E., Hunt, S., Kamau, E.M., Nzila, A., 2005. 2,4-Diaminopteridine-Based Compounds as Precursors for De Novo Synthesis of Antifolates: a Novel Class of Antimalarials. Antimicrobial Agents and Chemotherapy, 49, 3652–3657. https://doi.org/10.1128/AAC.49.9.3652-3657.2005

Negreira, N., de Alda, M.L., Barceló, D., 2014. Cytostatic drugs and metabolites in municipal and hospital wastewaters in Spain: Filtration, occurrence, and environmental risk. Science of The Total Environment 497–498, 68–77. https://doi.org/10.1016/j.scitotenv.2014.07.101

Negreira, N., Mastroianni, N., López de Alda, M., Barceló, D., 2013. Multianalyte determination of 24 cytostatics and metabolites by liquid chromatography–electrospray–tandem mass spectrometry and study of their stability and optimum storage conditions in aqueous solution. Talanta 116, 290–299. https://doi.org/10.1016/j.talanta.2013.04.070

Olalla, A., Negreira, N., López de Alda, M., Barceló, D., Valcárcel, Y., 2018. A case study to identify priority cytostatic contaminants in hospital effluents. Chemosphere 190, 417–430. https://doi.org/10.1016/j.chemosphere.2017.09.129

Parrella, A., Lavorgna, M., Criscuolo, E., Russo, C., Fiumano, V., Isidori, M., 2014. Acute and chronic toxicity of six anticancer drugs on rotifers and crustaceans. Chemosphere 115, 59–66. https://doi.org/10.1016/j.chemosphere.2014.01.013

Rabii, F.W., Segura, P.A., Fayad, P.B., Sauvé, S., 2014. Determination of six chemotherapeutic agents in municipal wastewater using online solid-phase extraction coupled to liquid chromatography-tandem mass spectrometry. Science of The Total Environment 487, 792–800. https://doi.org/10.1016/j.scitotenv.2013.12.050

Sabry, S.M., Abdel-Hady, M., Elsayed, M., Fahmy, O.T., Maher, H.M., 2003. Study of stability of methotrexate in acidic solution Spectrofluorimetric determination of methotrexate in pharmaceutical preparations through acid-catalyzed degradation reaction. Journal of Pharmaceutical and Biomedical Analysis 32, 409–423. https://doi.org/10.1016/S0731-7085(03)00239-5

Sanderson, H., 2003. Probabilistic hazard assessment of environmentally occurring pharmaceuticals toxicity to fish, daphnids and algae by ECOSAR screening. Toxicology Letters 144, 383–395. https://doi.org/10.1016/S0378-4274(03)00257-1

Turci, R., Sottani, C., Spagnoli, G., Minoia, C., 2003. Biological and environmental monitoring of hospital personnel exposed to antineoplastic agents: a review of analytical methods. Journal of Chromatography B 789, 169–209. https://doi.org/10.1016/S1570-0232(03)00100-4

Widemann, B.C., Sung, E., Anderson, L., Salzer, W.L., Balis, F.M., Monitjo, K.S., McCully, C., Hawkins, M., Adamson, P.C., 2000. Pharmacokinetics and metabolism of the methotrexate metabolite 2, 4-diamino-N(10)-methylpteroic acid. The Journal of Pharmacology and Experimental Therapeutics, 294, 894–901.

Yin, J., Shao, B., Zhang, J., Li, K., 2010. A Preliminary Study on the Occurrence of Cytostatic Drugs in Hospital Effluents in Beijing, China. Bulletin of Environmental Contamination and Toxicology 84, 39–45. https://doi.org/10.1007/s00128-009-9884-4

Zounková, R., Odráška, P., Dolealová, L., Hilscherová, K., Maršálek, B., Bláha, L., 2007. Ecotoxicity and genotoxicity assessment of cytostatic pharmaceuticals. Environmental Toxicology and Chemistry 26, 2208. https://doi.org/10.1897/07-137R.1

Chapitre 6. Discussion générale

Cette dernière partie revient sur les principaux résultats de ce travail en les discutant par rapport aux réponses attendues vis-à-vis de notre question principale sur la validité de la démarche d'éco-conception. A savoir : est-ce que l'insertion d'un atome d'oxygène remplaçant le groupe N-méthyl dans la structure du méthotrexate (MTX) rend la molécule plus facilement fragmentable donc moins persistante et donne lieu à des produits de transformations (TPs) différents et éventuellement moins toxiques ?

6.1. Evolution des molécules mères et des produits de transformation au cours des processus de dégradation

6.1.1. Stabilité comparée des deux composés

Les comparaisons de persistance des molécules mères MTX et OMTX ont été menées dans deux grands types de conditions : celles favorisant leur photodégradation (**Chapitre 3**) ou leur biodégradation (**Chapitre 4**). Il a été vérifié que d'autres processus de dégradation n'étaient pas impliqués lors de ces études spécifiques, par des témoins gardés à l'obscurité ou non ensemencés. Une attention particulière a également été prêtée à l'élimination par adsorption. Globalement, lors de l'étude de biodégradation en présence de boue activée, l'analogue OMTX s'est dégradé légèrement plus rapidement que le MTX en condition biotique (**Chapitre 4.2**), mais même si cette différence est statistiquement significative elle ne permet pas de conclure à un réel intérêt du OMTX sur le MTX. De même, sous une irradiation proche des conditions environnementales (300-450 nm) il n'y a pas eu de différence de cinétique observable. A l'inverse, le OMTX s'est révélé plus sensible que le MTX sous 254 nm, condition représentative des traitements de potabilisation, en se dégradant 30 fois plus vite que le MTX.

La photodégradation en milieu naturel et la biodégradation par des boues de station d'épuration dépend aussi de nombreux facteurs environnementaux non pris en compte dans nos conditions expérimentales. On a vu la difficulté de tirer des conclusions génériques à partir d'études expérimentales différentes où des facteurs comme la concentration du composé, mais aussi la composition de la matrice, la présence d'autres contaminants, la présence de matière organique dans les eaux ou encore l'apport de substrat, l'âge des boues ne sont pas comparables (Onesios et al., 2009 ; Kosjek et al., 2015). En particulier, la concentration initiale semble contrôler un mécanisme d'auto-sensibilisation lors de la photodégradation, tel que décrit par Hsu et al. (2019) mais cela a été observé uniquement pour le MTX

lors des tests sur algues. Nos résultats sont donc à relativiser mais il semble que la persistance du OMTX soit inférieure à celle du MTX dans les processus de photodégradation sous irradiation UV. La modification de structure aurait un réel intérêt pour éliminer les résidus de ce composé en traitement d'eau potable. Et en corollaire, elle n'aggrave pas la persistance du composé dans les autres conditions étudiées.

6.1.2. Voies de transformation : similitude et différences entre les produits de transformation

Les voies de dégradation des deux molécules se sont avérées similaires. En conditions abiotiques, nous avons observé la formation majoritaire de dérivés ptéridines par clivage C-N ou C-O (pour MTX et OMTX, respectivement), donnant lieux à des TPs communs aux deux molécules tels que le TP206 et TP208 déjà connus de la littérature dans le cas du MTX (Lai et al., 2017; Calza et al., 2014; Lutterbeck et al., 2015) et au TP193 seulement vu pour OMTX, ainsi que des équivalents pour l'autre partie de la molécule (*N*-methylaminobenzoic et benzoyl-glutamyl) avec TP280, TP266 et TP267, pour MTX et OMTX respectivement. D'autres TPs ont pu être identifiés, résultant de la modification de la partie acide glutamique donnant les TP408a et b, TP4242a et b ou encore le TP250 résultant d'un clivage C-N en plus de cette modification chez MTX et TP429 a et b, TP413, TP411 a et b pour OMTX ou bien de la perte de cette partie acide glutamique pour les molécules avec TP324 pour MTX et TP311 pour OMTX.

Toujours en condition abiotique, un isomère du OMTX a pu être observé mais reste non identifié, tandis qu'un isomère du MTX semble s'être formé lors des tests d'écotoxicité sur algues, résultant alors d'une photodégradation ou bien du métabolisme de l'algue et des bactéries associées aux cultures.

Enfin, nous avons observé la N-déméthylation spécifique à la molécule MTX donnant lieu au TP440 connu sous le nom d'aminopterine (Nduati et al., 2005).

Il faut souligner que les TPs majoritaires de la photodégradation (dérivés ptéridines) se sont avérés être minoritaires dans le cas de la biodégradation. Notons également que la structure proposée avec un niveau de confiance 2b selon Schymanski et al. (2014) pour le TP206, en accord avec les études de photodégradation de Calza et al. (2014) et Lai et al. (2017) a été remis en question suite à l'utilisation de l'étalon analytique 2,4-Diamino-6-pteridinecarboxylic Acid, AMPC (**Chapitre 4.1**). En effet, la masse, la fragmentation et le temps de rétention de cet étalon ont conduit aux mêmes informations que ceux du TP206. L'AMPC est quant à lui proposé par Kosjek et al. (2015) et Kanjal et al. (2020). Cependant, les auteurs cités ne donnent pas d'indication quant à son niveau de confiance lors de son identification et

ne proposent pas de schéma de fragmentation. Il n'est donc pas exclu que la masse 206 ne corresponde qu'à une seule et même structure qui serait celle de l'AMPC.

L'hydroxylation formant le 7-OH-MTX classé comme potentiellement nocif pour l'environnement par Besse et al. (2012) suite à l'étude de Kiffmeyer et al. (1998) qui le qualifiait de composé majeur et persistant lors de la biodégradation du MTX par des boues de station d'épuration, a pu être suivi et quantifié grâce au développement de méthodes décrit au **Chapitre 4.1**. Ce produit est minoritaire et transitoire dans l'expérimentation de biodégradation en boues activées ce qui est en accord avec l'étude de Kosjek et al. (2015). Dans l'étude du OMTX, son équivalent, le 7-OH-OMTX suit une dynamique similaire. Une fois de plus, il reste à souligner que les conditions expérimentales peuvent mener à des résultats contrastés d'une étude à l'autre.

La voie de biodégradation majoritaire du MTX comme de son analogue s'est avérée être l'hydrolyse de la partie glutamate. Le DAMPA est produit à partir du MTX en accord avec le travail de Kosjek et al. (2015) et son équivalent ODAMPA est formé à partir du OMTX comme nous l'avons proposé au **Chapitre 4.1.** Ces deux produits de transformation ne sont pas observés en photodégradation. Ils présentent la même dynamique atteignant une conversion de la molécule mère de plus 90% en seulement quelques heures et sont toujours présents au bout de 48 h d'incubation. Si le MTX et son analogue sont rapidement biodégradés, ce n'est donc pas le cas de leur produit majoritaire qui semblent être plus persistant et peu biodégradable. Il serait intéressant d'étudier leur biodégradabilité grâce au système Oxitop selon les recommandations de l'OCDE pour le « Closed Bottle Test 301D » (OECD, 1992).

De même en photodégradation, certains TPs majoritaires formés comme TP206 pour MTX ou TP193 pour OMTX pourraient s'avérer persistants puisqu'ils semblent s'accumuler dans le milieu. Si l'autre dérivé ptéridine TP208 semble se dégrader, il faudrait poursuivre les irradiations plus longtemps pour le vérifier. Calza et al. (2014) identifient des sous-produits du TP208 (M178 et M162) en photolyse directe et Lutterbeck et al. (2015) proposent aussi le M178 comme sous-produits du TP206 (en UV/H_2O_2) indiquant que ces composés peuvent être à leur tour dégradés lors de traitements poussés.

Finalement, si la dégradation du OMTX est bien plus rapide sous 254 nm, elle n'est pas modifiée sous 300-450 nm. En 3h, la majorité est convertie en dérivé ptéridine pour le MTX et pour le OMTX, il reste encore une proportion de TPs presque aussi importante ayant subi une modification de la partie acide glutamique et non un clivage (TP441, TP411a et b et TP429a et b). Contrairement à ce qui était

attendu, l'auto immolation de la molécule n'a pas spécialement été favorisée par le remplacement du groupe *N*-méthyl par un oxygène dans la structure du MTX ; tout au moins dans les deux conditions proches de conditions environnementales que nous avons considérées: irradiation à 300-450nm et dégradation par les boues de station d'épuration.

6.2. Effets des molécules mères et des produits de transformation

6.2.1. Effets comparés des deux composés

Les deux molécules mères ont un effet cytotoxique proche, en accord avec leur mode d'action pharmaceutique. Concernant leurs effets écotoxicologiques, on attend un effet similaire ou moindre pour le OMTX.

L'étude de biodégradation, menée à une concentration au moins 250 fois supérieure aux concentrations environnementales et dans des conditions de laboratoire éloignées de la réalité, n'avait pas pour objectif de conclure sur un effet des molécules sur les boues de station d'épuration en conditions réelles. Elle visait plutôt à observer une potentielle différence d'impact, que ce soit entre les molécules mères, ou entre molécules mères et les mélanges de TPs formés par biodégradation. Les indicateurs suivis lors de cette expérimentation n'ont pas permis d'observer un impact majeur et donc de mettre en évidence des différences d'impact entre molécules. Un temps de contact plus long, avec un apport en contaminant en continu auraient peut-être pu permettre d'observer des effets (Yao et al., 2012; Alvarino et al., 2014; Amariei et al., 2017). Un tel système aurait cependant été compliqué à mettre en place en raison des contraintes expérimentales. De plus, l'accès aux boues de station d'épuration était très limité.

Concernant l'évaluation des effets écotoxicologiques sur des organismes aquatiques, la bactérie *A. fischeri* s'est avérée peu sensible aux deux molécules. La littérature concernant l'effet du MTX sur cet organisme était déjà très hétérogène avec des informations contradictoires. Au contraire, la sensibilité de l'algue *R. subcapitata* a permis de révéler une différence indéniable entre les effets du MTX et du OMTX. Le support analytique, nécessaire au vu de la photosensibilité des molécules, a permis de conforter cette observation avec un effet 47 fois plus faible du OMTX sur la croissance de l'algue. Des expérimentations sur d'autres organismes sensibles, comme par exemple sur *Lemna minor* (Białk-Bielińska et al., 2017) complèteraient ces premières évaluations.

6.2.2. Effet de la transformation des deux composés sur les effets écotoxicologiques

Les suivis de cytotoxicité lors de la photo- comme de la biodégradation ont permis de mettre en évidence la perte du mode d'action initial de la part des TPs, que les TPs majoritaires soient des dérivés ptéridines issus du clivage de la molécule dans le cas de la photodégradation, ou issus de l'hydrolyse du glutamate formant le DAMPA et ODAMPA, dans le cas de la biodégradation. Le DAMPA était déjà connu pour perdre cette action cytotoxique (Widemann et al., 2000). Cependant Nduati et al. (2005) ont rapporté qu'il pouvait être actif sur des parasites, en étant à nouveau converti en MTX. On peut par ailleurs supposer que certains TPs minoritaires pourraient exercer un effet similaire au MTX : c'est en particulier le cas pour le TP440, dont la similarité de mode d'action par rapport au MTX a été démontrée (Bertino, 1993). Cependant, il est probable que la faible concentration de ces TPs minoritaires n'impacte pas la viabilité des cellules étudiées. De façon générale, une dégradation plus rapide permet d'accélérer la perte de l'effet cytotoxique, comme il a été vu de manière très marquée sous irradiation à 254 nm : la comparaison des deux composés donne un avantage non négligeable au OMTX dans cette condition particulière. De plus, comme nous l'avons vu dans le **Chapitre 3.2** (Figure 3.2- 4b), la cytotoxicité est moindre pour le OMTX et ses photoproduits quand on compare les deux composés à un degré d'avancement de la dégradation similaire. Donc même si l'auto-immolation initialement prévue pour conduire à la perte de l'activité cytotoxique a été peu observée, on peut retenir que les TPs issus de la modification de la partie acide glutamique, bien que présents en plus grande proportion chez OMTX que chez MTX, n'ont pas entraîné d'effet cytotoxique et les TPs du OMTX sont même moins cytotoxiques que ceux du MTX.

Concernant les effets écotoxicologiques sur organismes aquatiques, un test, simple, rapide et miniaturisé comme le test d'inhibition de la luminescence de la bactérie *A. fischeri*, n'est pas assez sensible pour permettre de mettre en évidence l'évolution des effets au cours de la dégradation. Ce résultat se démarque d'autres études qui ont suivi la toxicité des produits formés en exposant la bactérie au mélange issus de photo ou biodégradation (Calza et al. 2014; Lutterbeck et al., 2015; Lai et al., 2017).

Enfin, lors de l'exposition des molécules MTX et OMTX à la lumière nécessaire à la réalisation du test sur l'algue *R. subcapitata,* un phénomène d'auto-sensibilisation a pu être mis en évidence dans le cas du MTX. Celui-ci, en accord avec la littérature (Hsu et al., 2019), est lié à la formation des dérivés ptéridines dont la présence en tant que TPs majoritaires a été démontrée à l'issue du test. Ce phénomène n'a pas été observé pour le OMTX. La dégradation totale du MTX dans certains des échantillons, associée à une toxicité élevée, soulève des questions sur l'impact potentiel des TPs sur les algues. L'effet observé

peut être dû au contact entre les algues et le MTX lui-même (même pendant une durée potentiellement courte), mais aussi au mélange de celui-ci et de ses TPs. Un des TP majeur semble être un isomère du MTX et pourrait être impliqué dans la toxicité observée. Il serait intéressant de l'identifier, en mettant en œuvre des techniques analytiques complémentaires (comme de la RMN, mais cela implique la production d'une quantité conséquente de ce composé et sa purification). De plus, afin de distinguer, la part d'effets causés par le MTX de ceux causés par les TPs, un suivi analytique régulier aurait été nécessaire. Enfin, des tests en conditions semi-statiques afin d'assurer un contact continu avec le MTX permettraient de connaître les effets du MTX lui-même comme le proposent Białk-Bielińska et al. (2017). Ces auteurs ont ainsi démontré que le MTX a plus d'effet que les TPs. Bien évidemment, des tests sur les composés individuels tels que les dérivés ptéridines, ou le DAMPA, apporteraient des éléments de compréhension supplémentaires, mais ce n'est pas aisément réalisable. La limitation majeure est l'accessibilité des TPs, en raison de leur prix élevé ou de la nécessité de les obtenir expérimentalement (par synthèse ou expériences à vocation préparative puis fractionnement). Par exemple, le peu de données sur la toxicité du 7-OH-MTX pourrait être en partie dû à cette contrainte. Dans leur travail, Białk-Bielińska et al. (2017) n'ont pas testé le composé au-dessus de 10 mg/L. Pour d'autres TPs, comme le DAMPA, disponible et peu coûteux, une telle contrainte n'existe probablement pas. Par contre, pour évaluer les effets du ODAMPA, il faudrait procéder à des synthèses spécifiques et produire suffisamment de composés. Les perspectives proposées n'ont pas pu être réalisées dans le cadre de la thèse en raison d'une disponibilité limitée des étalons et du temps imparti.

6.3. Conclusion

Ce travail a permis de mettre en avant qu'une modification de structure d'un composé pharmaceutique, peut, tout en conservant une action thérapeutique similaire (ce qui semble le cas de la molécule choisie OMTX d'après son activité *in vivo* et sa métabolisation étudiée *in vitro,* mais sous réserve de test plus poussés) modifier son comportement et son impact d'un point de vue environnemental.

Il serait nécessaire de compléter l'expérimentation et pallier certaines des limitations que nous venons d'évoquer par des approches *in silico* qui reposent sur des outils combinant chimie computationnelle, modélisation statistique (ex QSARs, QSBRs), méthode de classification et bases de données. De telles approches font partie intégrante du développement pharmaceutique. Elles peuvent par exemple permettre de donner des indications quant au devenir des molécules ou encore déterminer la toxicité des composés identifiés et donc de mieux évaluer et comparer les impacts liés aux molécules mères et aux TPs. Par exemple, l'outil EnviPath, prédit les voies de réaction d'une molécule en se basant sur les règles de biotransformation de la littérature (Latino et al., 2017). Rastogi et al. (2014) ont identifié les parties de la structure impliquées dans une biodégradation plus avancée grâce à l'utilisation d'un logiciel appelé MetaPC. L'outil TyPol (Typologie des Polluants) peut également être utilisé pour évaluer le devenir et les effets d'un composé organique à partir de ses propriétés moléculaires par similarité avec des composés ayant des propriétés comparables et pour lesquels des paramètres environnementaux et écotoxicologiques sont connus (Servien et al., 2014; Benoit et al., 2017, Traoré et al., 2018). Il permet de regrouper des composés qui ont des comportements et/ou des effets similaires. L'utilisation de cet outil de classification pourrait être envisagée afin d'observer des changements de classe entre la molécule mère et les TPs et de comparer les TPs du MTX avec ceux du OMTX.

Au démarrage du projet EDIFIS en 2016, l'éco-conception de molécules pharmaceutiques était encore peu étudiée. Récemment, Leder et al. (2021) ont proposé une démarche pour évaluer un dérivé éco-conçu d'un antibiotique, dans laquelle la place des tests de toxicité in silico est prépondérante (Figure 6- 1), l'approche expérimentale concernant l'évaluation des propriétés environnementales rejoint les propositions faites dans le cadre de cette thèse.

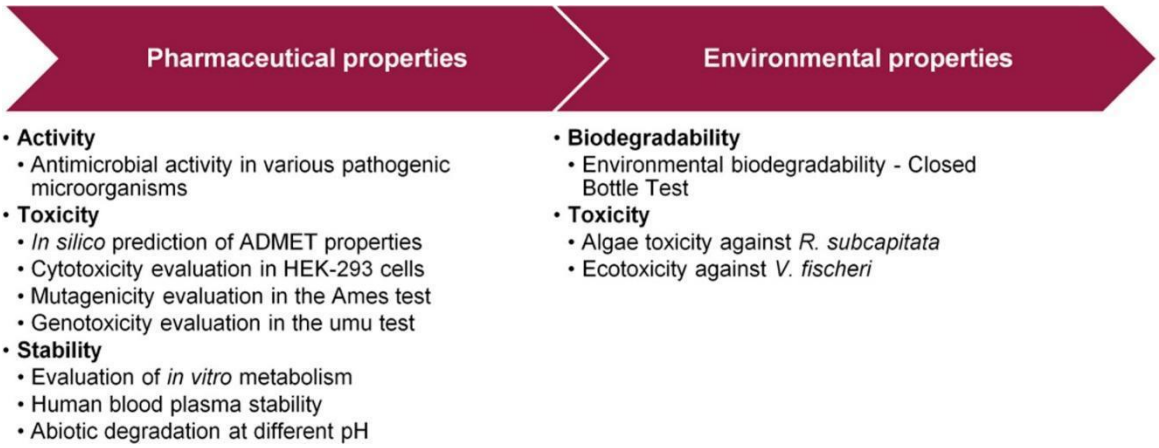


Figure 6- 1 Méthodologie de l'évaluation toxicologique et écotoxicologique d'un analogue éco-conçu selon Leder et al. (2021)

Comme nous l'avons vu dans le **Chapitre 1**, la réglementation concernant l'évaluation environnementale des pharmaceutiques est amenée à évoluer, pour prendre en compte le devenir et les effets des molécules et pourraient un jour prendre en compte ceux des TPs. La démarche d'éco-conception est donc bien une solution innovante qui permet d'anticiper ces problématiques, en proposant de nouvelles molécules qui présentent, a priori, un comportement mieux connu. Le travail de cette thèse, appliqué à l'exemple d'un anticancéreux, a permis d'aborder différentes méthodologies et de souligner leurs limites quant à l'évaluation du devenir et des effets des molécules.

6.4. Références

Alvarino, T., Katsou, E., Malamis, S., Suarez, S., Omil, F., Fatone, F., 2014. Inhibition of biomass activity in the via nitrite nitrogen removal processes by veterinary pharmaceuticals. Bioresource Technology 152, 477–483. https://doi.org/10.1016/j.biortech.2013.10.107

Amariei, G., Boltes, K., Rosal, R., León, P., 2017. Toxicological interactions of ibuprofen and triclosan on biological activity of activated sludge. Journal of Hazardous Materials 334, 193–200. https://doi.org/10.1016/j.jhazmat.2017.04.018

Benoit, P., Mamy, L., Servien, R., Li, Z., Latrille, E., Rossard, V., Bessac, F., Patureau, D., Martin-Laurent, F., 2017. Categorizing chlordecone potential degradation products to explore their environmental fate. Science of The Total Environment 574, 781–795. https://doi.org/10.1016/j.scitotenv.2016.09.094

Bertino, J.R., 1993. Karnofsky memorial lecture. Ode to methotrexate. Journal of Clinical Oncology 11, 5–14. https://doi.org/10.1200/JCO.1993.11.1.5

Besse, J.-P., Latour, J.-F., Garric, J., 2012. Anticancer drugs in surface waters. Environment International 39, 73–86. https://doi.org/10.1016/j.envint.2011.10.002

Białk-Bielińska, A., Mulkiewicz, E., Stokowski, M., Stolte, S., Stepnowski, P., 2017. Acute aquatic toxicity assessment of six anti-cancer drugs and one metabolite using biotest battery – Biological effects and stability under test conditions. Chemosphere 189, 689–698. https://doi.org/10.1016/j.chemosphere.2017.08.174

Calza, P., Medana, C., Sarro, M., Rosato, V., Aigotti, R., Baiocchi, C., Minero, C., 2014. Photocatalytic degradation of selected anticancer drugs and identification of their transformation products in water by liquid chromatography–high resolution mass spectrometry. Journal of Chromatography A 1362, 135–144. https://doi.org/10.1016/j.chroma.2014.08.035

Hsu, M.-H., Tsai, C.-J., Lin, A.Y.-C., 2019. Mechanism and pathways underlying the self-sensitized photodegradation of methotrexate under simulated solar irradiation. Journal of Hazardous Materials 373, 468–475. https://doi.org/10.1016/j.jhazmat.2019.03.095

Kanjal, M.I., Muneer, M., Abdelhaleem, A., Chu, W., 2020. Degradation of methotrexate by UV/peroxymonosulfate: Kinetics, effect of operational parameters and mechanism. Chinese Journal of Chemical Engineering 28, 2658–2667. https://doi.org/10.1016/j.cjche.2020.05.033

Kiffmeyer, T., Götze, H.-J., Jursch, M., Lüders, U., 1998. Trace enrichment, chromatographic separation and biodegradation of cytostatic compounds in surface water. Fresenius' Journal of Analytical Chemistry 361, 185–191. https://doi.org/10.1007/s002160050859

Kosjek, T., Negreira, N., de Alda, M.L., Barceló, D., 2015. Aerobic activated sludge transformation of methotrexate: Identification of biotransformation products. Chemosphere 119, S42–S50. https://doi.org/10.1016/j.chemosphere.2014.04.081

Lai, W.W.-P., Hsu, M.-H., Lin, A.Y.-C., 2017. The role of bicarbonate anions in methotrexate degradation via UV/TiO 2 : Mechanisms, reactivity and increased toxicity. Water Research 112, 157–166. https://doi.org/10.1016/j.watres.2017.01.040

Latino, D.A.R.S., Wicker, J., Gütlein, M., Schmid, E., Kramer, S., Fenner, K., 2017. Eawag-Soil in enviPath: a new resource for exploring regulatory pesticide soil biodegradation pathways and half-life data. Environmental Science: Processes & Impacts 19, 449–464. https://doi.org/10.1039/C6EM00697C

Leder, C., Suk, M., Lorenz, S., Rastogi, T., Peifer, C., Kietzmann, M., Jonas, D., Buck, M., Pahl, A., Kümmerer, K., 2021. Reducing Environmental Pollution by Antibiotics through Design for Environmental Degradation. ACS Sustainable Chemistry & Engineering 9, 9358–9368. https://doi.org/10.1021/acssuschemeng.1c02243

Lutterbeck, C.A., Baginska, E., Machado, Ê.L., Kümmerer, K., 2015. Removal of the anti-cancer drug methotrexate from water by advanced oxidation processes: Aerobic biodegradation and toxicity studies after treatment. Chemosphere 141, 290–296. https://doi.org/10.1016/j.chemosphere.2015.07.069

Nduati, E., Hunt, S., Kamau, E.M., Nzila, A., 2005. 2,4-Diaminopteridine-Based Compounds as Precursors for De Novo Synthesis of Antifolates: a Novel Class of Antimalarials. Antimicrobial Agents and Chemotherapy 49, 3652–3657. https://doi.org/10.1128/AAC.49.9.3652-3657.2005

Onesios, K.M., Yu, J.T., Bouwer, E.J., 2009. Biodegradation and removal of pharmaceuticals and personal care products in treatment systems: a review. Biodegradation 20, 441–466. https://doi.org/10.1007/s10532-008-9237-8

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

Schymanski, E.L., Jeon, J., Gulde, R., Fenner, K., Ruff, M., Singer, H.P., Hollender, J., 2014. Identifying Small Molecules via High Resolution Mass Spectrometry: Communicating Confidence. Environmental Science & Technology 48, 2097–2098. https://doi.org/10.1021/es5002105

Servien, R., Mamy, L., Li, Z., Rossard, V., Latrille, E., Bessac, F., Patureau, D., Benoit, P., 2014. TyPol – A new methodology for organic compounds clustering based on their molecular characteristics and environmental behavior. Chemosphere 111, 613–622. https://doi.org/10.1016/j.chemosphere.2014.05.020

Traoré H, Crouzet O., Mamy L., Sireyjol C., Rossard V., Servien R., Latrille E., Martin-Laurent F., Patureau D., Benoit P. 2018. Clustering pesticides according to their molecular properties and their impacts by considering additionnal ecotoxicological parameters in the TyPol method. Environmental Science and Pollution Research 25:4728–4738. https://doi.org/10.1007/s11356-017-0758-8

Widemann, B.C., Sung, E., Anderson, L., Salzer, W.L., Balis, F.M., Monitjo, K.S., McCully, C., Hawkins, M., Adamson, P.C., 2000. Pharmacokinetics and metabolism of the methotrexate metabolite 2, 4-diamino-N(10)-methylpteroic acid. The Journal of Pharmacology and Experimental Therapeutics 294, 894–901.

Yao, Y., Lu, Z., Min, H., Gao, H., Zhu, F., 2012. The effect of tetrahydrofuran on the enzymatic activity and microbial community in activated sludge from a sequencing batch reactor. Ecotoxicology 21, 56–65. https://doi.org/10.1007/s10646-011-0765-3

OECD, 1992. OECD Guidelines for the Testing of Chemicals. Ready Biodegradability 301D: Closed Bottle Test; OECD Pub.,

Annexe 1: Stabilité des composés MTX et OMTX à pH3 et pH7

Méthode :

Des solutions de 40µM de MTX et OMTX ont été préparées et ajustées à un pH de 3 ou 7 par ajout de $HClO_4^-$ et de NaOH (0.5M). Les essais, protégés de la lumière, sont plongés dans un bain thermostaté à 80°C. Durant 4h, des prélèvements sont effectués et les échantillons sont refroidis dans la glace avant d'être injectés en HPLC-UV selon la méthode détaillée dans le **Chapitre 3.1**. Pas de réplicas.

Résultats en Figure A-1 :

A un pH de 7, les deux molécules sont stables durant les 4h, ce qui correspondrait à approximativement 8 à 12 h à une température de 20°C (loi d'Arrhenius).

A un pH de 3, le MTX est stable, en accord avec la bibliographie[16]. Au contraire le OMTX révèle une grande instabilité dès les premières minutes, il est quasiment totalement dégradé en seulement deux heures (<10% résiduel).

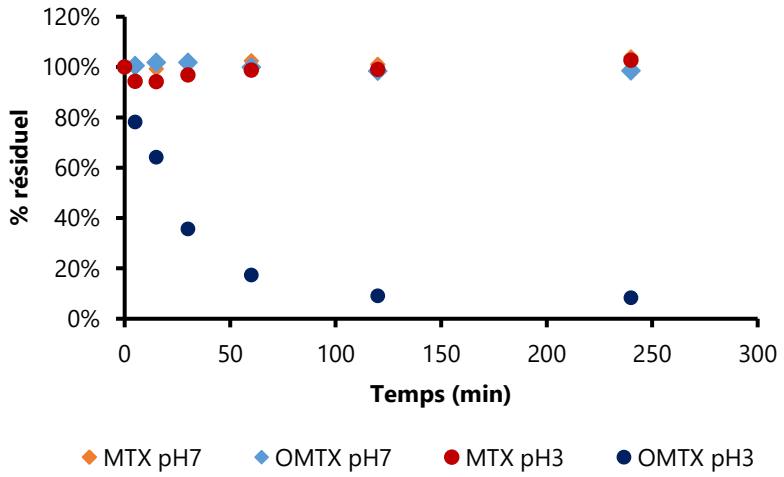


Figure A-1 Evolution de la concentration de MTX et OMTX à pH3 et pH7 et à 80°C (quantité de résidus en de MTX ou OMTX % des quantités initiales)

Conclusion :

Le pH acide nécessaire à la réaction de Fenton entraine la dégradation de la molécule OMTX dès la préparation de l'expérimentation. Comme nous l'avons vu dans le **Chapitre 2**, le MTX est efficacement dégradé par des procédés Fenton, le OMTX quant à lui y serait d'autant plus sensible. Cependant le travail de cette thèse se concentrera plutôt sur des traitements moins spécifiques. De plus, le procédé Fenton mène à une meilleure minéralisation des composés, tandis que les conditions de photodégradation que nous étudierons dans le **Chapitre 3** ont pour objectif d'observer et d'identifier une large variété de produits de transformation.

[16] Negreira, N., Mastroianni, N., López de Alda, M., Barceló, D., 2013. Multianalyte determination of 24 cytostatics and metabolites by liquid chromatography–electrospray–tandem mass spectrometry and study of their stability and optimum storage conditions in aqueous solution. Talanta 116, 290–299. https://doi.org/10.1016/j.talanta.2013.04.070

Annexe 2: Extrait du Supplémentary Information de l'article "Re-designing Environmentally Persistent Pharmaceutical Pollutant through programmed inactivation: the case of methotrexate" (Espinosa et al., submitted)

1. Synthesis

1.1 General

All chemical reagents were of analytical grade, obtained from Acros, Alfa Aesar, or Aldrich, and used without further purification. Solvents were obtained from SDS or VWR-Prolabo. Dichloromethane (DCM) was freshly distilled under CaH_2 and used immediately. Tetrahydrofurane (THF) was freshly distilled under sodium and used immediately. Flash chromatography was performed using silica gel (35–70 µm, Merck). Evaporation of solutions was performed under reduced pressure at temperature below 40 °C using rotary evaporator. Analytical TLC was performed using Silica Gel 60 F254 pre-coated aluminum plates (Merck). Spots were visualized by treatment with phosphomolybdic acid (PMA) revelator (for molecules containing boron atom) followed by heating and/or by absorbance of UV light at 254 nm, fluorescent light at 360 nm or diode on silica. NMR spectra were collected on Bruker DPX 250 (^{1}H at 250 MHz and ^{13}C at 62.5 MHz), AV 300 (^{1}H at 300 MHz and ^{13}C at 75 MHz and ^{11}B at 96 MHz) or AV 360 (^{1}H at 360 MHz and ^{13}C at 90 MHz) spectrometers and analyzed using MestReNova software. Chemical shifts are reported in ppm (δ) and coupling constants in Hz (J). NMR spectra were performed in $CDCl_3$ or $(CD_3)_2SO$. High-resolution mass spectrometry (HRMS) analyses were performed by electrospray with positive or negative (ESI^+ or ESI^-).

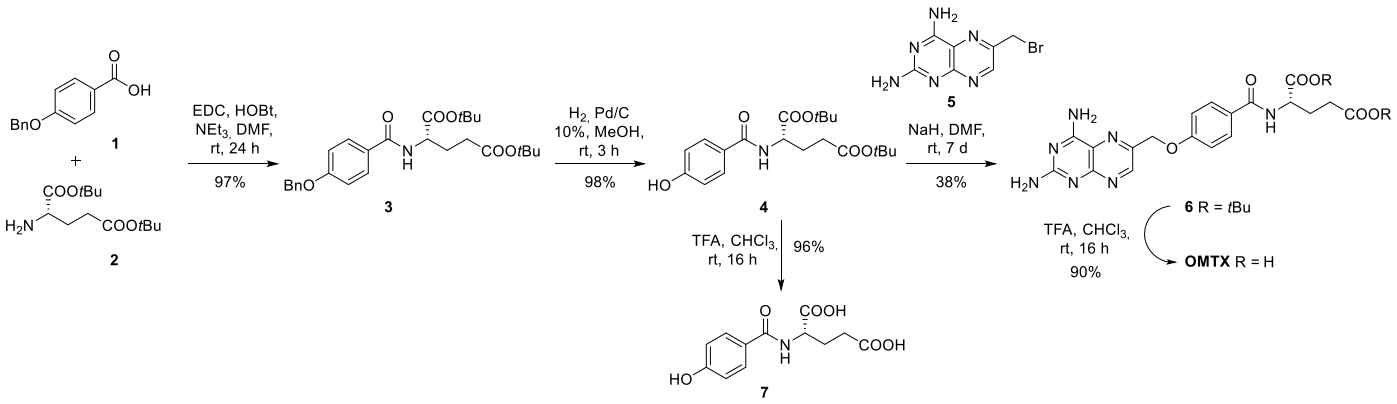
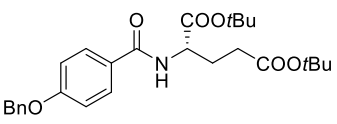


Figure S1. Synthetic pathway to ether analog **OMTX** and transformation product **7**.

Di-*tert*-butyl (4-(benzyloxy)benzoyl)-L-glutamate. 3.

p-benzyloxybenzoic acid **1** (1 g, 4.38 mmol, 1 eq.) was dissolved in anhydrous DMF (20 mL) and triethylamine (1.83 mL, 13.14 mmol, 3 eq.) was added dropwise. The mixture was stirred for five minutes at room temperature and then HOBt (710 mg, 5.26 mmol, 1.2 eq.), EDC (0.93 mL, 5.26 mmol, 1.2 eq.) and

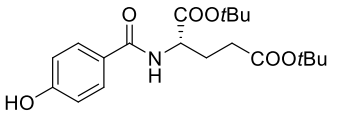
di-*tert*-butyl L-glutamate **2** (1.3 g, 4.38 mmol, 1 eq.) were added sequentially. The reaction mixture was stirred at room temperature until consumption of the reagents monitoring the reaction progress by TLC (cyclohexane/ethyl acetate, 7/3). Then, solvent was removed in vacuum, and the residue was partitioned between EtOAc and aqueous $NaHCO_3$ saturated solution. The aqueous layer was extracted with EtOAc (3 x 25 mL), and the combined organic layers were washed with brine, dried with $MgSO_4$, filtered and evaporated. The crude material was purified by column chromatography (cyclohexane/ethyl acetate, 8/2) to yield the corresponding peptide ester **3** (2 g, 97%) as a white solid.



^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 7.78 (d, *J* = 8.8 Hz, 2H, 2×CH_{Ar}), 7.47-7.27 (m, 5H, 5×CH_{Ar}), 6.98 (d, *J* = 8.9 Hz, 2H, 2×CH_{Ar}), 6.91 (d, *J* = 7.5 Hz, 1H, N*H*), 5.10 (s, 2H, PhCH_2), 4.66 (td, *J* = 7.9, 4.6 Hz, 1H, NHC*H*COO), 2.37-2.28 (m, 2H, CH_2COO), 2.27-2.16 (m, 1H, CH*H*CH_2COO), 2.09-1.97 (m, 1H, CH*H*CH_2COO), 1.48 (s, 9H, C(CH_3)$_3$), 1.41 (s, 9H, C(CH_3)$_3$). **^{13}C NMR** (75 MHz, $CDCl_3$) δ ppm: 172.8 (*C*OO^{t}Bu), 171.6 (*C*OO^{t}Bu), 166.6 (*C*ONH), 161.6 (*C*$_{Ar}$), 136.5 (*C*$_{Ar}$), 129.1 (*CH*$_{Ar}$), 128.8 (*CH*$_{Ar}$), 128.3 (*CH*$_{Ar}$), 127.6 (*CH*$_{Ar}$), 126.5 (O*C*$_{Ar}$), 114.7 (*CH*$_{Ar}$), 82.5 (*C*(CH$_3$)$_3$), 80.9 (*C*(CH$_3$)$_3$), 70.2 (O*C*H$_2$), 52.9 (NH*C*H), 31.8 (*C*H$_2$COO), 28.2 (C(*C*H$_3$)$_3$), 28.2 (C(*C*H$_3$)$_3$), 27.7 (*C*H$_2$). **MS ESI$^+$:** [M+H]$^+$ 470.3, [M+Na]$^+$ 492.2, [2M+Na]$^+$ 961.5. **HRMS**: (ESI+-MS, m/z) calcd for $C_{27}H_{35}NaNO_6$ [M+Na]+: 492.2357, found: 492.2360.

Di-*tert*-butyl (4-hydroxybenzoyl)-L-glutamate. 4.

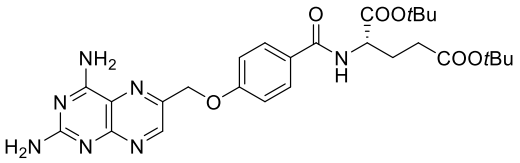
To a well stirred solution of **3** (2 g, 4.48 mmol, 1 eq.) in MeOH (40 mL) under Ar atmosphere, Pd/C (448 mg, 10%) was added. The atmosphere was replaced by H_2 using a balloon, and the suspension was stirred at room temperature for 2.5 hours. After that period of time, the reaction was filtered by a short pad of Celite®, the pad was washed several times with CH_2Cl_2, and the volatiles were removed under reduced pressure to provide **4** (1.64 g, 96%) as a white solid.



^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 7.64 (d, *J* = 8.7 Hz, 2H, 2xCH_{Ar}), 7.09 (d, *J* = 7.5 Hz, 1H, N*H*), 6.83 (d, *J* = 8.7 Hz, 2H, 2xCH_{Ar}), 4.63 (td, *J* = 8.0, 4.6 Hz, 1H, NHC*H*COO), 2.52-2.27 (m, 2H, *CH*$_2$COO), 2.27-2.12 (m, 1H, *CH*HCH$_2$COO), 2.13-1.99 (m, 1H, CH*H*CH$_2$COO), 1.47 (s, 9H, C(*CH*$_3$)$_3$), 1.40 (s, 9H, C(*CH*$_3$)$_3$). **^{13}C NMR** (75 MHz, $CDCl_3$) δ ppm: 173.1 (*C*OO^{t}Bu), 171.7 (*C*OO^{t}Bu), 167.8 (*C*ONH), 160.5 (*C*$_{Ar}$), 129.2 (*CH*$_{Ar}$), 124.7 (*C*$_{Ar}$), 115.6 (*CH*$_{Ar}$), 82.8 (*C*(CH$_3$)$_3$), 81.3 (*C*(CH$_3$)$_3$), 53.2 (NH*C*H), 31.8 (*C*H$_2$), 28.1 (C(*CH*$_3$)$_3$), 28.1 (C(*CH*$_3$)$_3$), 27.4 (*C*H$_2$). **MS ESI$^+$**: [M+Na]$^+$ 402.2, [2M+Na]$^+$ 781.4. **HRMS**: (ESI+-MS, m/z) calcd for $C_{20}H_{29}NaNO_6$ [M+Na]+: 402.1887, found: 402.1889.

Di-*tert*-butyl (4-((2,4-diaminopteridin-6-yl)methoxy)benzoyl)-L-glutamate. 6.

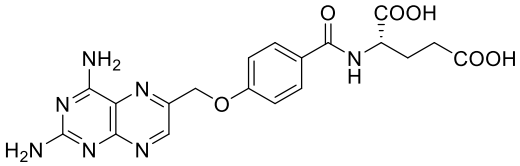
To a well stirred solution of **4** (2 g, 5.27 mmol, 2 eq.) in DMF anh. (10 mL) under Ar atmosphere, NaH (60% mineral oil, 211 mg, 5.27 mmol, 2 eq.) was added in portions and the suspension was stirred for 30 minutes at room temperature. Then, 6-(bromomethyl)pteridine-2,4-diamine hydrobromide **5** (90%, 986 mg, 2.64 mmol, 1 eq.) previously dissolved in DMF anh. (13 mL) was added dropwise and the reaction was stirred at rt for 7 days. After that time, MeOH was added to quench potential excess of NaH that could not have reacted and the product was precipitated by the addition of H_2O (70 mL). The precipitate was washed with H_2O (80 mL) and filtered by a short pad of silica gel, first eluted with cyclohexane:ethyl acetate, 6:4 (300 mL) to remove **4** and then with CH_2Cl_2/MeOH (9/1, 100 mL) to elute the expected compound. The fraction containing **6** was repurified by column chromatography CH_2Cl_2/MeOH (10/0 to 8/2), to afford **6** (555 mg, 38%) as bright yellow solid.



^{1}H NMR (400 MHz, DMF-d_7) δ ppm: 9.07 (s, 1H, CH_{Ar}), 8.62 (d, *J* = 7.6 Hz, 1H, CON*H*), 8.13 (d, *J* = 8.8 Hz, 2H, 2×CH_{Ar}), 8.01 (bs, 2H, NH_2), 7.33 (d, *J* = 8.8 Hz, 2H, 2×CH_{Ar}), 6.97 (bs, 2H, NH_2), 5.45 (s, 2H, OCH_2), 4.65-4.61 (m, 1H, NHC*H*), 2.57 (t, *J* = 7.2 Hz, 2H, CH_2COO), 2.30-2.24 (m, 1H, CH*H*CH$_2$COO), 2.20-2.14 (m, 1H, C*H*HCH$_2$COO), 1.56 (s, 9H, C(CH_3)$_3$), 1.54 (s, 9H, C(CH_3)$_3$). **^{13}C NMR** (100 MHz, DMF-d_7) δ ppm: 172.6 (*C*OO^{t}Bu), 172.3 (*C*OO^{t}Bu), 167.1 (*C*OHN), 164.7 (*C*$_{Ar}$), 164.4 (*C*$_{Ar}$), 162.8 (*C*$_{Ar}$), 157.0 (*C*$_{Ar}$), 151.1 (*C*H$_{Ar}$), 144.4 (*C*$_{Ar}$), 130.2 (*C*H$_{Ar}$), 128.0 (*C*$_{Ar}$), 122.6 (*C*$_{Ar}$), 115.2 (*C*H$_{Ar}$), 81.4 (*C*(CH$_3$)$_3$), 80.6 (*C*(CH$_3$)$_3$), 69.9 (O*C*H$_2$), 53.7 (NH*C*H), 30.2 (*C*H$_2$), 28.2 (C(*C*H$_3$)$_3$), 28.1 (C(*C*H$_3$)$_3$), 27.2 (*C*H$_2$). **MS ESI$^+$**: [M+H] $^+$ 554.3, [M+Na] $^+$ 576.3. **HRMS**: (ESI+-MS, m/z) calcd for $C_{27}H_{35}NaN_7O_6$ [M+Na]+: 576.2541, found: 576.2540.

(4-((2,4-diaminopteridin-6-yl)methoxy)benzoyl)-L-glutamic acid. OMTX.

A well stirred solution of **6** (435 mg, 0.786 mmol, 1 eq.) in $CHCl_3$ (14 mL) under Ar atmosphere at 0°C was treated with TFA (11 mL, 142 mmol, 180 eq.) dropwise and the reaction mixture was stirred at room temperature for 16 h. Then, the solvent was removed under reduced pressure and the brown residue was triturated with Et_2O (5 mL) to provide a pale yellow solid. The crude product was washed with Et_2O (30 mL) and CH_2Cl_2/MeOH (8/2, 60 mL) to afford **OMTX** (311 mg, 90%) as bright yellow solid. The analytical data of **OMTX** were in complete agreement with literature data.[17]

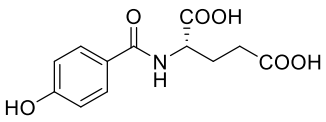


^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 8.96 (s, 1H, CH_{Ar}), 8.48 (d + bs, *J* = 7.6 Hz, 3H, CON*H* + NH_2), 7.90 (d, *J* = 8.8 Hz, 2H, 2×CH_{Ar}), 7.57 (bs, 2H, NH_2), 7.17 (d, *J* = 8.8 Hz, 2H, 2×CH_{Ar}), 5.33 (s, 2H, OCH_2), 4.41-4.36 (m, 1H, NHC*H*), 2.08 (t, *J* = 7.2 Hz, 2H, CH_2COO), 2.12-2.07 (m, 1H, CH*H*CH$_2$COO), 2.00-1.90 (m, 1H, C*H*HCH$_2$COO).**^{13}C NMR** (100 MHz, DMSO-d_6) δ ppm: 173.9 (*C*OOH), 173.4 (*C*OOH), 166.0 (*C*OHN), 162.9 (*C*$_{Ar}$), 160.4 (*C*$_{Ar}$), 160.0 (*C*$_{Ar}$), 150.0 (*C*H$_{Ar}$), 145.5 (*C*$_{Ar}$), 129.4 (*C*H$_{Ar}$), 126.8 (*C*$_{Ar}$), 122.0 (*C*$_{Ar}$), 114.3 (*C*H$_{Ar}$), 68.5 (O*C*H$_2$), 52.0 (NH*C*H), 30.4 (*C*H$_2$COOH), 26.0 (*C*H$_2$). **MS ESI$^+$**: [M+H]$^+$ 442.1, [M+Na]$^+$ 464.1. **HRMS**: (ESI+-MS, m/z) calcd for $C_{19}H_{20}N_7O_6$ [M+H]$^+$: 442.1462, found: 442.1457.

(4-hydroxybenzoyl)-L-glutamic acid. 7. A well stirred solution of **4** (300 mg, 0.791 mmol, 1 eq.) in $CHCl_3$ (9 mL) under Ar atmosphere at 0°C was treated with TFA (7 mL, 95 mmol, 180 eq.) dropwise and the reaction mixture was stirred at room temperature for 16 h. Then, the solvent was removed under reduced pressure and the brown residue was triturated with Et_2O to afford **7** (202 mg, 96%) as white solid. The analytical data of **7** were in complete agreement with literature data.[18]

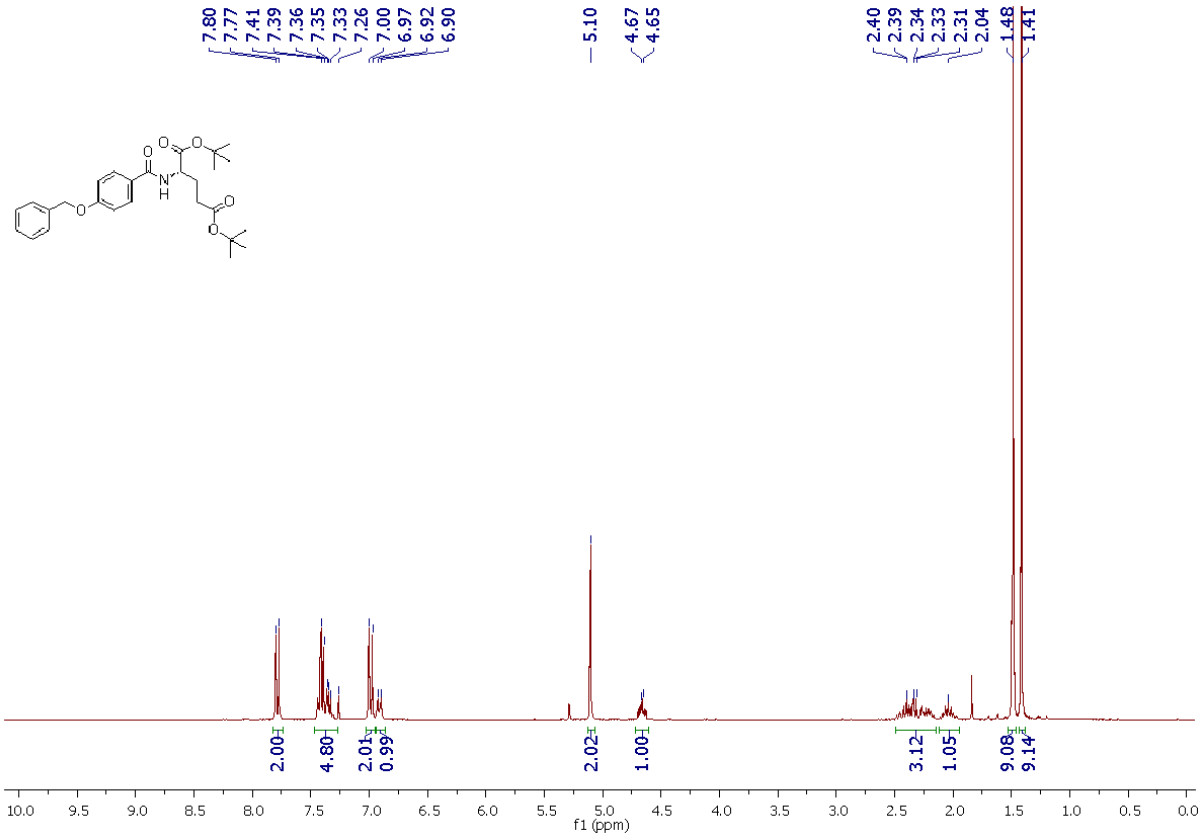
^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 8.11 (d, *J* = 7.6 Hz, 1H, CON*H*), 7.73 (d, *J* = 8.8 Hz, 2H, 2×CH_{Ar}), 7.80 (d, *J* = 8.8 Hz, 2H, 2×CH_{Ar}), 4.34-4.30 (m, 1H, NHC*H*), 2.30 (t, *J* = 7.2 Hz, 2H, CH_2COO), 2.08-2.02 (m, 1H, CH*H*CH$_2$COO), 1.96-1.90 (m, 1H, C*H*HCH$_2$COO).**^{13}C NMR** (100 MHz, DMSO-d_6) δ ppm: 174.7 (2x*C*OOH), 165.9 (*C*OHN), 160.3 (*C*$_{Ar}$), 129.3 (*C*H$_{Ar}$), 124.9 (*C*$_{Ar}$), 114.8 (*C*H$_{Ar}$), 52.5 (NH*C*H), 31.0

[17] Nair, M. G.; Campbell, P. T. *J. Med. Chem.* **1976**, 19(6), 825-829.
[18] Trennheuser, F.; Burkharda, G.; Becker, H. *Phytochemistry.* **1994**, 37(3), 899-903.

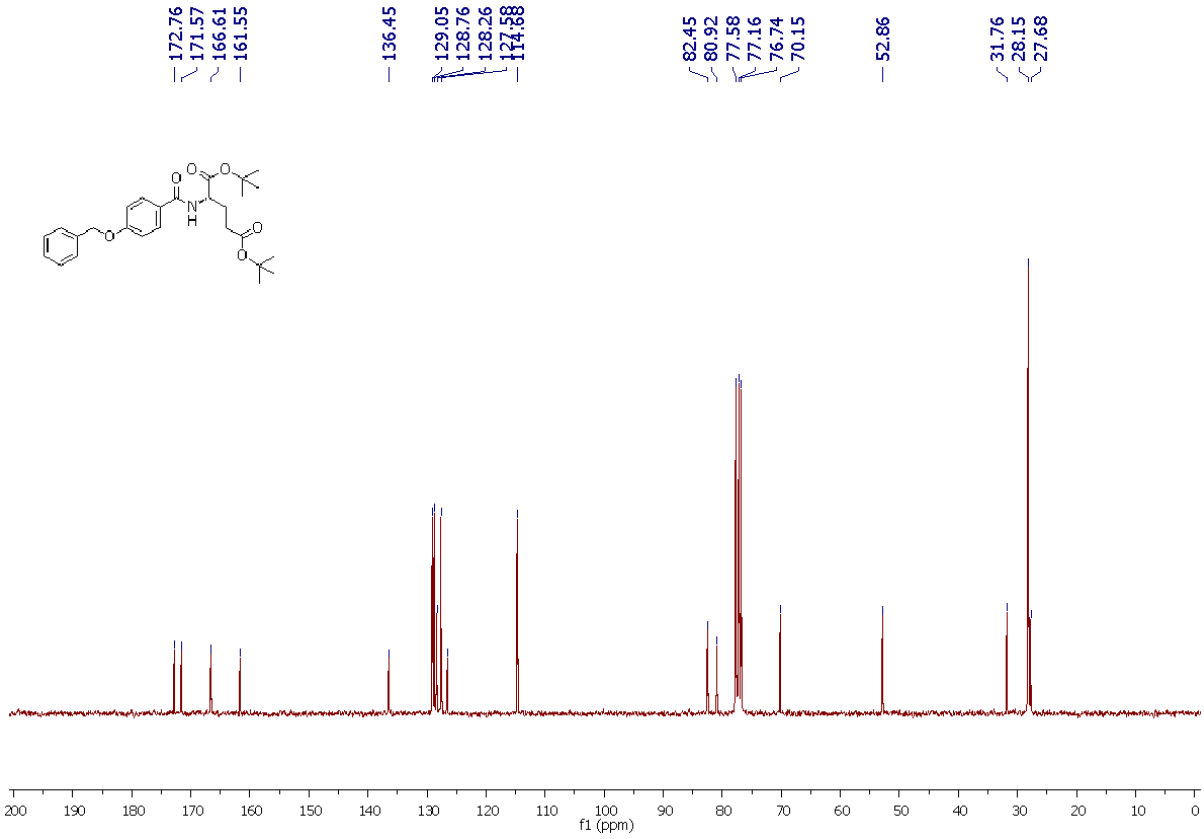


(CH$_2$COOH), 26.8 (CH$_2$). **MS ESI$^+$**: [M+H]$^+$ 268.1, [M+Na]$^+$ 290.1. **HRMS**: (ESI+-MS, m/z) calcd for $C_{12}H_{13}NaNO_6$ [M+Na]$^+$: 290.0635, found: 290.0630.

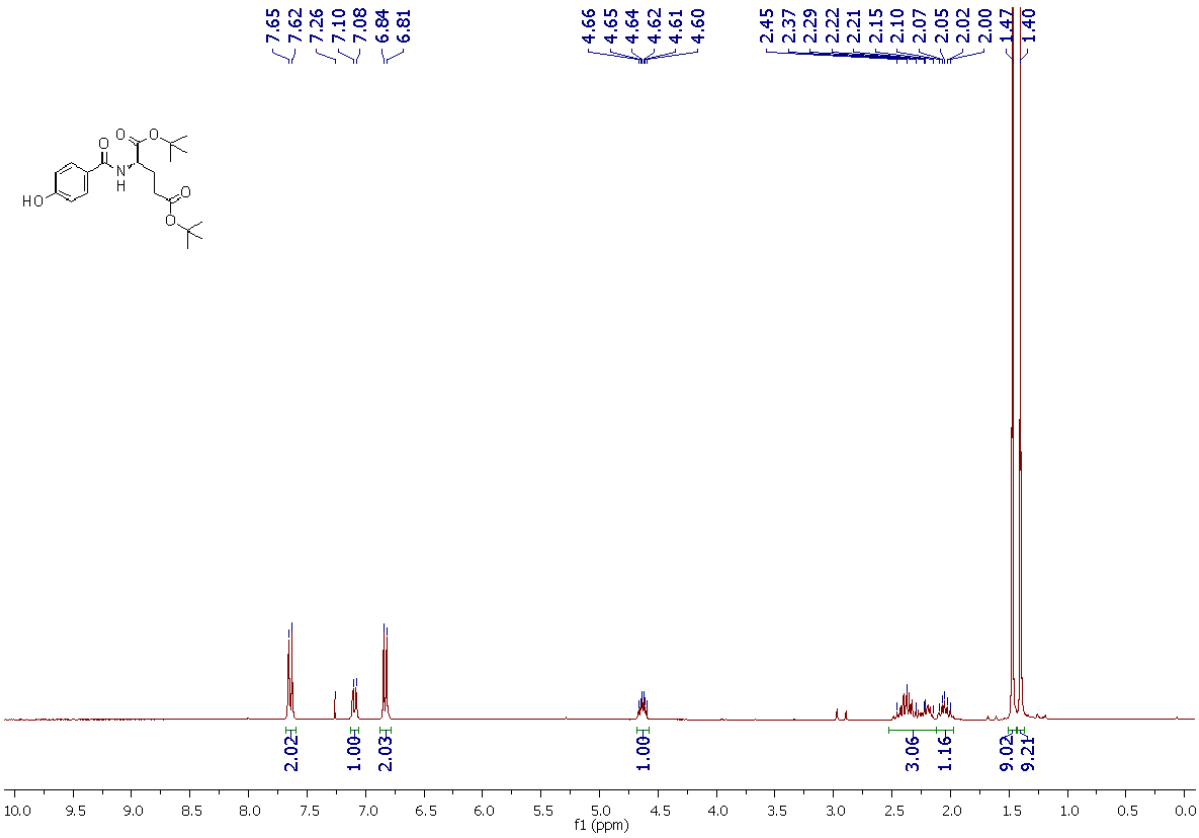
^{1}H NMR (300 MHz, CDCl$_3$) of compound 3



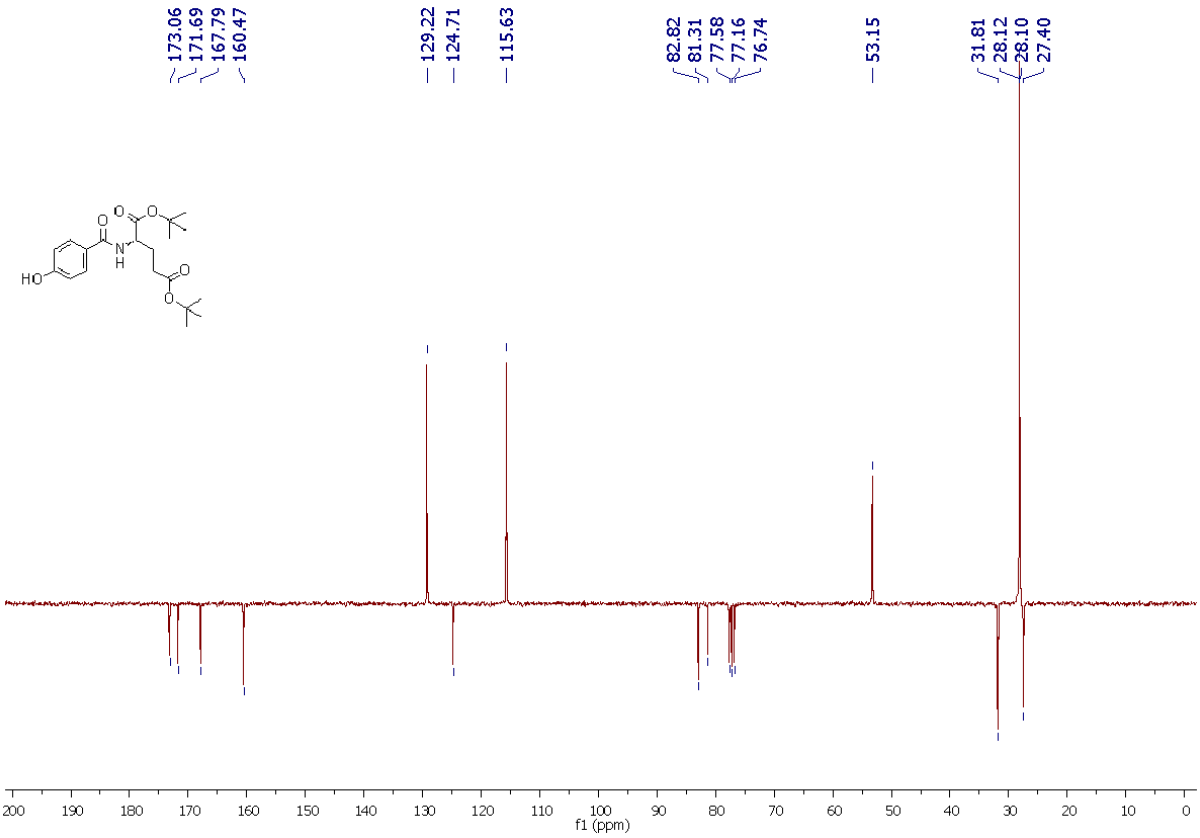
^{13}C NMR (75 MHz, $CDCl_3$) of compound 3



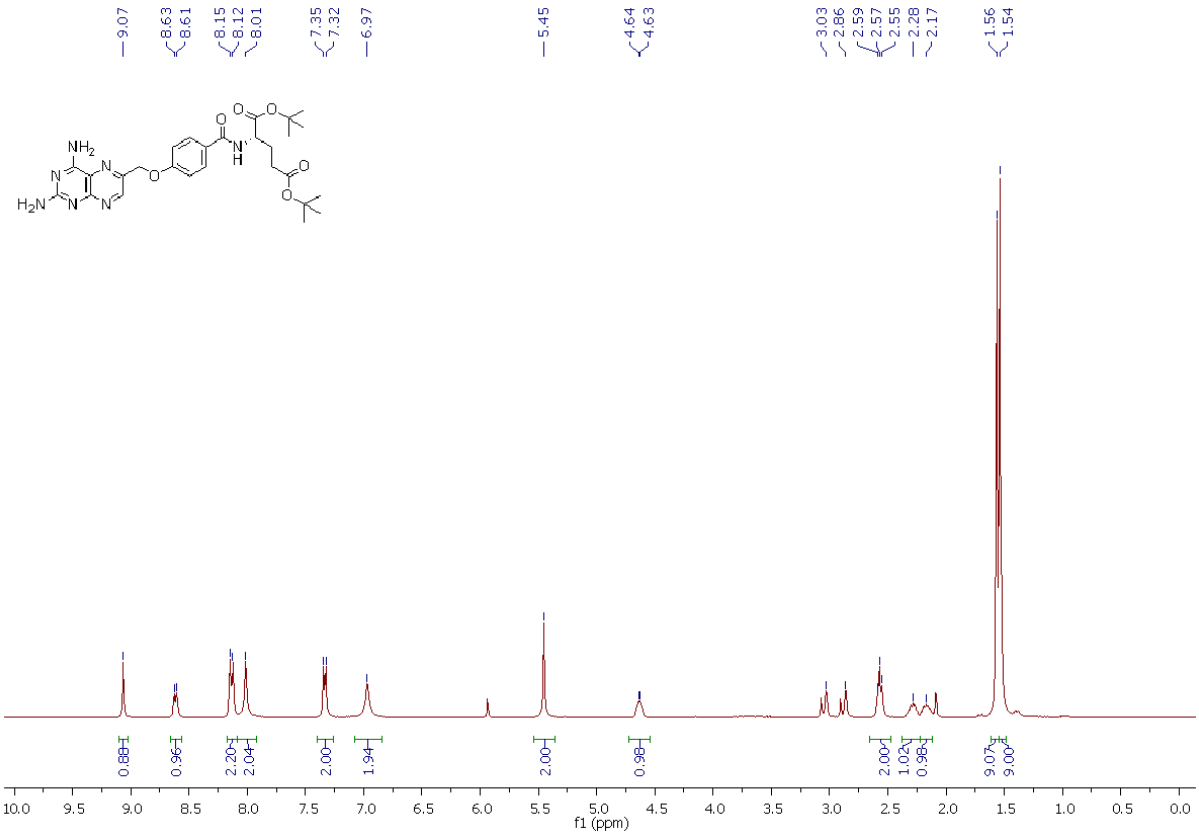
^{1}H NMR (300 MHz, $CDCl_3$) of compound 4



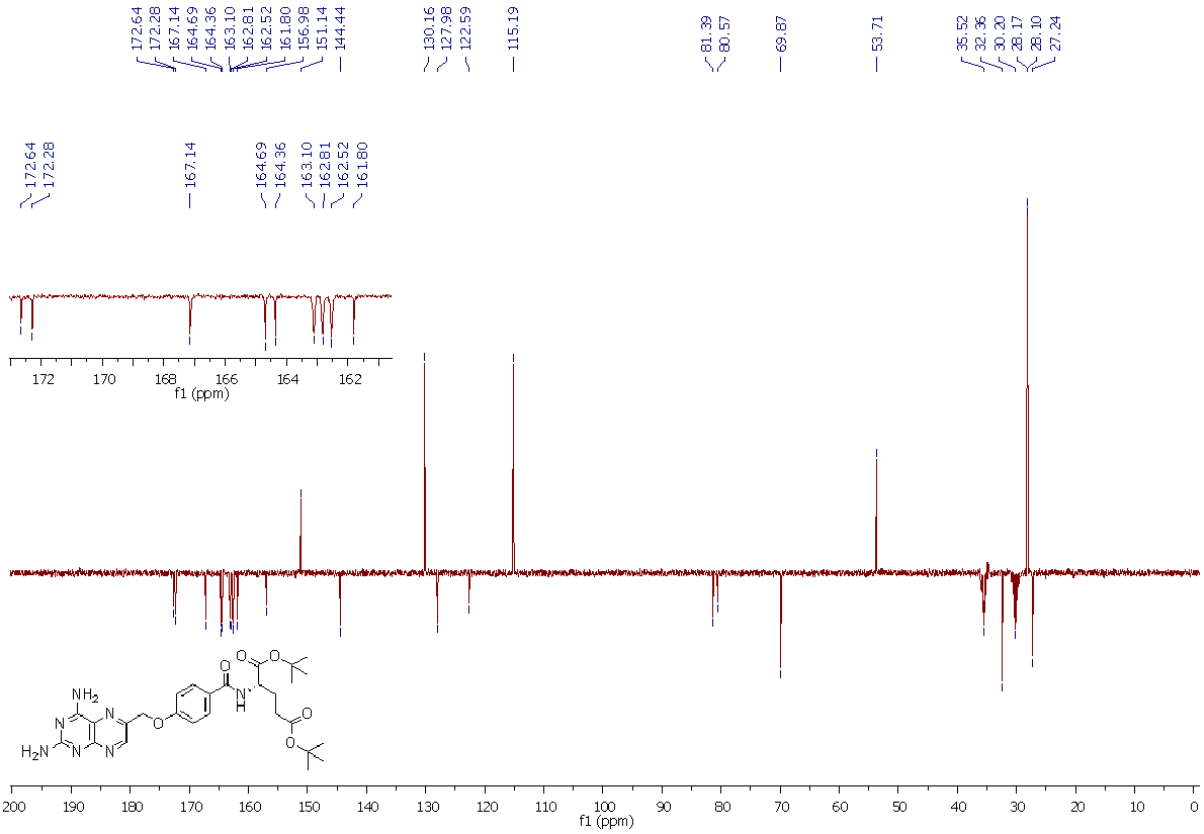
^{13}C NMR (75 MHz, $CDCl_3$) of compound 4



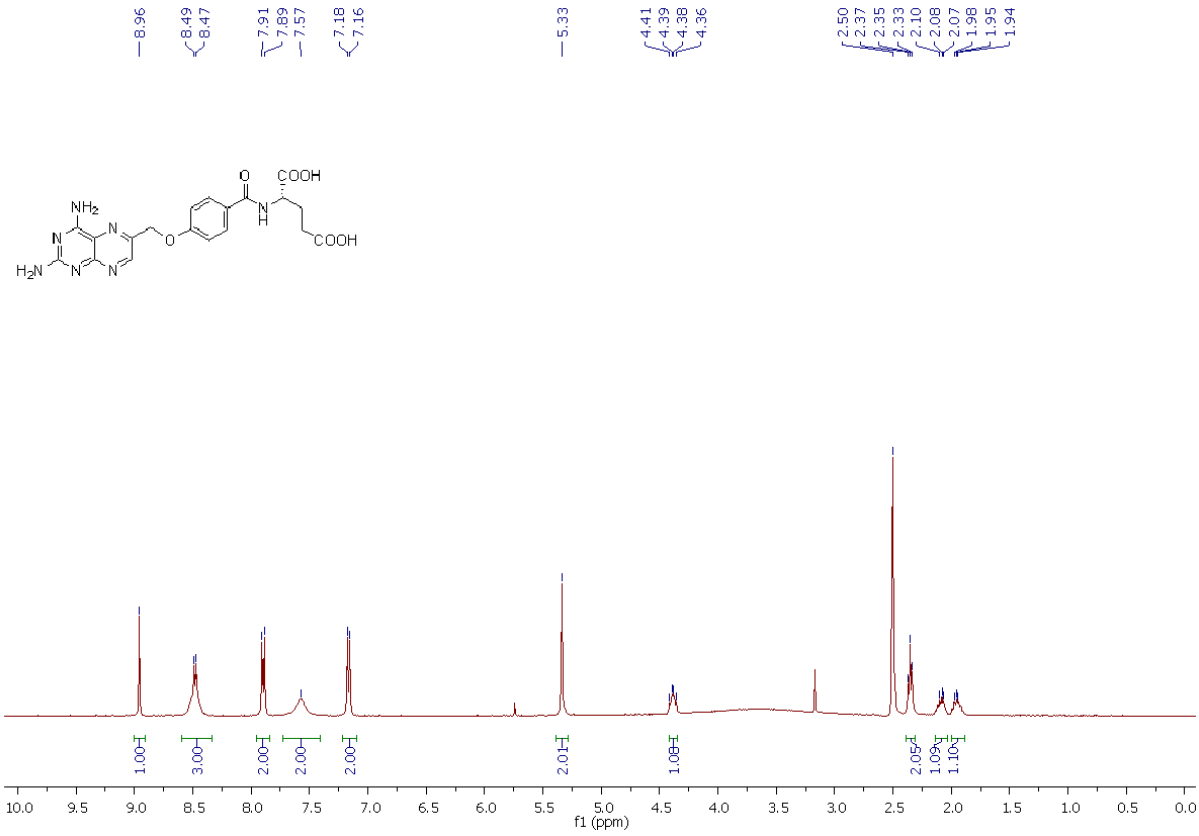
^{1}H NMR (400 MHz, DMF-d_7) of compound 6



^{13}C NMR (100 MHz, DMF-d_7) of compound 6



^{1}H NMR (400 MHz, DMSO-d_6) of OMTX



^{13}C NMR (100 MHz, DMSO-d_6) of OMTX

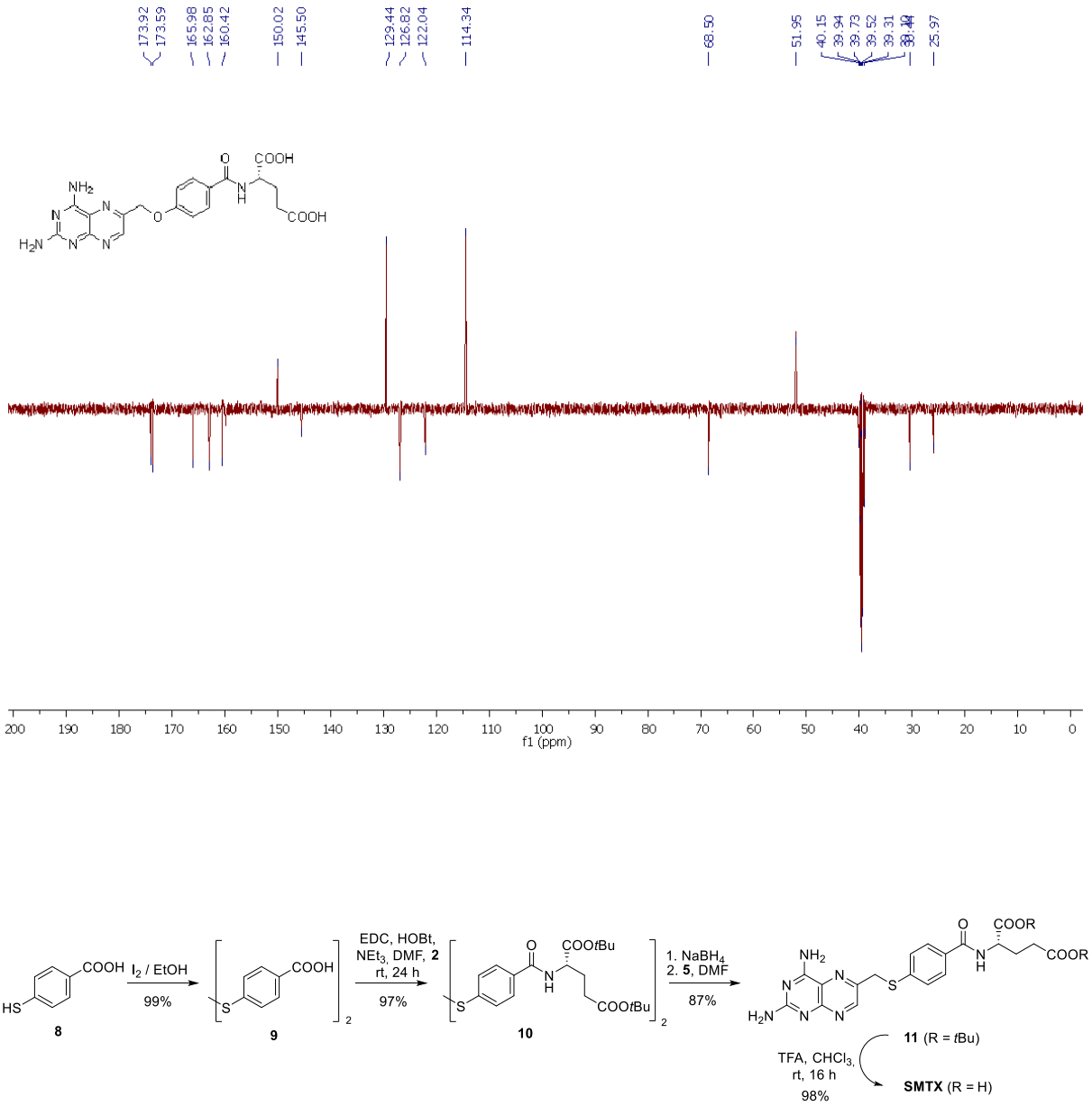
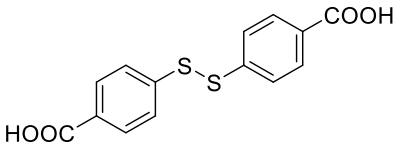


Figure S2. Synthetic pathway to **SMTX**.

4,4'-disulfanediyldibenzoic acid. 9.

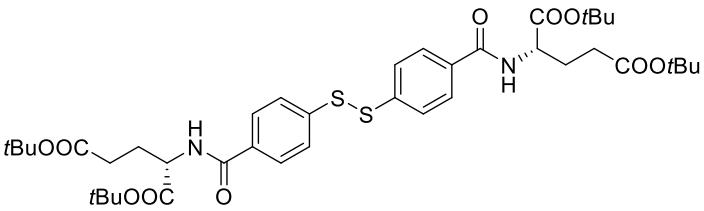
4-mercaptobenzoic acid **8** (1 g, 6.47 mmol, 1 eq.) was dissolved in absolute EtOH (50 mL) and a saturated solution of I_2 in EtOH was added dropwise until a white precipitate appeared. The mixture was centrifuged, washed (2 x 40 mL of cold EtOH) and dried under high vacuum to yield the corresponding disulfide **9** (980 mg, 99%) as a white solid. The analytical data of **9** were in complete agreement with literature data.[19]

[19] Pazos, E.; Garcia-Algar, M.; Penas, C.; Nazarenus, M.; Torruella, A. Pazos-Perez, N.; Guerrini, L.; Eugenio Vázquez, M.; Garcia-Rico, E.; Mascareñas, J. L.; Alvarez-Puebla, R. A. *J. Am. Chem. Soc.* **2016**, 138(43), 14206-14209.



^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 7.93 (d, *J* = 8.6 Hz, 2H, 4×H_{Ar}), 7.63 (d, *J* = 8.6 Hz, 4H, 4×H_{Ar}). **^{13}C NMR** (75 MHz, $CDCl_3$) δ ppm: 166.70 (*C*OOH), 140.80 (C_{Ar}), 130.4 (CH_{Ar}), 129.75 (C_{Ar}), 126.1 (CH_{Ar}). **HRMS**: (ESI+-MS, m/z) calcd for $C_{14}H_{10}NaO_4S_2$ [M+Na]+: 328.9913, found: 328.9902.

Tetra-*tert*-butyl 2,2'-((4,4'-disulfanediylbis(benzoyl))bis(azanediyl))(2S,2'S)-diglutarate. 10. Diacid **9** (0.91 g, 2.97 mmol, 1 eq.) was dissolved in anhydrous DMF (15 mL) and triethylamine (2.48 mL, 17.82 mmol, 6 eq.) was added dropwise at 0°C. The mixture was stirred for ten minutes at room temperature and then HOBt (963 mg, 7.13 mmol, 2.4 eq.), EDC (1.25 mL, 7.13 mmol, 2.4 eq.) and di-*tert*-butyl L-glutamate **2** (1.93 g, 6.53 mmol, 2.2 eq.) were sequentially added. The reaction mixture was stirred at room temperature until consumption of the reagents monitoring the reaction progress by TLC (cyclohexane/ethyl acetate, 6/4). Then, solvent was removed in vacuum, and the residue was partitioned between EtOAc and aqueous $NaHCO_3$ saturated solution. The aqueous layer was extracted with EtOAc (3 x 25 mL), and the combined organic layers were washed with brine, dried with $MgSO_4$, filtered and evaporated. The crude material was purified by column chromatography (cyclohexane/ethyl acetate, 8/2) to yield the corresponding peptide ester **10** (2 g, 85%) as a white solid. The analytical data of **10** were in complete agreement with literature data.[20]

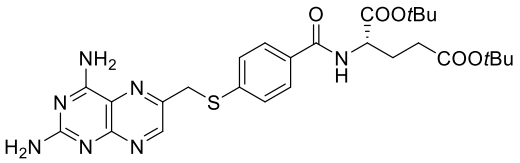


^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 7.68 (d, *J* = 8.6 Hz, 4H, 4×H_{Ar}), 7.41 (d, *J* = 8.5 Hz, 4H, 4×H_{Ar}), 7.20 (d, *J* = 7.7 Hz, 2H, 2×NH), 4.60 (td, *J* = 8.2, 4.5 Hz, 2H, 2×C*H*NH), 2.36-2.10 (m, 6H, 2×CH_2, 2×C*H*HCH$_2$COO), 2.03-1.90 (m, 2H, 2×CH*H*CH$_2$COO), 1.42 (s, 18H, 2×C(CH_3)$_3$), 1.35 (s, 18H, 2×C(CH_3)$_3$). **^{13}C NMR** (75 MHz, $CDCl_3$) δ ppm: 172.5 (*C*OO^{t}Bu), 171.4 (*C*OO^{t}Bu), 166.2 (*C*ONH), 140.5 (C_{Ar}), 132.5 (C_{Ar}), 127.9 (CH_{Ar}), 126.5 (CH_{Ar}), 82.4 (*C*(CH$_3$)$_3$), 52.9 (NH*C*H), 31.6 (*C*H$_2$), 28.0 (C(*C*H$_3$)$_3$), 28.0 (C(*C*H$_3$)$_3$), 27.3 (*C*H$_2$). **MS ESI$^+$**: [M+H] $^+$ 789.3, [M+Na] $^+$ 811.3. **HRMS**: (ESI+-MS, m/z) calcd for $C_{40}H_{56}N_2NaO_{10}S_2$ [M+Na]+: 811.3269, found: 811.3253.

Di-*tert*-butyl (4-(((2,4-diaminopteridin-6-yl)methyl)thio)benzoyl)-L-glutamate. 11.

To a well stirred solution of **10** (500 mg, 0.634 mmol, 1 eq.) in absolute EtOH (6 mL) under Ar atmosphere, $NaBH_4$ (43 mg, 1.14 mmol, 1.8 eq.) was added and the suspension was stirred at room temperature for 2 h (complete reduction of the disulfide monitored by TLC (cyclohexane/ethyl acetate, 6/4). Then, 6-(bromomethyl)pteridine-2,4-diamine hydrobromide **5** (90%, 237 mg, 0.634 mmol, 1 eq.) previously dissolved in anhydrous DMF (6 mL) was added dropwise and the reaction was stirred at rt for 2 days. After that time, the solvent was evaporated under reduced pressure and the crude was purified by column chromatography CH_2Cl_2/MeOH (10/0 to 97/3), to afford **11** (333 mg, 87%) as bright yellow solid.

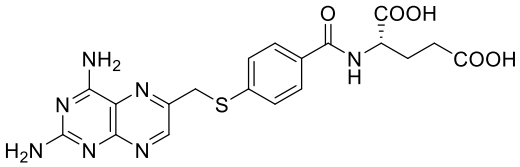
[20] Singh, S. K.; Hynes, J. B. *J. Heterocyclic Chem.* **1991**, 28, 977-981.



^{1}H NMR (300 MHz, DMSO-d_6) δ ppm: 8.76 (s, 1H, C*H*$_{Ar}$), 8.56 (d, *J* = 7.5 Hz, 1H, CON*H*), 7.80 (d, *J* = 8.4 Hz, 2H, 2×C*H*$_{Ar}$), 7.63 (d, *J* = 39.4 Hz, 2H, NH_2), 7.51 (d, *J* = 8.5 Hz, 2H, 2×C*H*$_{Ar}$), 6.68 (bs, 2H, NH_2), 4.49 (s, 2H, SC*H*$_2$), 4.29 (dd, *J* = 12.7, 9.5 Hz, 1H, NHC*H*), 2.32 (t, *J* = 7.4 Hz, 2H, C*H*$_2$), 2.07-1.83 (m, 2H, C*H*$_2$), 1.39 (s, 9H, COO^{t}Bu), 1.37 (s, 9H, COO^{t}Bu). **^{13}C NMR** (75 MHz, DMSO-d_6) δ ppm: 171.6 (*C*OO^{t}Bu), 171.1 (*C*OO^{t}Bu), 166.1 (*C*OHN), 162.9 (*C*$_{Ar}$), 162.7 (*C*$_{Ar}$), 154.9 (*C*$_{Ar}$), 150.6 (*C*H$_{Ar}$), 144.7 (*C*$_{Ar}$), 140.0 (*C*$_{Ar}$), 131.0 (*C*$_{Ar}$), 128.1 (*C*H$_{Ar}$), 126.8 (*C*H$_{Ar}$), 121.1 (*C*$_{Ar}$), 80.6 (*C*(CH$_3$)$_3$), 79.8 (*C*(CH$_3$)$_3$), 52.5 (NH*C*H), 34.8 (S*C*H$_2$), 31.4 (*C*H$_2$), 27.7 (C*C*H$_3$), 27.7 (C*C*H$_3$), 25.9 (*C*H$_2$). **MS ESI$^+$**: $[M+H]^+$ 570.2, $[M+Na]^+$ 592.2. **HRMS**: (ESI+-MS, m/z) calcd for $C_{27}H_{36}N_7O_5S$ $[M+H]^+$: 570.2493, found: 570.2484.

(4-(((2,4-diaminopteridin-6-yl)methyl)thio)benzoyl)-L-glutamic acid. SMTX.

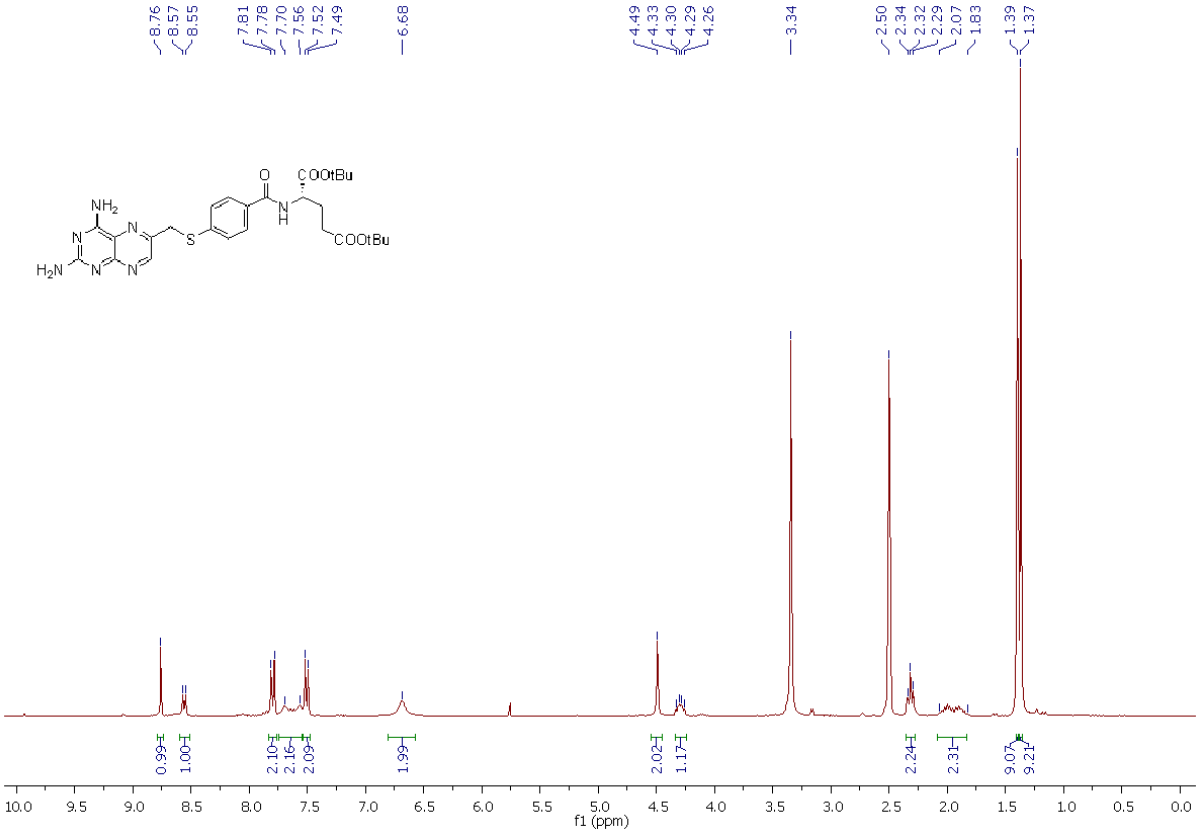
A well stirred solution of **11** (310 mg, 0.544 mmol, 1 eq.) in $CHCl_3$ (10 mL) under Ar atmosphere at 0°C was treated with TFA (8.3 mL, 104 mmol, 180 eq.) dropwise and the reaction mixture was stirred at room temperature for 16 h. Then, the solvent was removed under reduced pressure and the brown residue was triturated with Et_2O (40 mL) to provide a pale yellow solid. The crude product was washed with Et_2O (40 mL) and MeOH (10 mL) to afford **SMTX** (244 mg, 98%) as bright yellow solid. The analytical data of **SMTX** were in complete agreement with literature data.[21]



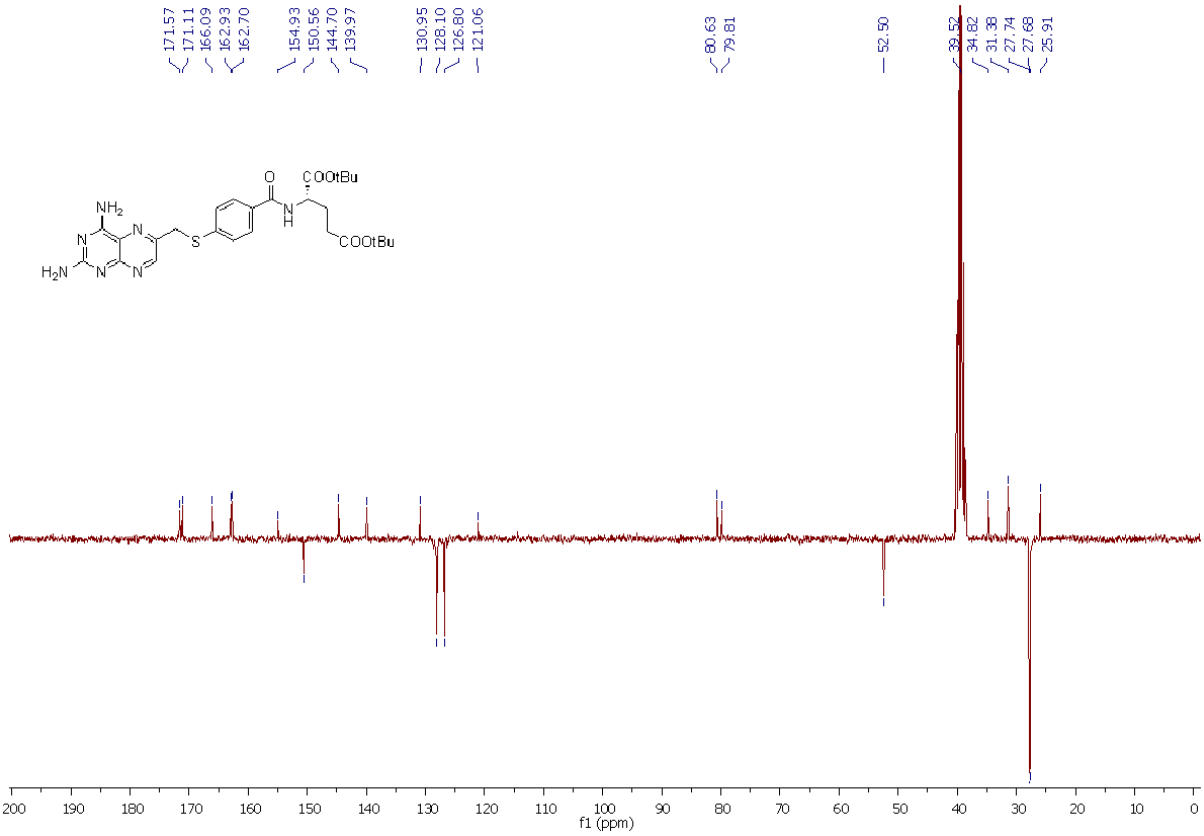
^{1}H NMR (300 MHz, DMSO-d_6) δ ppm: 9.20 (d, *J* = 32.1 Hz, 2H, N*H*$_2$), 8.92 (s, 1H, C*H*$_{Ar}$), 8.60 (d, *J* = 7.6 Hz, 2H, N*H*$_2$), 7.82 (d, *J* = 8.5 Hz, 2H, 2×C*H*$_{Ar}$), 7.51 (d, *J* = 8.5 Hz, 2H, 2×C*H*$_{Ar}$), 4.58 (s, 2H, SC*H*$_2$), 4.37 (m, 1H, NHC*H*), 2.33 (t, *J* = 7.4 Hz, 2H, CH_2), 2.13-2.05 (m, 1H, CH*H*), 1.98-1.88 (s, 1H, CH*H*). **^{13}C NMR** (75 MHz, DMSO-d_6) δ ppm: 173.9 (*C*OOH), 173.5 (*C*OOH), 166.0 (*C*OHN), 162.7 (*C*$_{Ar}$), 156.0 (*C*$_{Ar}$), 150.3 (*C*H$_{Ar}$), 149.9 (*C*$_{Ar}$), 145.6 (*C*$_{Ar}$), 139.3 (*C*$_{Ar}$), 131.2 (S*C*$_{Ar}$), 128.2 (*C*H$_{Ar}$), 127.0 (*C*H$_{Ar}$), 121.9 (*C*$_{Ar}$), 52.0 (NH*C*H), 34.6 (S*C*H$_2$), 30.4 (*C*H$_2$COOH), 25.9 (*C*H$_2$). **MS ESI$^+$**: $[M+H]^+$ 458.1, $[M+Na]^+$ 480.1. **HRMS**: (ESI+-MS, m/z) calcd for $C_{19}H_{20}N_7O_5S$ $[M+H]^+$: 458.1241, found: 458.1228.

[21] Kim, Y. H.; Gaumont, Y.; Kisliuk, R. L.; Mautner, H. G. *J. Med. Chem.* **1975**, 18(8), 776-780.

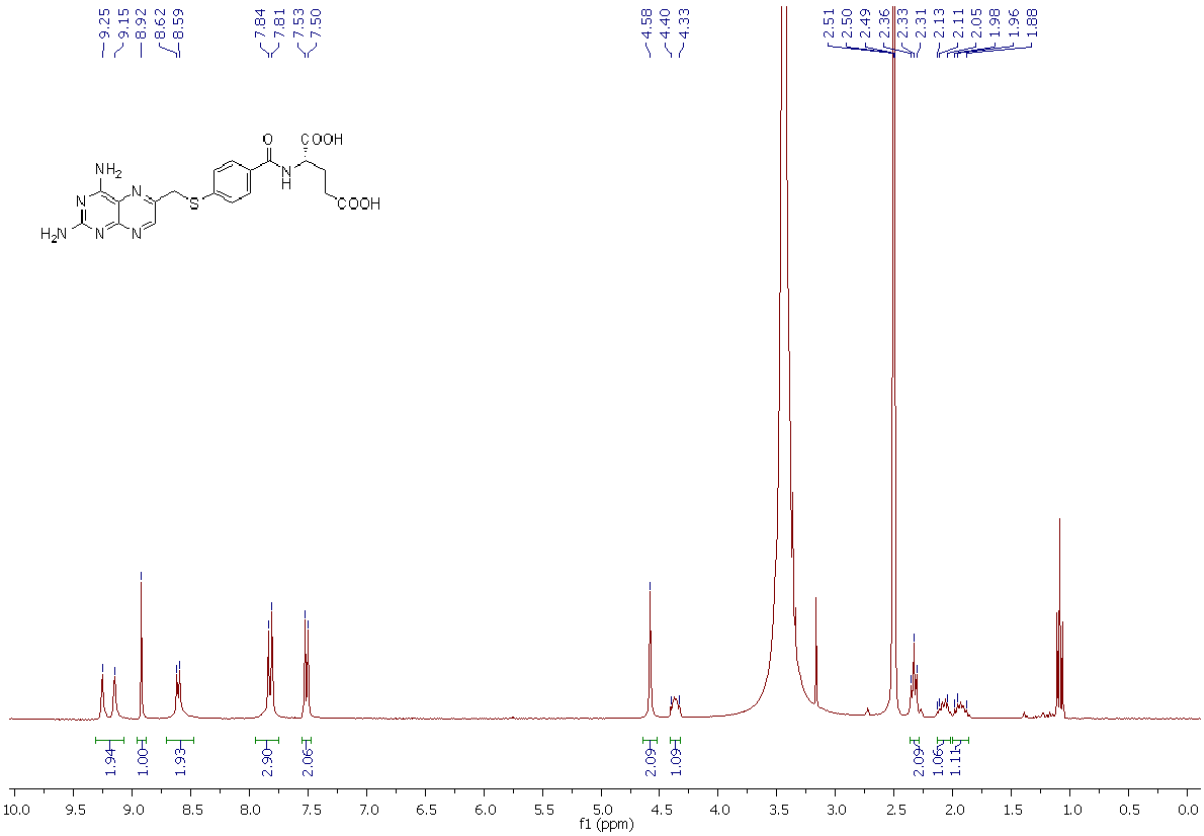
^{1}H NMR (300 MHz, DMSO-d_6) of compound 11



^{13}C NMR (75 MHz, DMSO-d_6) of compound 11



^{1}H NMR (300 MHz, DMSO-d_6) of compound SMTX



^{13}C NMR (75 MHz, DMSO-d_6) of compound SMTX

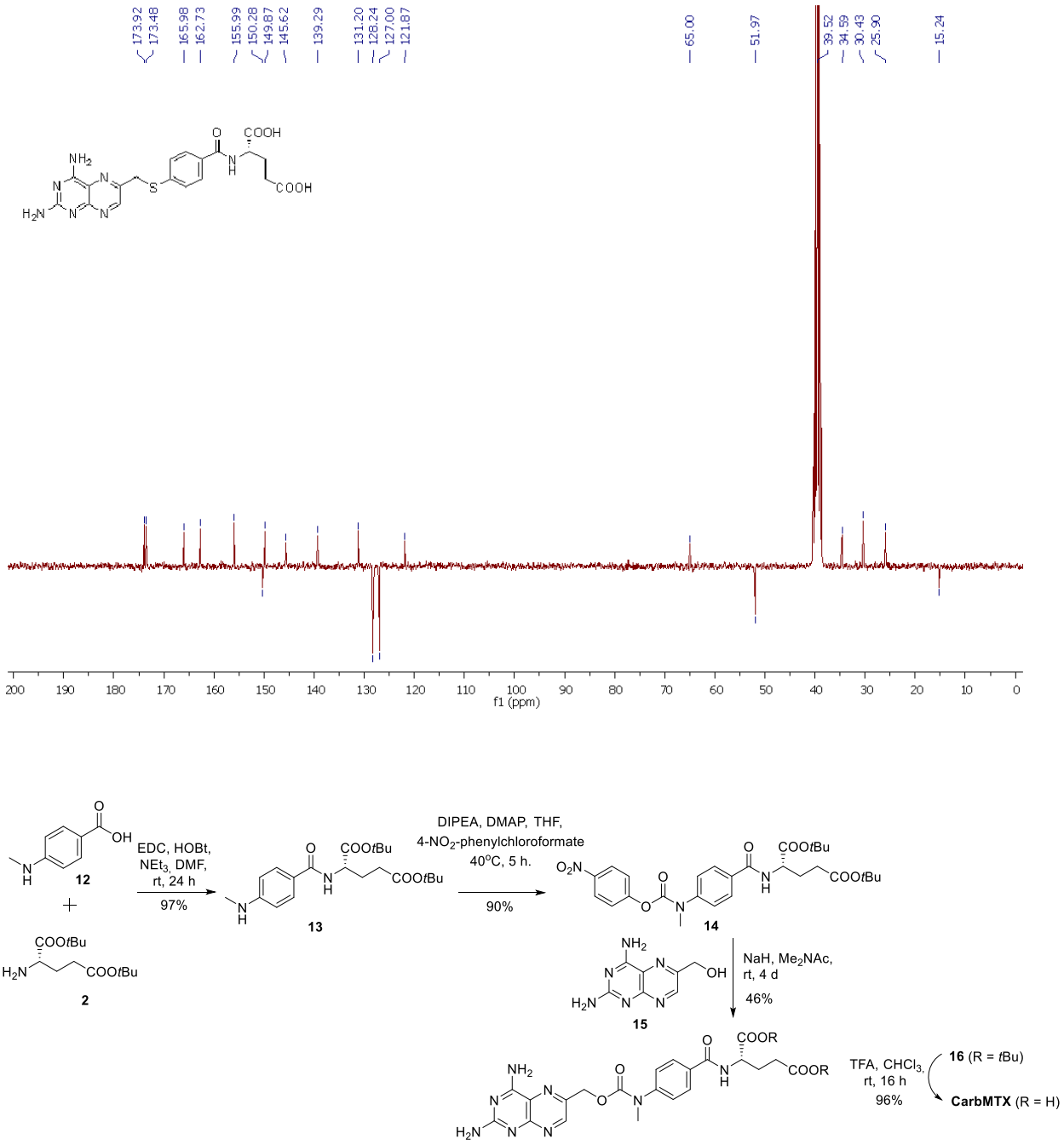
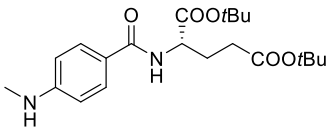


Figure S3. Synthetic pathway to **CarbMTX**.

Di-*tert*-butyl (4-(methylamino)benzoyl)-L-glutamate. 13.

4-(methylamino)benzoic acid **12** (756 mg, 5 mmol, 1 eq.) was dissolved in anhydrous DMF (25 mL) and triethylamine (2.1 mL, 15 mmol, 3 eq.) was added dropwise. The mixture was stirred for five minutes at

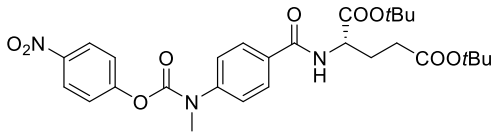
room temperature and then HOBt (811 mg, 6 mmol, 1.2 eq.), EDC (1.06 mL, 6 mmol, 1.2 eq.) and di-*tert*-butyl L-glutamate **2** (1.48 g, 5 mmol, 1 eq.) were sequentially added. The reaction mixture was stirred under Ar at room temperature until consumption of the reagents monitoring the reaction progress by TLC. Then, solvent was removed in vacuum, and the residue was partitioned between EtOAc and aqueous $NaHCO_3$ saturated solution. The aqueous layer was extracted with EtOAc (3 x 25 mL), and the combined organic layers were washed with brine, dried with $MgSO_4$, filtered and evaporated. The crude material was purified by column chromatography (cyclohexane/ethyl acetate, 7/3) to yield the corresponding peptide ester **13** (1.9 g, 97%) as a white solid. The analytical data of **13** were in complete agreement with literature data.[22]



^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 7.61 (d, *J* = 8.7 Hz, 2H, 2×CH_{Ar}), 6.79 (d, *J* = 7.6 Hz, 1H, NH), 6.48 (d, *J* = 8.7 Hz, 2H, 2×CH_{Ar}), 4.62 (td, *J* = 8.0, 4.6 Hz, 1H, NHC*H*), 4.41 (s, 1H, NH), 2.77 (d, *J* = 3.0 Hz, 3H, CH_3NH), 2.40-2.10 (m, 3H, CH_2, C*H*HCH$_2$COO), 2.02-1.90 (m, 1H, CH*H*CH$_2$COO), 1.42 (s, 9H, C(CH_3)$_3$), 1.36 (s, 9H, C(CH_3)$_3$). **^{13}C NMR** (75 MHz, $CDCl_3$) δ ppm: 172.6 (*C*OO^{t}Bu), 171.7 (*C*OO^{t}Bu), 167.0 (*C*ONH), 152.2 (C_{Ar}), 128.7 (*CH*$_{Ar}$), 121.4 (C_{Ar}), 111.2 (*CH*$_{Ar}$), 82.1 (*C*(CH$_3$)$_3$), 80.6 (*C*(CH$_3$)$_3$), 52.5 (NH*C*H), 31.6 (*C*H$_2$), 30.1 (*C*H$_3$), 28.0 (C(*C*H$_3$)$_3$), 27.9 (C(*C*H$_3$)$_3$), 27.7 (*C*H$_2$). **MS ESI$^+$**: $[M+H]^+$ 393.2, $[M+Na]^+$ 415.2, $[2M+Na]^+$ 807.5. **HRMS**: (ESI$^+$-MS, m/z) calcd for $C_{21}H_{32}N_2NaO_5$ $[M+Na]^+$: 415.2203, found: 415.2202.

Di-*tert*-butyl(4-(methyl((4-nitrophenoxy)carbonyl)amino)benzoyl)-L-glutamate.14.

To a well stirred dispersion of 4-nitrophenyl chloroformate (1.16 g, 5.73 mmol, 1.5 eq.) and DMAP (93 mg, 0.76 mmol, 0.2 eq.) in anhydrous THF (15 mL), was added dropwise a previously prepared solution of **13** (1.5 g, 3.82 mmol, 1 eq.) and DIPEA (2 mL, 11.46 mmol, 3 eq.) in anhydrous THF (20 mL) under Ar atmosphere. The reaction mixture was stirred at 40°C for 2 days under Ar. Then, aqueous $NaHCO_3$ saturated solution (50 mL) was added and the product was extracted with EtOAc (2 x 25 mL) and CH_2Cl_2 (2 x 25 mL), the combined organic layers were washed with brine, dried with $MgSO_4$, filtered and evaporated. The crude material was purified by flash chromatography (CH_2Cl_2 to CH_2Cl_2/EtOAc, 95/5) to yield **14** (1.93 g, 90%) as a white solid.

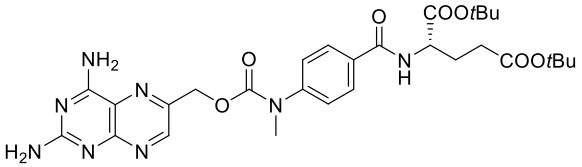


^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 8.23 (d, *J* = 9.1 Hz, 2H, 2×CH_{Ar}), 7.87 (d, *J* = 8.6 Hz, 2H, 2×CH_{Ar}), 7.41 (d, *J* = 8.4 Hz, 2H, 2×CH_{Ar}), 7.30 (d, *J* = 8.6 Hz, 2H, 2×CH_{Ar}), 7.15 (d, *J* = 7.4 Hz, 1H, CONH), 4.65 (td, *J* = 7.8, 4.5 Hz, 1H, C*H*NH), 3.45 (s, 3H, CH_3), 2.49 – 2.15 (m, 3H, CH_2 + C*H*H), 2.10-1.98 (m, 1H, C*H*H), 1.48 (s, 9H, C(CH_3)$_3$), 1.40 (s, 9H, C(CH_3)$_3$). **^{13}C NMR** (75 MHz, $CDCl_3$) δ ppm: 172.9 (*C*OO^{t}Bu), 171.3 (*C*OO^{t}Bu), 166.2 (*C*ONH), 156.0 (C_{Ar}), 152.3 (C_{Ar}), 145.3 (C_{Ar}), 145.1 (C_{Ar}), 128.3 (*CH*$_{Ar}$), 126.2 (C_{Ar}), 125.2 (*CH*$_{Ar}$), 122.2 (*CH*$_{Ar}$), 115.8 (*CH*$_{Ar}$), 82.7 (*C*(CH$_3$)$_3$), 81.1 (*C*(CH$_3$)$_3$), 53.1 (*CH*NH), 31.7 (*C*H$_2$), 28.1 (C(*C*H$_3$)$_3$), 28.1 (C(*C*H$_3$)$_3$), 27.4 (*C*H$_2$). **MS ESI$^+$**: $[M+H]^+$ 558.2, $[M+Na]^+$ 580.2, $[2M+Na]^+$ 1137.5. **HRMS**: (ESI$^+$-MS, m/z) calcd for $C_{28}H_{35}N_3NaO_9$ $[M+Na]^+$: 580.2266, found: 580.2265.

Di-*tert*-butyl (4-((((2,4-diaminopteridin-6-yl)methoxy)carbonyl)(methyl)amino)benzoyl)-L-glutamate. 16.

[22] Peiro Cadahia, J.; Clausen, M. H.; Bondebjerg, J.; Hansen, C. A. *PCT Int. Appl. WO2018037120* (**2018**).

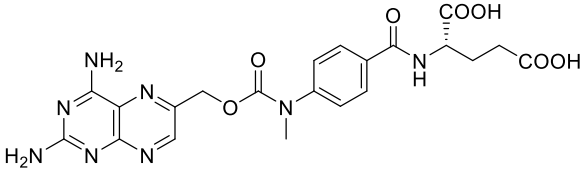
To a well stirred solution of 2,4-diamino-6-(hydroxymethyl)pteridine hydrochloride **15** (205 mg, 0.897 mmol, 1 eq.) in anhydrous Me_2NAc (8 mL) under Ar atmosphere at 0°C, NaH (60% mineral oil, 71 mg, 1.793 mmol, 2 eq.) was added in portions and the suspension was stirred for 20 minutes. Then, **14** (1 g, 1.793 mmol, 2 eq.) was added and the mixture was stirred in dark at rt for 3 days. Then, two more equivalents of NaH were added and the mixture was stirred in dark at rt for 1 more day. After that time, the product was precipitated by the addition of H_2O (40 mL). The precipitate was washed with H_2O (3 x 40 mL) and purified by column chromatography CH_2Cl_2/MeOH (100/0 to 95/5), to afford **16** (250 mg, 46%) as bright yellow solid.



^{1}H NMR (300 MHz, DMSO-d_6) δ ppm: 8.73 (s, 1H, CH_{Ar}), 8.59 (d, *J* = 7.6 Hz, 1H, CON*H*), 7.86 (d, *J* = 8.6 Hz, 2H, 2×CH_{Ar}), 7.65 (d, *J* = 27.4 Hz, 2H, NH_2), 7.48 (d, *J* = 8.6 Hz, 2H, 2×CH_{Ar}), 6.73 (bs, 2H, NH_2), 5.24 (s, 2H, OCH_2), 4.37-4.25 (m, 1H, NHC*H*), 3.30 (s, 3H, CH_3), 2.33 (t, *J* = 7.4 Hz, 2H, CH_2), 2.08-1.84 (m, 2H, CH_2), 1.40 (s, 9H, C(CH_3)$_3$), 1.37 (s, 9H, C(CH_3)$_3$). **^{13}C NMR** (75 MHz, DMSO-d_6) δ ppm: 171.6 (*C*OO^tBu), 171.1 (*C*OO^tBu), 166.1 (*C*OHN), 163.2 (C_{Ar}), 162.9 (C_{Ar}), 155.7, 154.2, 149.9 (*C*H$_{Ar}$), 145.6 (C_{Ar}), 143.1 (C_{Ar}), 131.0 (C_{Ar}), 128.1 (*C*H$_{Ar}$), 124.9 (*C*H$_{Ar}$), 121.5 (C_{Ar}), 80.7 (*C*(CH_3)$_3$), 79.8 (*C*(CH_3)$_3$), 66.1 (O*C*H$_2$), 52.5 (NH*C*H), 38.7 (*C*H$_3$), 31.4 (*C*H$_2$), 27.8 (C(*C*H$_3$)$_3$), 27.7 (C(*C*H$_3$)$_3$), 25.9 (*C*H$_2$). **MS ESI$^+$**: [M+H]$^+$ 611.3, [M+Na]$^+$ 633.3. **HRMS**: (ESI$^+$-MS, m/z) calcd for $C_{28}H_{35}N_3NaO_9$ [M+H]$^+$: 611.2936, found: 611.2937.

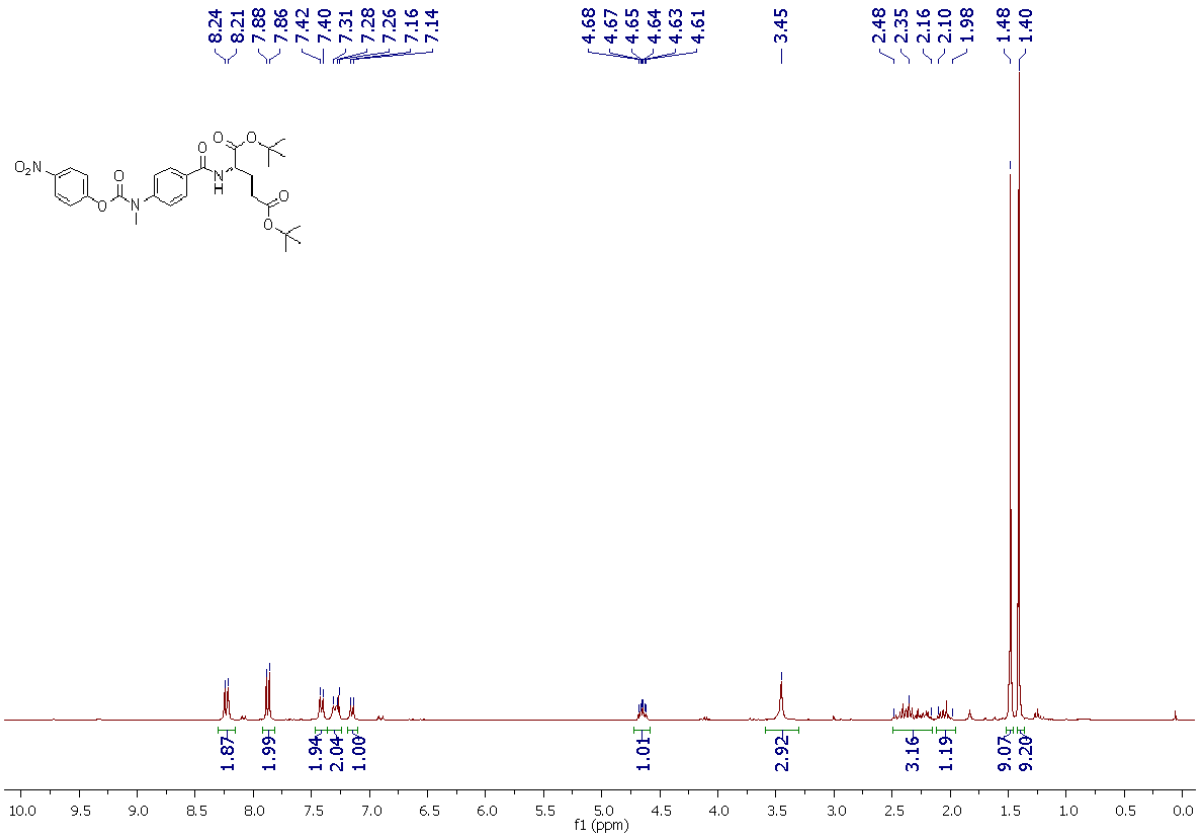
(4-((2,4-diaminopteridin-6-yl)methoxy)benzoyl)-L-glutamic acid. CarbMTX.

A well stirred solution of **16** (240 mg, 0.393 mmol, 1 eq.) in $CHCl_3$ (7 mL) under Ar atmosphere at 0°C was treated with TFA (5.5 mL, 71 mmol, 180 eq.) dropwise and the reaction mixture was stirred at rt for 16 h. Then, the solvent was removed under reduced pressure and the brown residue was triturated with Et_2O (5 mL) to provide a pale yellow solid. The crude product was washed with Et_2O (2 x 20 mL) and CH_2Cl_2/MeOH (95/5, 3 x 40 mL) to afford **CarbMTX** (189 mg, 96%) as bright yellow solid.

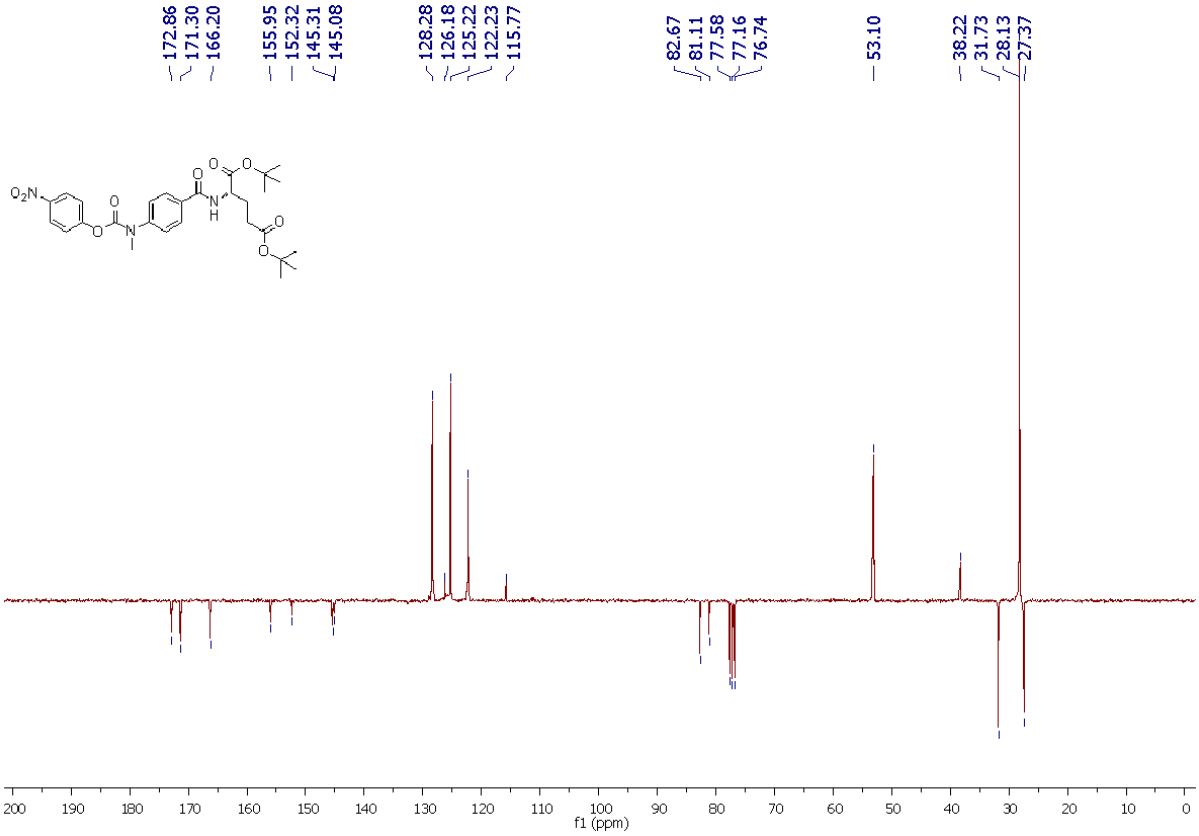


^{1}H NMR (300 MHz, DMSO-d_6) δ ppm: 8.79 (s, 1H, CH_{Ar}), 8.63 (dd, *J* = 7.7 Hz, 3.2 Hz, 2H, NH_2), 8.47 (dd, *J* = 24.0 Hz, 2H, NH_2), 7.87 (d, *J* = 8.6 Hz, 2H, 2×CH_{Ar}), 7.48 (d, *J* = 8.6 Hz, 2H, 2×CH_{Ar}), 5.28 (s, 2H, OCH_2), 4.39 (m, 1H, NHC*H*), 3.31 (s, 3H, CH_3), 2.35 (t, *J* = 7.4 Hz, 2H, CH_2), 2.15-2.03 (m, 1H, CH*H*), 2.01-1.89 (s, 1H, CH*H*). **^{13}C NMR** (75 MHz, DMSO-d_6) δ ppm: 173.8 (*C*OOH), 173.4 (*C*OOH), 165.9 (*C*OHN), 162.7 (C_{Ar}), 154.0 (C_{Ar}), 149.5 (*C*H$_{Ar}$), 145.6 (C_{Ar}), 128.0 (*C*H$_{Ar}$), 124.9 (*C*H$_{Ar}$), 121.8 (C_{Ar}), 65.7 (*C*H$_2$), 51.9 (NH*C*H), 37.2 (*C*H$_3$), 30.3 (*C*H$_2$), 25.8 (*C*H$_2$). **MS ESI$^+$**: [M+H]$^+$ 499.2, [M+Na]$^+$ 521.1. **HRMS**: (ESI$^+$-MS, m/z) calcd for $C_{21}H_{23}N_8O_7$ [M+H]$^+$: 499.1684, found: 499.1674.

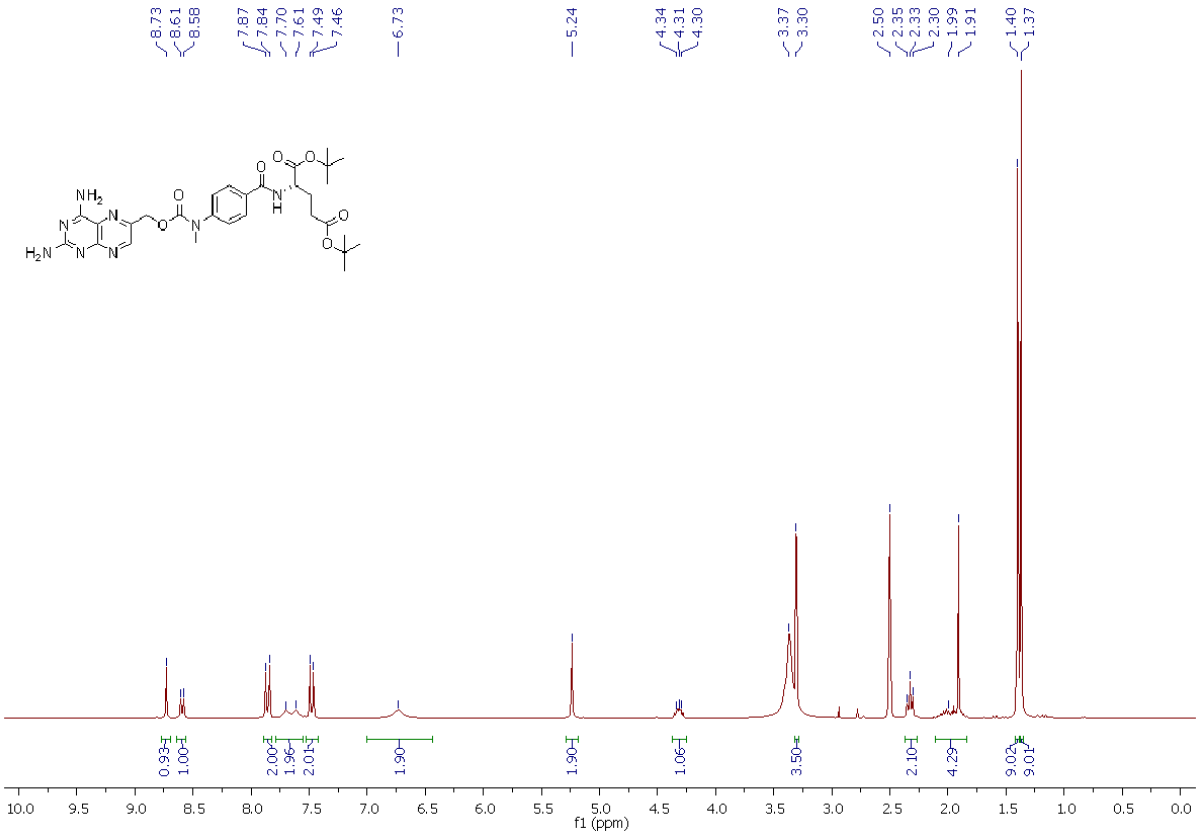
^{1}H NMR (300 MHz, $CDCl_3$) of compound 14


8.24
8.21
7.88
7.86
7.42
7.40
7.31
7.28
7.26
7.16
7.14
4.68
4.67
4.65
4.64
4.63
4.61
3.45
2.48
2.35
2.16
2.10
1.98
1.48
1.40
1.87
1.99
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3.16
1.19
9.07
9.20
f1 (ppm)

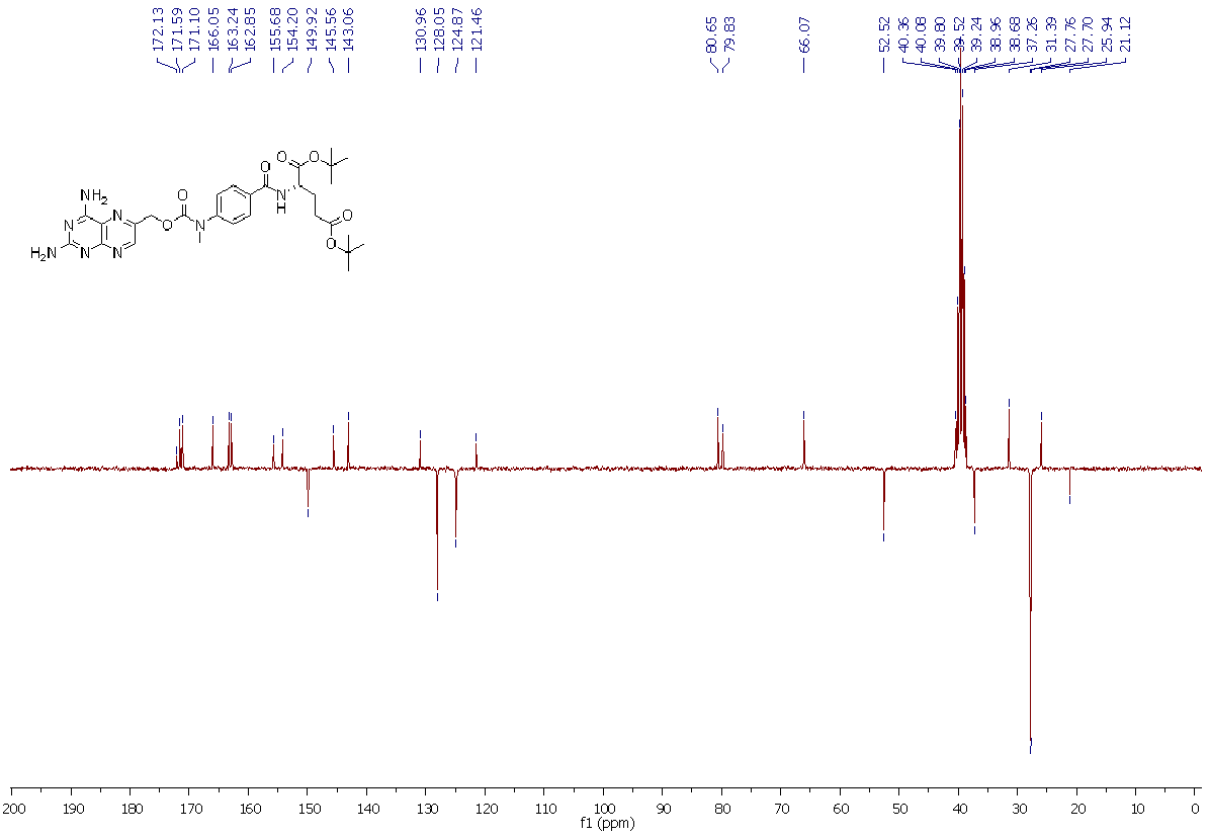
^{13}C NMR (75 MHz, $CDCl_3$) of compound 14



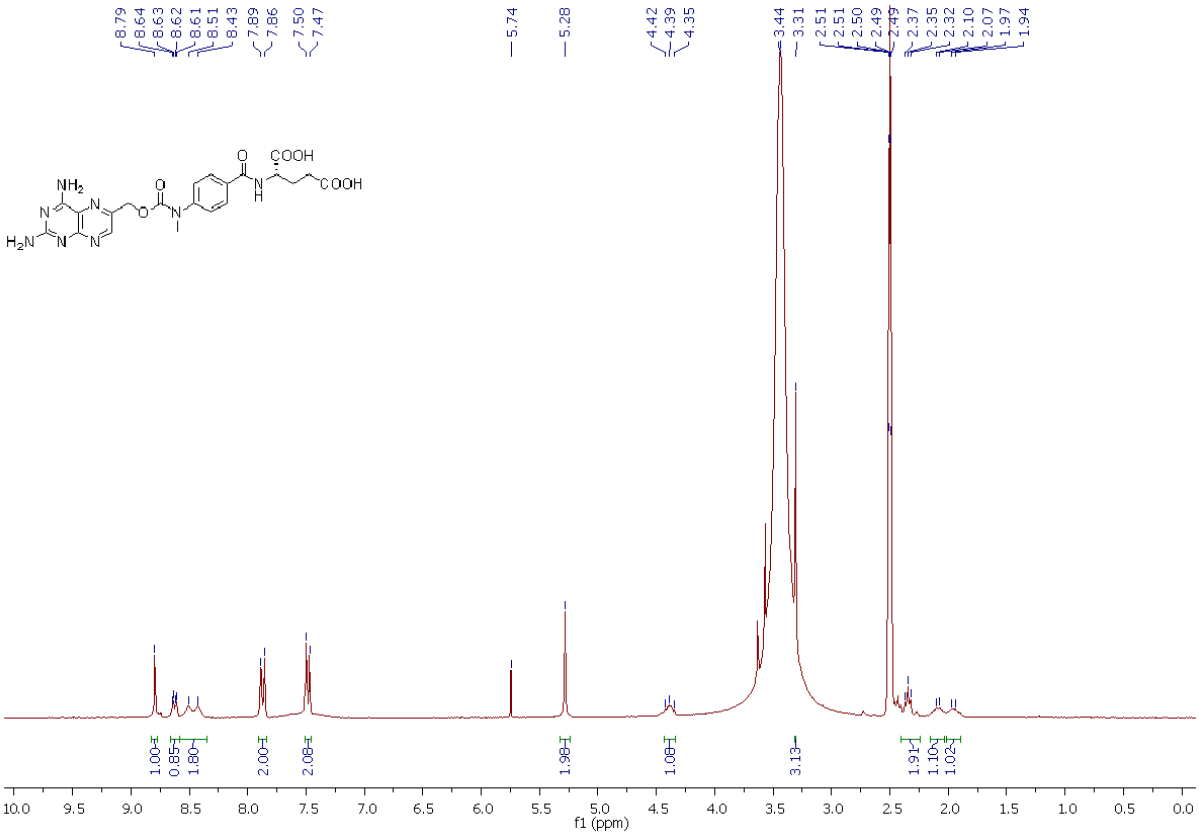
^{1}H NMR (300 MHz, DMSO-d_6) of compound 16



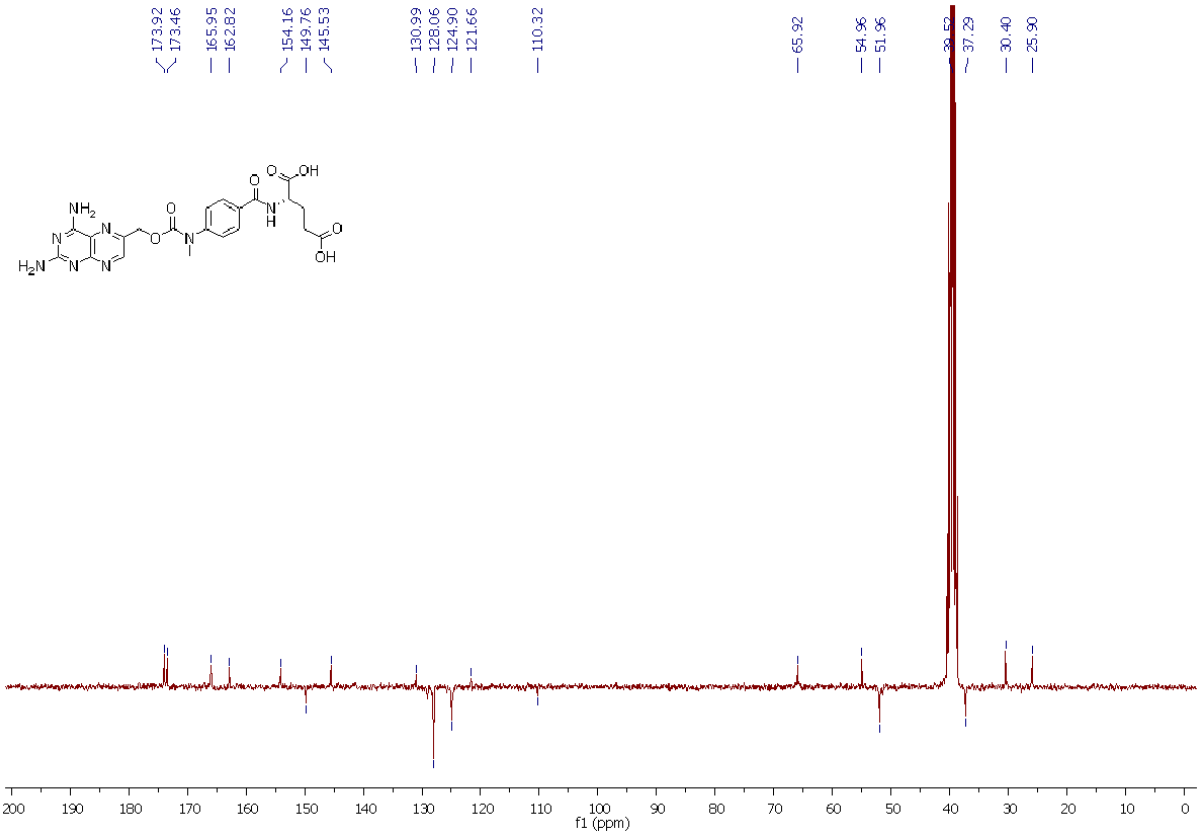
^{13}C NMR (75 MHz, DMSO-d_6) of compound 16



^{1}H NMR (300 MHz, DMSO-d_6) of CarbMTX



^{13}C NMR (75 MHz, DMSO-d_6) of CarbMTX



Title: Toward the development of sustainable pharmaceutical compounds: Characterizing the degradation and the ecotoxicological effects of an eco-designed anticancer drug analog

Keywords: Pharmaceutical, eco conception, water, photo- and biodegradation, structural identification, transformation products

Abstract:

Pharmaceuticals are increasingly reported to contaminate natural waters because of their partial elimination by wastewater treatment plant and can have an impact on aquatic organisms up to human health through potable water in the case of the most persistent ones. Additionally, their transformation products (TPs) are often unidentified and their impacts are insufficiently assessed.

To limit this contamination, one promising lever is the ecoconception, considering environmental fate and impact since the development of the molecule design. An eco-designed molecule would then be a molecule which degrades easily and which would produce harmless TPs once excreted in the environment.

The validity of this innovative approach was assessed using the cytotoxic methotrexate (MTX) as drug model, due to its potent action, its reported presence in wastewater effluent and its ability to produce a harmful metabolite (7-hydroxy-methotrexate), comparing to an analog (OMTX) synthesized with the hypothesis it will be less persistent and produce less toxic TPs.

This hypothesis was assessed through i) photodegradation studies where 11 TPs were evidenced for MTX and 12 for OMTX, revealing similar degradation pathways but faster degradation kinetics under 254 nm for OMTX; ii) a biodegradation study by an activated sludge where no major effects were observed on the microbial community. 8 additional TPs were evidenced for MTX, the harmful 7-hydroxy-mehtotrexate was found minor and transient, and 4 TPs for OMTX which was biodegraded faster; iii) cytotoxicity tests conducted during photo and biodegradation indicating TPs lost the original pharmaceutical action and finally iv) an acute aquatic ecotoxicological assessment in favor of OMTX.

Titre : Vers le développement de composés pharmaceutiques durables : Caractérisation de la dégradation et évaluation écotoxicologique d'un analogue d'anticancéreux eco-conçu

Mots-clés : Pharmaceutiques, éco conception, eau, photo- et biodégradation, identification structurale, produits de transformation

Résumé :

Les composés pharmaceutiques sont fréquemment détectés dans les eaux naturelles en raison de leur élimination partielle par les stations d'épuration. Ils peuvent avoir un impact sur les organismes aquatiques comme sur la santé humaine, en particulier les plus persistants dont des traces peuvent se retrouver dans l'eau potable. De plus, leurs produits de transformation (TPs) ne sont pas souvent identifiés, et leurs impacts rarement évalués.

Un levier prometteur est l'écoconception, prenant en compte le devenir et l'impact environnemental dès le développement des molécules pharmaceutiques. Une molécule éco-conçue serait alors une molécule qui se dégrade facilement et qui produirait des TPs inoffensifs une fois excrétés dans l'environnement.

Cette approche innovante a été évaluée en utilisant l'anticancéreux méthotrexate (MTX) comme modèle, en raison de son action cytotoxique, de sa présence dans les eaux usées et de l'apparition d'un métabolite nocif et persistant (le 7-hydroxy-methotrexate), par rapport à un analogue (OMTX) synthétisé selon l'hypothèse qu'il sera moins persistant et produira des composés moins toxiques.

Cette hypothèse a été testée via l'étude i) de la photodégradation avec la mise en évidence de 11 TPs du MTX et de 12 du OMTX, révélant des voies de dégradation similaires mais une dégradation plus rapide à 254 nm pour OMTX ; ii) de la biodégradation par une boue activée et l'impact en retour sur les communautés microbiennes ne montrant pas d'effet écotoxicologique majeur. 8 TPs additionnels ont été mis en évidence pour le MTX, le 7-OH-MTX est apparu mineur et transitoire, et 4 TPs pour l'OMTX, biodégradé plus rapidement ; iii) de la cytotoxicité au cours de la photo et de la biodégradation montrant une perte de l'action pharmaceutique d'origine et enfin iv) l'évaluation de l'écotoxicité aiguë sur des organismes aquatiques en faveur de l'OMTX.

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