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ORIGINAL PAPER

Degradation and toxicity of mitoxantrone and chlorambucil in water

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Abstract The combination of liquid chromatography coupled to mass spectrometry (LC–MS) and acute toxicity studies with *Daphnia magna* was used to elucidate the water stability of the cytostatic compounds, mitoxantrone and chlorambucil, and their transformation products (TPs). Both compounds were rapidly degraded in water with the subsequent formation of bioactive TPs. Mitoxantrone suffered a rapid change in its conformation with the formation of four toxic TPs, which were unaltered and stable in water along the 2-day studied period. LC–MS analyses were allowed to identify the conformational changes of mitoxantrone that included the loss of the two amino alcohols (*N*-ethylethanolamina) $[C_4H_{10}NO]^+$, the loss of *N*-ethylethanolamina $[C_4H_{10}NO]^+$ and *N*-methylethanolamina $[C_3H_8NO]^+$, the loss of CH_2OH from the original molecule and the formation of mitoxantrone dicarboxilic acid. Chlorambucil was also rapidly degraded in water loosing a hydroxyl group and forming a bioactive TP that further degraded within the following 12 h. The degradation of chlorambucil was also related to an exponential loss of toxic activity towards *D. magna* survival. The present results indicate that LC–MS methods should target TPs since field concentrations of these compounds measured in water may not reflect their toxicity.

Keywords Mitoxantrone · Chlorambucil · Degradation · Toxicity · Water

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Introduction

Risk assessment of xenobiotics is conventionally based on toxicity responses resulting from test animals exposed to them. It often assumes that the toxicity of a compound increases with dose and exposure time, but it seldom considers that dose and exposure period may act independently. Accordingly, when monitoring the risk of pollutants present in a particular environment, it is recommended to relate measured concentration levels to toxicity (van Leeuwen and Hermens 1995). Nevertheless, in many cases, it is difficult to associate exposure doses with toxicity, for example when considering pulse exposures (Ashauer et al. 2007). There is also the case of xenobiotics having delayed toxicity, which means that they impair organisms live processes long after pulse exposures (Reynaldi and Liess 2005; Schulz and Liess 2000). Finally, many xenobiotics degrade fast in the environment, but their transformation products (TPs) are equally or even more toxic than the parental compounds (Chambers et al. 1989). Exceptions to the classical rule are going to increase in the near future when assessing environmental hazards of labile emerging pollutants.

Cytostatic compounds are an emerging group of environmental contaminants whose presence, effects, and risk are highly unknown. Cytostatic compounds are a heterogeneous group of pharmaceuticals used to treat cancer and other diseases that act inhibiting cell division and hence the development of tumours (Villasana et al. 2010). In this study, mitoxantrone and chlorambucil have been studied and represent two drugs synthesized in the 1970–1980 s that are still used today worldwide. Mitoxantrone belongs to the series of anthraquinones and stops tumour growth by cross-linking guanine bases in DNA double-helix strands—directly attacking DNA (Kizek et al. 2012). Mitoxantrone

is used in metastatic breast and ovarian cancer, acute myeloid and lymphoblastic leukaemia and non-Hodgkin's lymphoma and has a potent antiviral, antibacterial, immunomodulatory and antitumour activities (Zee-Cheng and Cheng 1978). Chlorambucil is an antineoplastic agent used to treat lymphocyte leukaemia that belongs to the group of alkylating agents (Rai et al. 2000). Mitoxantrone is excreted in 6–11 % by urine and in 25 % by faeces and the rest in metabolized form (BDI Pharma 2012). Like other pharmaceuticals, both parental products or metabolites are excreted and discharged to the sewage works via urban or hospital effluents (Gómez-Canela et al. 2012) and reach waste water treatment plant (WWTP), where cytostatic can undergo treatment and be released to surface waters by the effluents (Kosjek and Heath 2011; Llewellyn et al. 2011; Negreira et al. 2013a, b). Due to their unspecific toxicity, cytostatics may affect aquatic organisms (Buerge et al. 2006). So far, little is known on the toxicity and stability of these compounds in water. This is important for both analytical and toxicological evaluation because a risk analysis cannot be performed for this emerging family of contaminants.

HPLC methods were developed in the 1980 and 1990 s to study the toxicokinetics of cytostatic compounds in plasma (Payet et al. 1988). In more recent studies, HPLC methods coupled to mass spectrometry have been developed (Ferrando-Climent et al. 2013; Kosjek et al. 2013; Kovalova et al. 2012; Negreira et al. 2013a, b; Nussbaumer et al. 2011), although in most cases, they refer only to the identification of the parental compound. The degradation or metabolization of a particular chemical with biological activity not necessarily means that such metabolite losses its activity. For example, many organophosphorous pesticides need to be oxidized to their oxon metabolites to be active (Lacorte et al. 1997). One way to study the stability and biological activity of a compound is to combine mass spectrometry analytical methods with toxicity assays performed across different exposure periods. Among the available toxicity methods, the acute toxicity test of *Daphnia magna* (*D. magna*) is one of the most widely used assays in aquatic toxicology due to its reliability and sensitivity (OECD 2004).

The aim of this study was to determine the stability and related biological activity of mitoxantrone and chlorambucil in water. Liquid chromatography coupled to mass spectrometry in scan mode provided precise information on the structural changes of both chemicals during incubation experiments, whereas toxicity assays provided information on the biological activity of active TPs of such drugs. The combined approach serves as a proxy for other chemicals, directed to define the environmental behaviour and risk of emerging pollutants, considering also their TPs.

Materials and methods

Chemicals and reagents

Pure reference compounds mitoxantrone ($C_{22}H_{28}N_4O_6$) and chlorambucil ($C_{14}H_{19}NO_2Cl_2$) of 98 % purity were purchased from Sigma-Aldrich (St. Louis, USA). Mitoxantrone has a pKa of 11.44 and water solubility range of 5–10 mg L^{-1} (25 °C). Chlorambucil has a pKa of 5.75 and a high solubility with a value of 1.24e4 mg L^{-1} (25 °C). Stock standard solutions were prepared at a concentration of 1,000 ng μL^{-1} in methanol for mitoxantrone and in acetone for chlorambucil, and working solutions at 100 ng μL^{-1}. Reconstituted hard water was prepared according to American Society for Testing Materials (ASTM water). Methanol, acetonitrile (SupraSolv grade) and HPLC water (LiChrosolv grade) were supplied by Merck (Darmstadt, Germany). Ammonium formate ≥99 % was purchased from Sigma-Aldrich (St. Louis, USA), and formic acid was supplied by Merck (Darmstadt, Germany).

Stability in water

Mitoxantrone and chlorambucil were spiked in ASTM hard water at 2 and 10 ng μL^{-1} and were kept in the dark at ambient temperature. Aliquots were analysed at time 0, 24 and 48 h. Analyses were carried out by direct sample injection using an AcQuity ultra-performance liquid chromatography (UPLC) system equipped with a quaternary pump and connected to a triple quadrupole MS/MS system (Waters, USA). Data were acquired and processed using MassLynx 4.1 software package. A ZORBAX Eclipse XDB-C18 Narrow-Bore column 2.1 × 150 mm, 5 µm from Agilent Technologies (Santa Clara, USA), was used. The mobile phase consisted of solvent A 5 mM ammonium formate in water and solvent B 5 mM ammonium formate in methanol. The initial mobile phase composition was 70 % A and 30 % B (5 min), to 70 % B in 3 min and held for 10 min, and to 100 % B in 3 min. These conditions were held for 4 min, and then, the initial conditions were regained in 5 min with an equilibration time of 2 min. The flow rate was set at 0.3 mL min^{-1}. To optimize ionization and to establish mass spectral features, individual mitoxantrone and chlorambucil were first analysed by flow injection analysis (FIA) in UPLC–MS in positive ESI mode. Full-scan data acquisition was performed scanning from *m/z* 100 to 600. A profile mode was used to determine fragmentation patterns and sensitivity, using a scan time of 2 s with a step size of 0.1 u and a pause between each scan of 2 ms. Optimized parameters were source temperature (from 125 to 150 °C), cone voltage (from 5 to 50 V) and collision energy (from 5 to 50 eV). Other parameters used are shown in Table 1.

Table 1 HPLC–MS/MS chromatographic and mass spectrometric conditions used to determine the wto studied cytostatic agents

	Mitoxantrone/chlorambucil
Column	ZORBAX Eclipse XDB-C18 Narrow-Bore 2.1 × 150 mm, 5 µm
Chromatographic parameters	
Injection volume	10 µL
Flow rate	300 µL min^{-1}
Mobile phase A	H_2O with 5 mM NH_4COOH
Mobile phase B	MeOH with 5 mM NH_4COOH
Acquisition time	30 min
Source parameters	
Scan range	*m/z* 100–600
Capillary	3.5 kV
Extractor	3 V
Polarity	Positive (ESI+)
Source Temperature	150 °C
Desolvation Temperature	350 °C
Cone gas flow	1 L h^{-1}
Desolvation gas flow	550 L h^{-1}
Collision gas flow	0.19 mL min^{-1}
Analyser parameters	
LM1 resolution	15.00
HM1 resolution	15.00
Ion energy 1	0.50
MS mode entrance	10.00
MS mode collision energy	3.00
MS mode exit	10.00
LM2 resolution	15.00
HM2 resolution	15.00
Ion energy 1	0.50
MS mode entrance	1.00
MS mode collision energy	20.00
MS mode exit	0.50
Gain	1.00
Multiplier	−651.31

Toxicity tests using *D. magna*

Acute toxicity of the studied compounds to *D. magna* was conducted using two approaches. Firstly, a conventional acute assay was used where animals were exposed to freshly prepared solutions, and their survival was monitored at 24, 48, 72 and 96 h. Secondly, animals were exposed to freshly prepared (time 0) and aged (2, 4, 8 and 24 h old) test concentrations of mitoxantrone and chlorambucil, and their survival was monitored at 48 h. In the first case, the inclusion of different exposure periods allowed to assess time-dependant toxicity, which is always desirable when extrapolating short laboratory exposures to long-term field exposures (Barata et al. 1999). In the second case, performing acute test with freshly prepared and aged water will allow evaluating the bioactivity of TPs. Test was conducted following standardized protocols (OECD 1981). Mitoxantrone concentrations ranging from 0.1 to 40 ng μL^{-1} were prepared directly in ASTM water. Chlorambucil stock solutions ranging from 7 to 300 ng μL^{-1} were prepared in ASTM water using acetone as a carrier (1 mL L^{-1}). Ageing of water was conducted in the laboratory in ASTM at 20 °C in the darkness to prevent photolysis, if any. Assays were conducted in 50 mL of test medium with 10 animals, per triplicate, and were started at <24-h old neonates and ended at 48 h. Measured endpoint was immobility. Lethal median concentration effects and its 95 % CI were estimated fitting immobility concentration responses to the Hill regression model (Eq. 1) using least-square regression methods (Thienpont et al. 2013).

$$I(c_i) = \frac{1}{1 + \left(\frac{C_i}{LC_{50}}\right)^{-\text{Hill}}} \quad (1)$$

where $I(c_i)$ is proportion of immobile animals at concentration c_i, c_i concentration of compound (i), LC_{50} median lethal concentration and Hill shape constant.

Results and discussion

Stability tests

The stability of mitoxantrone and chlorambucil in water was first assessed during a period of 48 h, to determine hydrolysis and eventual formation of TPs. LC–MS in scan acquisition mode was used (Figs. 1, 2). Mitoxantrone was analysed at a cone voltage of 42 V that yielded the major intensity of the protonated molecule at *m/z* 445 $[M + H]^+$ and yielded fragments ions used for identification purposes. This fragmentation pattern was also observed by Zhang et al. (2010) who analysed mitoxantrone in rat plasma with HPLC–MS/MS, and the molecular ion at *m/z* 445 and one fragment ion at *m/z* 88 $[C_4H_{10}NO]^+$ were identified at 40 V of cone voltage. For chlorambucil, the optimum cone voltage was 30 V, which produced the molecular ion at *m/z* 304 $[M + H]^+$. Davies et al. (1999) report the same molecular ion and a fragment ion at *m/z* 192 with HPLC–MS/MS.

Rapid degradation of mitoxantrone occurred in water, and this compound disappeared completely at time 0 (t_0). Figure 1 shows the LC–MS/MS chromatogram in scan

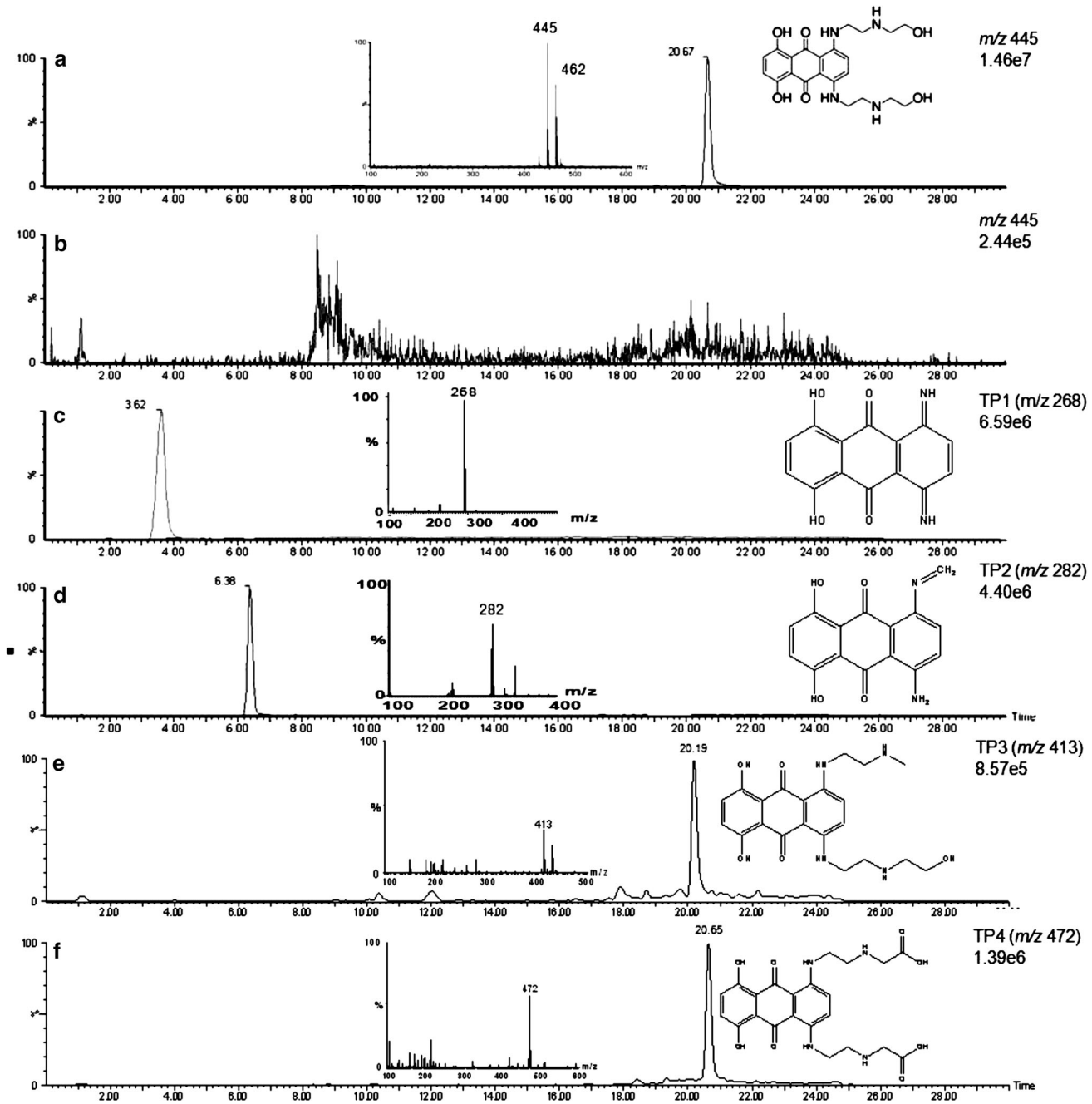

Fig. 1 LC–MS chromatogram and mass spectra of a standard of mitoxantrone at 10 ng μL^{-1} (**a**); chromatographic profile of mitoxantrone (protonated molecule) in a spiked water at t_0 (**b**); mitoxantrone transformation product 1 (*m/z* = 268) at t_0 (**c**); mitoxantrone transformation product 2 (*m/z* = 282) at t_0 (**d**); mitoxantrone transformation product 3 (*m/z* = 413) at t_0 (**e**); and mitoxantrone transformation product 4 (*m/z* = 472) at t_0 (**f**)

mode where the trace of mitoxantrone at 20.67 min is not present. At t_0, 4 TPs were already produced, which were identified. TP1 at *m/z* 268 was identified as 1,4-dihydroxy-5,8-diiminoanthracene-9,10(5*H*,8*H*)-dione and corresponds to the loss of the two amino alcohols (*N*-ethylethanolamina) $[C_4H_{10}NO]^+$ from the original molecule. This breaking of the aliphatic chains produces a relocation of the non-bonded electron pair of nitrogen, which makes an electronic rotation to get the stability of the structure (Fig. 2c). This effect can be observed in mitoxantrone's preparation by Murdock and Durr in (1986) and was reported recently by De Leoz et al. in a modified preparation of this drug (De Leoz et al. 2006; Murdock and Durr 1986). TP2 was identified at *m/z* 282 as 1,4-dihydroxy-5-imino-8-(methyleneamino)anthracene-9,10(5*H*,8*H*)-dione and corresponds to the loss of *N*-ethylethanolamina $[C_4H_{10}NO]^+$ and *N*-methylethanolamina

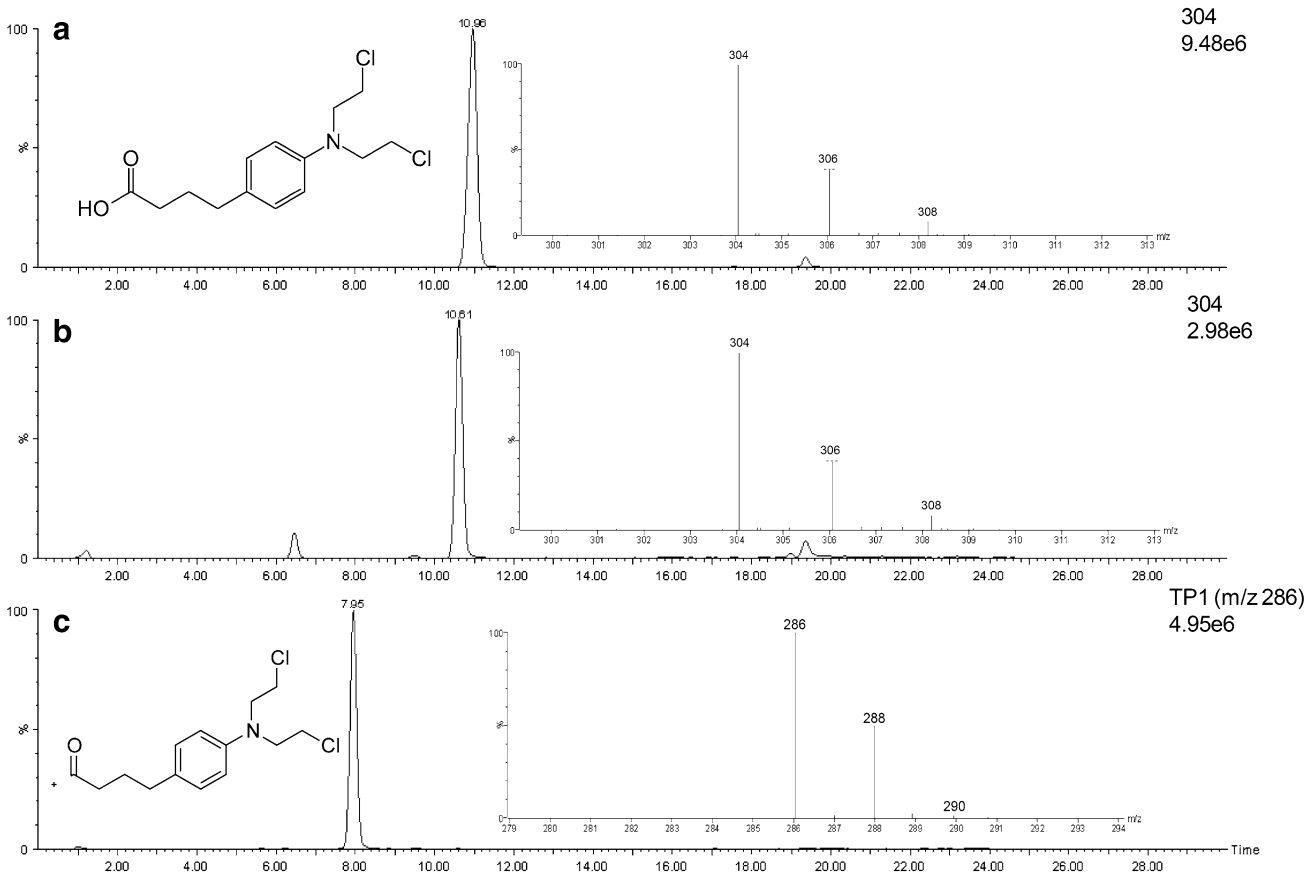

Fig. 2 LC–MS chromatogram and mass spectra of a standard of chlorambucil at 10 ng μL^{-1} (**a**); chlorambucil (protonated molecule) in a spiked water sample at time 0 (t_0) (**b**); and chlorambucil transformation product 1 (*m/z* = 286) in a spiked water sample at t_0 (**c**)

$[C_3H_8NO]^+$ (Fig. 2d). TP3 was identified at *m/z* 413 and corresponds to the loss of CH_2OH from the original structure, and TP4 identified at *m/z* 472 corresponds to mitoxantrone dicarboxilic acid [14]. These 4 TPs were stable along the 48 h of the experiment, as shown in Fig. 3. The same degradation behaviour was observed at 10 and 2 ng μL^{-1} spiking level. Considering a total decomposition of mitoxantrone to the 4 TPs, at 10 ng μL^{-1} and t_0, TP1 was detected with 61.9 % of the total initial mitoxantrone concentration (6.19 ng μL^{-1}) and remained constant throughout the time until (t_{40}); TP2 was detected with 23.4 % of the initial mitoxantrone concentration (2.34 ng μL^{-1}); TP3 was detected at 0.58 ng μL^{-1} and TP4 at 0.88 ng μL^{-1}, and their concentration remained constant throughout time (Fig. 3). A similar pattern was observed at 2 ng μL^{-1} spiking level. At t_0, TP1 was detected with 64 % of the initial concentration (1.28 ng μL^{-1}), TP2 was detected with 3 % of the initial concentration (0.06 ng μL^{-1}), TP3 at 0.23 ng μL^{-1} and TP4 at 0.42 ng μL^{-1}, and all remained constant up to t_{40} (Fig. 3).

There are no studies that report the stability of mitoxantrone in water, but according to its basic pKa of 11.44 will preclude its dissociation in surface waters that often have pHs 7–8. Nevertheless, previous studies reported that mitoxantrone is stable in human plasma (Ehninger et al. 1985a) and urine (Payet et al. 1988). Ehninger et al. (1985a) demonstrated that the degradation of mitoxantrone does not involve a loss of activity, indicating that mitoxantrone metabolites are the active ingredient of the drug. The same authors estimated a terminal half-life of 214.8 h (approximately 9 days), monitored by HPLC with UV detection at 658 nm where the blue colour of the 2 identified metabolites was attributed to the presence of the anthracenedione ring system (Ehninger et al. 1985b). Payet et al. (1988) identified mitoxantrone and two mono- and dicarboxylic metabolites in human plasma and urine samples using HPLC methods. In the previous study, a half-live of 24 h was estimated for mitoxantrone.

Chlorambucil has also a pKa far below (5.75) the normal pH of surface waters, and accordingly to (Kosjek and Heath 2011), it should also be dissociated in ASTM hard water. Figure 2 shows the scan chromatograms for a standard solution and the spiked water where chlorambucil and a TP1 product are identified. This TP1 was identified at *m/z* 286 $[C_{14}H_{18}NOCl_2]^+$, corresponding to the loss of the hydroxyl group (Fig. 2). The chlorine

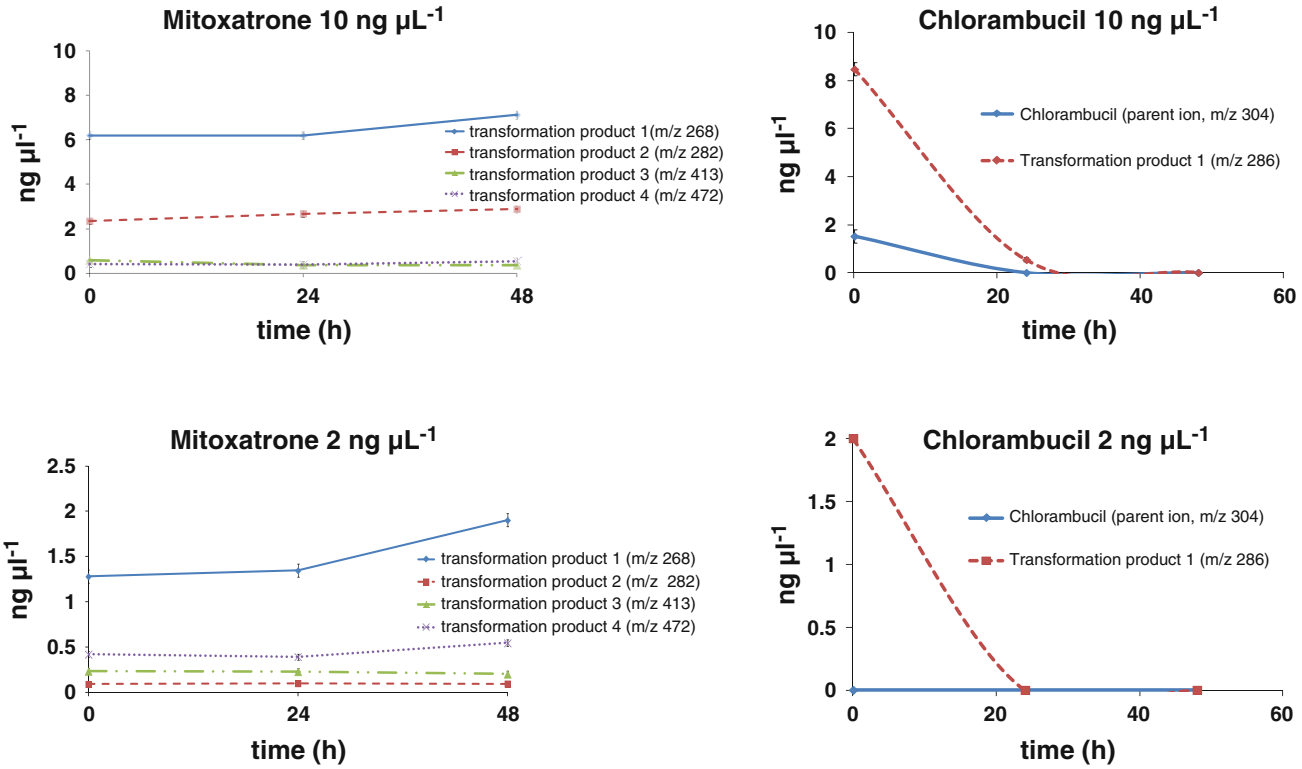

Fig. 3 Stability of target compounds in water at 2 and 10 ng μL^{-1}. *Error bars* are SE ($N = 3$). In some cases, *bars* were smaller than symbol size

isotopic pattern was observed at *m/z* 286/288/290. At t_0 and at concentration of 10 ng μL^{-1}, chlorambucil residue levels in water, monitored at *m/z* 304, decreased by 81 % of the initial (1.52 ng μL^{-1}) and degraded completely after 24 h. Assuming a total transformation of chlorambucil to TP1, its concentration was estimated to be 8 ng μL^{-1} at t_0 and decreased in time being completely degraded at 24 h (Fig. 3). At t_0 and at concentration of 2 ng μL^{-1}, the parent ion of chlorambucil was not detected while TP1 was identified, but its residue levels in water also decreased in time being undetected after 24 h (Fig. 3). Our results agree with those of Löf et al. (1997) that using HPLC coupled to UV detector reported that chlorambucil half-life in unbuffered water was approximately 30 min. Recently, Negreira et al. (2013a, b) determined the stability of 24 cytostatic compounds and metabolites in HPLC water at different temperatures, and after 24 h, chlorambucil was degraded by 80 % at 4 °C and completely degraded at 15 and 25 °C.

Toxicological results

Toxicity response of the studied compounds varied differently with exposure period (Table 2). Mitoxantrone toxicity increases exponentially with exposure time from 24 to 96 h being its LC_{50} at 96-h 29-fold more toxic than at 24 h (Table 2). Conversely, chlorambucil toxicity increased only slightly from 24 to 48 h being its LC_{50} at 48 or 96 h only 1.4 times more toxic than at 24 h (Table 2).

In the aged water experiment, toxicity of mitoxantrone increased slightly from freshly prepared to 2-h aged water and remained constant until 48 h (Table 3). On the

Table 2 Estimated lethal concentration effects (ng μL^{-1}) and 95 % CI of *D. magna* juveniles exposed to the test solutions during 24, 48, 72 and 96 h

Exposure period (h)	LC_{50} (ng μL^{-1})	95 % CI		N	r^2
Mitoxantrone					
24	20.3	16.9	23.7	24	0.85
48	5.2	4.3	6.1	24	0.94
72	1.4	1.1	1.6	24	0.99
96	0.7	0.5	0.8	28	0.97
Chlorambucil					
24	30.6	28.6	32.7	50	0.87
48	23.2	22.7	23.8	50	0.93
72	23.2	22.3	23.7	50	0.91
96	22.1	21.0	23.1	50	0.90

Estimates were obtained fitting mortality to the Hill regression curve. Sample site (N) and coefficients of determination (r^2) are also depicted. All regressions were significant at $P < 0.01$

Table 3 Estimated lethal concentration effects (ng μL^{1}) and 95 % CI of *D. magna* juveniles exposed to aged water of the tested solutions

Aged test water	LC_{50} (ng μL^{-1})	95 % CI		*N*	r^2
Mitoxantrone					
0	7.2	6.4	8.2	10	0.96
3	5.8	4.8	6.8	10	0.93
4	5.6	5.2	6.2	10	0.99
8	5.5	5.0	6.3	10	0.98
24	5.4	4.8	6.2	10	0.97
48	4.9	4.3	5.5	10	0.97
Chlorambucil					
0	23.9	19.4	28.4	10	0.95
3	40.8	27.5	54.1	10	0.81
4	131.3	84.1	178.5	10	0.92
8	354	158.4	549.6	10	0.91

Estimates were obtained fitting mortality to the Hill regression curve. Sample site (*N*) and coefficients of determination (r^2) are also depicted. All regressions were significant at $P < 0.01$

contrary, the toxicity of chlorambucil decreased exponentially with time (Table 3) being not toxic at all above initial nominal concentrations of 300 ng μL^{-1} in aged water more than 8 h. Comparison of toxicity with stability responses evidenced the following relationship. The concentration of the 4 TPs of mitoxantrone accounted for up to 100 % of the total mitoxantrone residues, and they were stable during 48 h. The same behaviour was observed for mitoxantrone toxicity in the aged water experiment; thus, mitoxantrone TPs rather than the parental compound are the active toxic compounds. There are no studies that have assessed the toxicity of mitoxantrone TPs in aquatic organisms (McGrath and Li 2008); thus, our data provide the first evidence that mitoxantrone per se is not stable in water, but its TPs are stable and toxic to *D. magna*. For chlorambucil, the concentration of TP1 accounted for more than 80 % of the total residues in freshly prepared solutions, but decreased exponentially being at 24-h 16-fold lower than initial concentrations. A similar behaviour was observed in the aged water toxicity experiment where toxicity of chlorambucil decreased exponentially being in aged water of 8-h 15-fold lower than initial values (Table 3). Therefore, TP1 of chlorambucil is likely to be the active metabolite. Unfortunately, there is no reported toxicological information of chlorambucil to aquatic organism, but environmental fate studies indicate that many pharmaceuticals degraded rapidly and can increase the formation of their TPs (Kovalova et al. 2012).

Time-dependant toxicity of mitoxantrone and chlorambucil supports their chemical instability as follows: Mitoxantrone's TP1 to TP4 were stable in water and toxic; thus, toxicity of this compound increased in an expected way with time as more transformation bioactive products will be taken up by *D. magna* juveniles and hence will be damaging their target sites. Conversely, chlorambucil TP was not stable in water, and it almost completely degraded at 24 h; consequently, toxicity of this compound did not increase with exposure time and decreased exponentially in aged water. Interestingly, chlorambucil LC_{50} at 48 h in Table 2 were similar to that of Table 3 in freshly prepared solutions (aged test water 0 h), but twofold lower than that obtained in aged water just 3 h. This means that short-term pulse exposures (<3 h) of chlorambucil were long enough to impair survival in *D. magna* at 48 h. A similar behaviour was reported for pyrethroid insecticides that showed delayed acute and chronic toxicity to aquatic invertebrate species after short pulses of just a few hours (Medina et al. 2004a, b; Reynaldi and Liess 2005; Schulz and Liess 2000).

Conclusion

This study has demonstrated that mitoxantrone and chlorambucil, two cytostatic compounds highly used to treat cancer, are labile in water, but do not necessarily lose toxicity since they are degraded to bioactive TPs. These findings have environmental implications, especially when undertaking monitoring studies, as follows: (1) the parental compound can be rapidly degraded in water, thus not being detected with LC–MS methods, especially if working with selected ion or reaction monitoring; and (2) the toxicity of mitoxantrone and chlorambucil is related to the TPs, which can be more stable in water than the parent compound. Therefore, sensitive methods to identify both parental compounds and TPs should be developed, focused on quadrupole with scan acquisition mode, time of flight or Orbitrap MS. In this study, we have contributed to define, in laboratory-controlled conditions, the stability and toxicity of two compounds in an attempt to define the aquatic behaviour of cytostatic contaminants. The results obtained herein can be a proxy for the analysis of other cytostatic compounds which can reach surface waters through urban or sewage treatment plant discharges.

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