**Cyclophosphamide and ifosfamide residues in aqueous environment: occurrence, treatment and toxicity**

Marjeta Česen1,2, Tina Kosjek1,2, Francesco Busetti3, Ester Heath1,2

1 Jožef Stefan Institute, Jamova cesta 39, 1000 Ljubljana, Slovenia

2 Jožef Stefan International Postgraduate School, Jamova cesta 39, 1000 Ljubljana, Slovenia

3Curtin University, Curtin Water Quality Research Centre (CWQRC), GPO Box U1987, Perth, WA 6845, Australia

Cyclophosphamide (CP) and ifosfamide (IF), although extensively used to successfully treat various cancers, are potentially cytotoxic, genotoxic, mutagenic and teratogenic and once in the aqueous environment could affect living organisms. The objectives of this study were to develop a method for the trace analysis of CP, IF and their selected metabolites, namely keto-cyclophosphamide (keto-CP), N-dechloroethyl-cyclophosphamide (N-decl-CP) and carboxy-cyclophosphamide (carboxy-CP) and evaluate their occurrence in Slovene wastewaters (at hospitals with oncological patients and corresponding wastewater treatment plants, WWTPs). Furthermore, the removal and transformation of parent compounds was studied using advanced treatments. Finally, the toxicity of the investigated compounds towards phytoplankton species was assessed with respect to OECD TG 201 guidelines.

Extraction of CP, IF and keto-CP from wastewater was performed using HLB Oasis (60 mg, 3 cc) cartridges, whereas for N-decl-CP and carboxy-CP ENV+ (100 mg, 3cc) cartridges were used. Optimal derivatization of the metabolites was achieved by N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide containing 1% tert-butyldimethylchlorosilane, while for CP and IF trifluoroacetic anhydride was used. Samples were analysed by gas chromatography-mass spectrometry.

Cyclophosphamide was detected in hospital wastewaters (14 - 22,000 ng L-1), WWTP influents (19 - 27 ng L-1) and effluent (17 ng L-1). IF was detected only in hospital wastewaters (48 - 6,800 ng L-1). **The detected concentrations of metabolites carboxy-CP, N-decl-CP and keto-CP were up to 13,202 ng L-1, 2,099 ng L-1 and 178 ng L-1 in hospital wastewaters, respectively, but below the LODs in WWTP influents and effluents.**

The average CP and IF removal efficiencies in spiked artificial wastewater (CP and IF: 10 µg L-1) used for biological treatment were 41% (CP) and 18% (IF). For hospital wastewater (CP and IF: 5 - 7 µg L-1), the average removal efficiencies were slightly higher (59% and 35% for CP and IF, respectively). An optimised abiotic treatment (UV/O3/H2O2 at 5 g L-1) gave the highest removal efficiencies, i.e. 98.8% and 94.0% for CP and IF, respectively. When hospital wastewater was used, the removal of CP and IF was 98.4% and 97.9%, respectively. Coupling biological treatment to UV/O3/H2O2 at 5 g L-1 removed over 99% of both compounds. **The formation of TPs under abiotic treatments, i.e. UV with and without H2O2 in miliQ revealed 4 TPs of CP and 3 TPs of IF, formed during UV and UV/H2O2 experiments. One novel TP (imino-ifosfamide), formed from IF, was identified in a sample treated with UV/H2O2.**

Toxicity evaluation with alga P*seudokirchneriella subcapitata* and cyanobacterium *Synechococcus leopoliensis* revealed NOECvalues of CP and IF towards both species >320 mg L-1. Further, we tested the toxicity of the metabolites towards *S. leopoliensis*. Results revealed NOEC values of N-decl-CP and keto-CP >320 mg L-1, whereas EC50for carboxy-CP was 21.1 mg L-1. The experimentally determined EC50 (11.5 mg L-1) value of a mixture of CP, IF, N-decl-CP, keto-CP and carboxy-CP was lower than predicted by the concentration addition model (21.1 mg L-1) suggesting a potentiating effect of a mixture compared to that of the compounds individually.

This is to our knowledge the first study indicating high potential of selected abiotic treatment to remove selected cytostatic residues albeit the high removal efficiencies for CP and IF (>99%) were achieved under exaggerated conditions. In addition, this is the first report addressing the ecotoxicity of CP, IF and their metabolites as single compounds and as a cocktail.

**ACKNOWLEDGEMENT:** This work was financially supported by the EU through the EU FP7 project CytoThreat (Fate and effects of cytostatic pharmaceuticals in the environment and the identification of biomarkers for an improved risk assessment on environmental exposure, grant agreement No.: 265264) and by the Slovenian Research Agency (Program groups P1-0143, Project L1-5457 and J1-6744 and Young researcher grant to M. Č.).