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Ecotoxicological evaluation of caffeine and its derivatives from a simulated chlorination step

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HIGHLIGHTS

- Processes of chlorination in the treatment of raw water
- STP chlorination of caffeine
- 8-Chlorocaffeine, the most toxic compound in the long term on rotifers
- *N,N′*-dimethylurea toxic to algae
- Antigenotoxicity of caffeine and two derivatives

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ABSTRACT

Caffeine is ubiquitous in surface and ground waters and it has been proposed as a marker of the anthropogenic pressure on the environment. Sewage treatment plants based on active sludges seem to be not very efficient in its complete removal from effluents while additional disinfection treatments by chlorination are able to do it. In a simulation of the chlorination step herein we report that caffeine is transformed in six by-products: 8-chlorocaffeine, 1,3-dimethyl-5-azabarbituric acid, *N,N′*-dimethylparabanic acid, *N,N′*-dimethyloxalamide, *N*-methylurea and *N,N′*-dimethylurea. The ecotoxicity of caffeine and identified compounds was evaluated on the rotifer *Brachionus calyciflorus* and the alga *Pseudokirchneriella subcapitata* to assess acute and chronic toxicity, while SOS Chromotest and Ames Test were used to detect the genotoxic potential of the investigated compounds. Moreover, we assessed the possible antigenotoxic effect of the selected compounds using SOS Chromotest after co-incubation with the standard genotoxin, 4-nitroquinoline 1-oxide. Chronic exposure to these compounds caused inhibition of growth population on the rotifer while the algae seemed to be unaffected. Results indicated that caffeine (**1**), *N,N′*-dimethyloxamide (**4**) and *N,N′*-dimethylparabanic acid (**5**) reduced β-galactosidase activity in comparison with positive control, both at 1 and 5 mg/L of 4-NQNO with a good dose–response.

1. Introduction

Caffeine is the main alkaloid of coffee plants and it is also present in tea leaves, in cacao pods and in about other 60 species as the ilex plant. A world release of about 100 million kg of caffeine (ICO) is estimated.

As result, caffeine, and in some cases its metabolite paraxanthine, has been found worldwide in surface and ground waters that is why caffeine is the most commonly proposed anthropogenic marker in surface waters and is a life-style compound just as nicotine (Buerge et al., 2003).

Caffeine has been found in waters of Wascana Creek (Waiser et al., 2011), Ontario and Lake Erie in Canada (Metcalfe et al., 2003), Llobregat and its tributaries (Huerta-Fontela et al., 2007, 2008) in ground waters near Barcelona, Spain (Teijon et al., 2010; Albaiges et al., 1986), and 72 surface waters in the United States (Focazio et al., 2008; Kolpin et al., 2004).

The presence of caffeine in water bodies reflects the fact that this compound is not completely removed in many sewage treatment plants. Martinez-Bueno et al. (2011), in a study run on a plant located in the southeast of Spain, have detected mean concentrations of caffeine and its metabolite paraxanthine in the influent (67.1 and 49.7 μg/L) and in the effluent (16.7 and 11.4 μg/L); concentrations of 135 μg/L of caffeine in the influent and 52 μg/L in the effluent (Yuo et al., 1999) have been found in the Lanzhou plant in China. The presence of caffeine has also been reported in the effluents of plants of the towns of Cantabria, Almeria (Gomez et al., 2010) as well as in the Zagreb plant in Croatia (Grung et al., 2007) and the Montreal plant in Canada (Blaise et al., 2006).

Caffeine removal is incomplete in sewage treatment plants which use conventional treatments based on activated biological sludges

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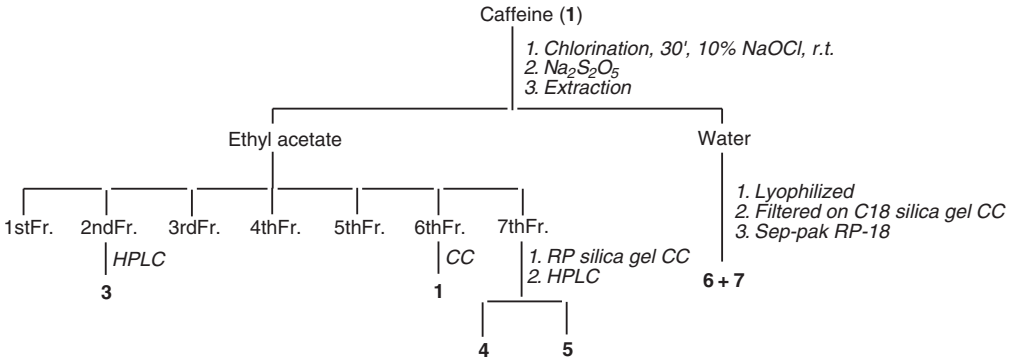


Scheme 1. Isolation of the different identified compounds.

(Yang et al., 2011). On the contrary, high or complete removal of caffeine has been reported by Huerta-Fontela et al. (2008), Stackelber et al. (2004) and Boleda et al. (2011) when chlorination is used as additional treatment in drinking water treatment plants. However Glassmeyer and Shoemaker (2005) reported no apparent change in caffeine even after 48 h of contact with hypochlorite via benchtop experiments. Sodium hypochlorite is the most widely used disinfectant because of its efficiency and cheapness. Despite these advantages, hypochlorite can react with organic matter to yield a wide variety of by-products (Christman et al., 1983; Helmer, 1999; Zarrelli et al., 2012; DellaGreca et al., 2009) that have been associated with adverse health effects (Clark et al., 2001; Nieuwenhuijsen et al., 2000; Nakamura et al., 2008; Sekizawa and Onodera, 2010).

Caffeine (**1**) chlorination by hypochlorite has been already investigated by Gould and Hay (1982) and Gould and Richards (1984). The authors studied the kinetics of the reaction in the pH range 5–9 and identified some by-products by GC–MS analysis. The authors concluded that the reaction is a quite slow process and that the reaction rate is strongly pH-dependent and it is influenced by caffeine — hypochlorite ratio more than the absolute concentration of reactants.

Some studies report the toxicological effects of caffeine on different freshwater species and results show that caffeine does not seem to be a threat for the aquatic environment at least in short term exposure due to the high concentrations required to determine a significant effect (Calleja et al., 1994; Moore et al., 2008). However, the continuous introduction of caffeine may cause subtle effects acting as a pseudo-persistent pollutant for its continuous release in the environment and little is known about its chronic effects (OECD, 2002). Furthermore, several studies reported the genotoxic potential and mutagenic potential of caffeine on animal models and results are inconsistent and inconclusive (Choundhury and Palo, 2004) even if caffeine showed antigenotoxic activity towards known genotoxins (Woziwodzka et al., 2011).

Then, for the worldwide presence of caffeine in the aquatic systems, it is important to evaluate the environmental impact of caffeine transformation products, since only the ecotoxicity of one by-product, *N,N′*-dimethylurea, is known (OECD, 2003). Therefore, the main aim of this work was to test caffeine and its derivatives obtained by a reaction between caffeine and sodium hypochlorite mimicking the chlorination step. We investigated the acute and chronic toxicities of caffeine and its six transformation products on organisms from two levels of the freshwater aquatic chain, the rotifer *Brachionus calyciflorus* and the alga *Pseudokirchneriella subcapitata*. Furthermore, the possible mutagenesis and genotoxicity of these compounds were performed using the Ames Test on *Salmonella typhimurium* and the SOS Chromotest on *Escherichia coli* PQ37, respectively, to detect point mutations and the induction of SOS DNA repair system. The SOS Chromotest on caffeine and some derivatives, previously co-incubated with the standard genotoxin 4-nitroquinoline 1-oxide, was performed to establish the possible antigenotoxic effect.

2. Material and methods

2.1. Apparatus

HPLC was performed on a Shimadzu LC-10AD by using UV–VIS detector Shimadzu RID-10A. A semipreparative HPLC was performed using a RP18 (LiChrospher 10 μm, 250 × 10 mm i.d., Merck) column with a flow rate of 1.2 mL min^{-1}. Column chromatography (CC) was carried out on Merck Kieselgel 60 (230–400 mesh). Electronic impact mass spectra (EI-MS) were obtained with a QP-5050A (Shimadzu) EI 70 eV spectrometer. ^{1}H- and ^{13}C-NMR spectra were recorded on a Varian INOVA-500 NMR instrument (^{1}H at 499.6 MHz and ^{13}C at 125.62 MHz), referenced with deuterated solvents ($CDCl_3$ or CD_3OD) at 25 °C. Proton-detected heteronuclear correlations were measured using a gradient heteronuclear single-quantum coherence (HSQC), optimized for $^{1}J_{HC} = 155$ Hz, a gradient heteronuclear multiple bond coherence (HMBC), optimized for $^{n}J_{HC} = 8$ Hz.

2.2. Chlorination procedure and product isolation

Caffeine **1** (1 g) dissolved in MilliQ water (1 L) was treated for 30 min with 10% hypochlorite (molar ratio 1:6; concentration spectroscopically determined λ_{max} 292 nm, ε 350 $dm^3 mol^{-1} cm^{-1}$) at room temperature. The pH of the solution, measured by a pH-meter at 5 min intervals, rises from the initial pH 6.8 to 9.3, after 5 min, and it remained at this value in the residue time. After 30 min, the reaction was quenched by sodium sulfite excess and lyophilized. The residue was distributed between ethyl acetate and water.

The ethyl acetate fraction (689 mg) was chromatographed on silica gel CC using a gradient of dichloromethane:acetone, to give seven fractions (Scheme 1). The 2nd (67 mg), eluted with dichloromethane, was purified by HPLC using a reversed phase column and eluting with 2:1:7 methanol:acetonitrile:water, to give compound **3** (15 mg). The 4th (59 mg), eluted with 99:1 dichloromethane:acetone, contained

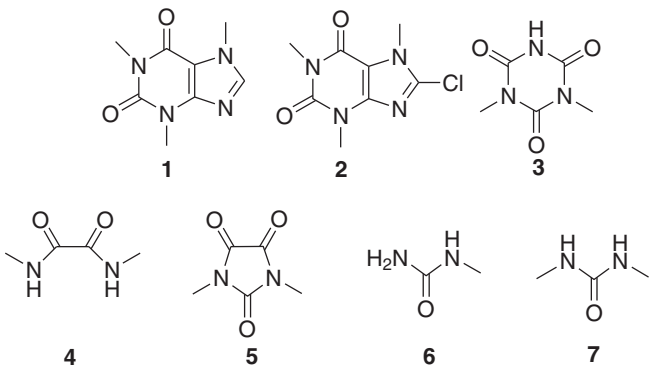
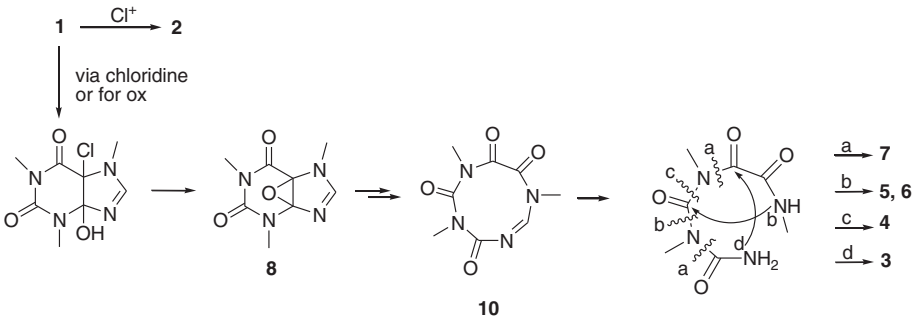


Fig. 1.



Scheme 2. Suggested pathways for the reaction of caffeine (**1**) with hypochlorite.

compound **2** (20 mg) that was purified by HPLC using a reversed phase column and eluting with 2:1:7 methanol:acetonitrile:water. The 6th (405 mg), eluted with 1:1 dichloromethane:acetone, was rechromatographed on silica gel CC eluting with a gradient of dichloromethane:methanol. The fraction, eluted with 90:10 dichloromethane:methanol, contained the unreacted caffeine **1** (373 mg). The 7th (105 mg), eluted with acetone, was filtered on reversed phase silica gel CC with water and acetonitrile. The fraction eluted with acetonitrile (150 mg) was filtered on Sep-Pak RP-18 with methanol and the eluate (133 mg) was purified by HPLC using a reversed phase column, eluting with 4:1:5 methanol:acetonitrile:water, to give **4** (21 mg) and **5** (38 mg).

The water fraction was lyophilized and filtered on reversed phase silica gel CC with water, methanol and acetonitrile. The fraction eluted with methanol (161 mg) was filtered on Sep-Pak RP-18 with water and the eluate (88 mg) was analyzed by NMR. It consisted of *N*-methylurea (**6**) and *N,N′*-dimethylurea (**7**), as evidenced by comparison of ^{1}H- and ^{13}C-NMR spectra of this mixture with those of authentic samples.

2.3. Test compounds

Caffeine, *N*-methylurea, *N,N′*-dimethylurea and 4-nitroquinoline 1-oxide (4-NQNO) were purchased by Sigma-Aldrich Chemicals (Milan, Italy). Compounds were dissolved in double-deionized water to make stock solutions. The test solutions were prepared by mixing the appropriate volume of the stock solutions to be tested and the test medium. Compound concentrations used in acute definitive tests were based on the yield of derivatives chemically separated, while concentrations in chronic definitive ones were based on results from range-finding tests (dilution factor = 10). To evaluate the potential antigenotoxic activity of all compounds, the caffeine and its derivatives (tested at the same concentrations used for the genotoxicity assay) were co-incubated at 22 °C for 3 h on a mechanical shaker with 1 and 5 μg/mL of a standard genotoxin, 4-NQNO.

2.4. Toxicity tests

Acute toxicity was detected on the rotifer *B. calyciflorus* (less than 2 h old) hatched from cysts supplied by MicroBioTest, Belgium. The hatching was carried out 16–18 h before the beginning of the test in synthetic freshwater (moderately hard medium) at 25 ± 1 °C and under continuous illumination (3000–4000 lx). Five or six concentrations (0.3 mL of test solution for each test well) of each compound were tested in six replicates with five animals. The multiwells were incubated at 25 °C in darkness for 24 h. The test parameter considered was mortality and the concentration that gave the 50% effect in 24 h was indicated as LC_{50} (ASTM E1440, 1991).

Chronic toxicity tests were carried out on *P. subcapitata* and *B. calyciflorus*. The test on *P. subcapitata* was performed in 96-well microplates according to Paixao et al. (2008). The algal suspension was taken from an exponentially growing pre-culture according to ISO 8692 (2004) and it was added to five or six concentrations of compounds to have a final algal concentration of 10^4 cells/mL. Compound concentrations, the positive control ($K_2Cr_2O_7$) and the negative control (0.3 mL per well) were tested in six replicates. Plates were incubated under continuous illumination (light source 6000 lx) at 25 ± 1 °C for 72 h on a microplate shaker (450 rpm). The algal growth has been monitoring for 72 h at 450 nm (SpectraFluor, Tecan, Switzerland). The plates were read immediately before the test and every 24 h during the incubation. The mean percentage inhibition of the specific growth rate in comparison with the negative control was estimated and EC_{50}-72 h values were calculated.

The chronic toxicity on *B. calyciflorus* was performed according to ISO 20666 procedure (ISO, 2008). Organisms used for the tests (less than 2 h old) were hatched in the same conditions of acute assay. Tests were performed in six replicates (one rotifer per well) of 0.9 mL of test solution (five concentrations). Organisms were fed with 0.1 mL of a fresh suspension of 10^7 cells/mL of *P. subcapitata*. Plates were incubated in darkness at 25 ± 1 °C for 48 h. The test parameter considered was the population growth inhibition compared to negative control, and the concentration that gave the 50% effect was calculated.

Caffeine and its derivatives were tested at least three times (three independent assays). Acute and chronic results were analyzed using Prism 5 (GraphPad Inc., CA, USA). L(E)C_{50} values were calculated by nonlinear regression with four parameter logistic equations for $p \leq 0.05$.

2.5. Ames Test/SOS Chromotest

The mutagenicity of caffeine and its derivatives was evaluated with the Ames Test on two *S. typhimurium* strains, the TA98 strain to test for frame-shift mutations, and the TA100 strain to test for base-pair substitutions in three independent experiments. We conducted the plate incorporation assay without metabolic activation (S9) to detect

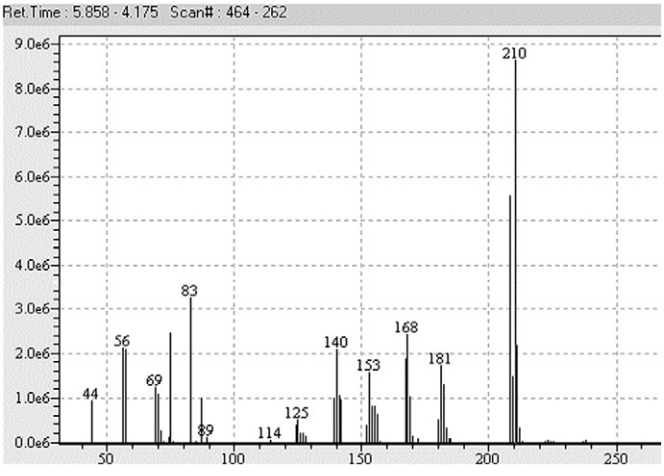


Fig. 2. MS spectrum of epoxide **8**.

Table 1
Acute and chronic L(E)C_{50} values in mg/L with confidence limits (95% probability).

Compound	*Brachionus calyciflorus* 24 h	*Brachionus calyciflorus* 48 h	*Pseudokirchneriella subcapitata* 72 h
1	1018 (933–1109)	104 (73–222)	N.E.[a] up to 150
2	N.E. up to 150	3.5 (0.9–13.3)	N.E. up to 150
3	N.E. up to 150	N.E. up to 50	N.E. up to 150
4	734 (706–763)	23.8 (13.8–41.1)	N.E. up to 150
5	N.E. up to 150	17.3 (12.8–23.4)	N.E. up to 150
6	1932 (1779–2311)	14.3 (3.4–24.6)	N.E. up to 150
7	1927 (1623–2286)	362 (112–662)	320 (170–602)

[a] N.E., no effect.

direct mutagenic effects of compounds (Maron and Ames, 1983). The plates were incubated at 37 °C for 72 h in the dark. The number of His^+ revertants was counted, and the mutagenic ratio (MR) was calculated as the ratio between the mean number of revertants of compound and negative control plates. A compound was considered mutagenic when the MR was at least twice than the negative control and a clear dose–response relationship was observed (US Environmental Protection Agency, 1983). The compound revertant number was compared to the corresponding negative control value.

The genotoxic and antigenotoxic activities were assessed with the SOS Chromotest (Quillardet and Hofnung, 1993) on the *E. coli* strain, PQ37. This strain carried a *sfiA*::*lacZ* fusion gene and a deletion of the normal lac region; thus, the β-galactosidase activity was strictly dependent on *sfiA* expression, which increased in response to specific DNA damaging agents. The assay was quantitative, and the dose–response curves presented a linear region. The β-galactosidase and alkaline phosphatase activities (constitutive in *E. coli* PQ37) were measured as ortho-nitrophenol and 4-nitrophenyl concentration at 420 nm (SpectraFluor, Tecan, Switzerland) for the ratio $R = \beta/\rho$ where β represents β-galactosidase activity and ρ, phosphatase alkaline activity. The results were expressed as induction factor (IF) for each dilution of compound, defined as $IF = R/R_0$, in which R_0 is the ratio measured in the negative control. The antigenotoxic activity against 4-NQNO was identified as inhibition $\% = IF_{\text{positive control}} - IF_{\text{sample}} / IF_{\text{positive control}} - IF_{\text{negative control}}$.

2.6. Validity criteria of biological tests

Acute toxicity test accuracy was measured using $K_2Cr_2O_7$ (Sigma-Aldrich Chemicals) in reference toxicant tests in order to verify that L(E)C_{50}s were in the range of known toxicities for *B. calyciflorus*. Toxicity tests with different concentrations of DMSO were performed to detect solvent toxicity. Acute toxicity data were processed when the effect in negative control was <10%. Chronic toxicity test on *B. calyciflorus* was valid if the reproduction in the control was observed in at least 87.5% of the replicates and if the average number of live female counted in the control was greater than or equal to 3 at the end of the test (ISO, 2008). Test on algae was valid if the number of algal cells in the negative control increased by at least a factor of 16 after 72 h of incubation and if $K_2Cr_2O_7$ EC_{50} was equal to 1.19 ± 0.27 mg/L (ISO, 2000). Temperature, hardness, dissolved oxygen, conductivity and pH were checked at the beginning and at the end of each test.

The adequacy of Ames Test data was evaluated considering: 1) the satisfactory bacterial strain checks, 2) the acceptability of positive (sodium azide for TA100, 2-nitrofluoren for TA98), negative and solvent controls that had to fall within empirical 95% laboratory-control confidence limits and, 3) the acceptability of dose–response relationship. The adequacy of SOS Chromotest data was evaluated considering the satisfactory check of *E. coli* PQ37 (alkaline phosphatase production) and the acceptability of positive (4-nitroquinoline *N*-oxide) and negative controls within 95% laboratory-control confidence limits.

3. Results

Caffeine dissolved in MilliQ water was treated with sodium hypochlorite. The reaction was run at pH 7 and was quenched by bisulfite addition after 2 h. The reaction solution was lyophilized and the residue was subsequently separated into an organic and into an aqueous fraction. Repeated chromatographic processes of the organic fraction led to the isolation of 8-chlorocaffeine (**2**), 1,3-dimethyl-5-azabarbituric acid (**3**), *N*,*N*′-dimethyloxalamide (**4**), and *N*,*N*′-dimethylparabanic acid (**5**), beside unreacted caffeine (**1**) (Fig. 1). Compounds **1**–**5** were

Table 2
SOS Chromotest results of **1**, **4** and **5** compounds co-incubated with increasing concentrations of 4-NQNO (antigenotoxic activity).

Compound		I.F.[a]		I.F.[a]
4-NQNO	1 mg/L	6.32 ± 1.89	5 mg/L	17.86 ± 2.31
	Concentration (mg/L) + 4-NQNO (1 mg/L)		Concentration (mg/L) + 4-NQNO (5 mg/L)	
1	0.82	6.09 ± 0.12	0.82	15.82 ± 0.25
	2.46	5.63 ± 0.21	2.46	15.78 ± 0.12
	7.40	4.28 ± 0.13	7.40	15.54 ± 0.15
	22.0	4.08 ± 0.08	22.0	15.38 ± 0.18
	66.6	4.06 ± 0.11	66.6	14.99 ± 0.08
	200.00	3.94 ± 0.10	200	14.97 ± 0.05
4	0.82	4.22 ± 0.13	0.82	17.96 ± 0.95
	2.46	3.89 ± 0.15	2.46	16.95 ± 0.56
	7.40	3.48 ± 0.11	7.40	15.53 ± 0.41
	22.00	3.28 ± 0.10	22	14.27 ± 0.27
	66.60	3.05 ± 0.11	66.6	13.01 ± 0.58
	200.00	2.66 ± 0.08	200.00	11.75 ± 0.35
5	0.82	5.62 ± 0.14	0.82	16.05 ± 0.56
	2.46	5.30 ± 0.12	2.46	14.78 ± 0.12
	7.40	4.82 ± 0.08	7.40	14.00 ± 0.89
	22.00	3.74 ± 0.17	22	12.36 ± 0.69
	66.60	3.26 ± 0.22	66.6	12.06 ± 0.35
	200.00	2.15 ± 0.68	200	9.38 ± 0.45

[a] Values are expressed as induction factor (I.F.) means from three independent samplings ± SD.

identified by comparison of ^{1}H- and ^{13}C-NMR spectra and GC–MS data with those of authentic samples and those reported in the literature (Doege and Eger, 2007; Neves et al., 2011; Kobayashi et al., 2010).

The aqueous layer consisted of *N*-methylurea (**6**) and *N*,*N*′-dimethylurea (**7**), as evidenced by comparison of ^{1}H- and ^{13}C-NMR spectra of this mixture with those of authentic samples.

Based on our results and literature data a mechanistic hypothesis for the formation of the by-products is shown in Scheme 2. Aromatic electrophilic substitution at C-8 of caffeine gives **2**. All other products could derive from epoxide **8**. The intermediacy via chlorohydrin of this epoxide that has been suggested by Klawonn et al. (2003) was confirmed here for the first time. Indeed, LC–MS analysis of the chlorination mixture showed a small peak at *m*/*z* 210 (Fig. 2) which could be in agreement with the molecular formula $C_8H_{10}N_4O_3$ of epoxide **8**. From epoxide **8**, probably through hydrolysis and oxidation as reported by Khurana et al. (2007), it is possible to get to macrocycle **10**. This compound has been suggested by Kolonko Kenneth et al. (1979) and by Dalmazio et al. (2005) as an intermediate in the ozonization and photo-Fenton reactions of caffeine, respectively. Subsequent steps of hydrolysis, oxidation and decarboxylation could lead to compound **11**.

As shown in Scheme 2, hydrolysis *a* leads to *N*,*N*′-dimethylurea (**7**), transamidation *b* affords *N*,*N*′-dimethylparabanic acid (**5**) and *N*-methylurea (**6**) and hydrolysis *c* leads to *N*,*N*′-dimethyloxalamide (**4**). Finally, reaction *d* gives 1,3-dimethyl-5-azabarbituric acid (**3**).

HPLC purified compounds **2**–**5** and authentic samples of *N*-methylurea (**6**) and *N*,*N*′-dimethylurea (**7**) were used in the ecotoxicological tests. In Table 1 the results of acute and chronic toxicity on the rotifer *B. calyciflorus* and of the chronic toxicity on the alga *P. subcapitata* are reported. LC_{50} values were found in the acute test on the rotifer for compounds **1**, **4**, **6**, and **7** at very high concentrations, in the order of hundreds and/or thousands of mg/L while no acute effect was found for the other compounds. A different trend was shown by chronic effect on the same organism. Caffeine showed a median inhibition of reproduction (EC_{50}) on rotifers at 104.0 mg/L while the chloro-derivative **2** was more toxic than the other compounds with an EC_{50} of 3.5 mg/L. Rotifers seemed to be more affected in the long term exposure than algae which did not show any toxicity effect up to 150 mg/L except for compound **7**.

The mutagenicity of caffeine and its derivatives was examined with the Ames Test. The results showed that only the chlorinated derivative **2** caused a significant increase in revertants with a MR > 2 and a clear dose–response relationship starting from 2.46 to 600 mg/L in TA98 and 7.40 to 600 mg/L in TA100.

Genotoxicity refers to the ability to interact with DNA and/or the cellular apparatus and these events can be reversible because of cellular repair processes. To measure genotoxicity, the SOS Chromotest was used and samples were considered genotoxic when the IF value was ≥2 and they showed a clear dose–effect relationship (Ruiz and Marzin, 1997). In the present study, all compounds investigated were not able to activate the SOS DNA repair system in *E. coli* PQ37; thus no data are shown for their IF values. For this reason we tried to detect a possible antigenotoxic activity of the compounds investigated. The results are shown in Table 2. Among all compounds investigated only caffeine (**1**), *N*,*N*′-dimethyloxamide (**4**) and *N*,*N*′-dimethylparabanic acid (**5**) reduced β-galactosidase activity in comparison with positive control, both at 1 and 5 mg/L of 4-NQNO, with a good dose–response effect.

4. Discussion

The use of the freshwater rotifer *B. calyciflorus* and of the microalga *P. subcapitata* as representative aquatic organisms in acute and chronic toxicity tests was justified because they have a widespread geographic distribution and a strong impact on several important ecological processes in waters. As expected, the acute toxicity of caffeine and its derivatives was found at very high concentrations to be of no environmental concern considering caffeine occurrence in surface water, in the order of μg/L. Our results agree with those of Calleja et al. (1994) who found acute toxicity of caffeine on *B. calyciflorus* in the same order of magnitude (Table 1). Chronic results on rotifers were more relevant even if EC_{50} values were obtained for concentration in the range of mg/L (8-chlorocaffeine the most toxic compound). Our results on algae confirm the few literature toxicological data about caffeine and *N*,*N*′-dimethylurea (**7**) which reported EC_{50} values >100 mg/L and of 560 mg/L, respectively, on *Scenedesmus subspicatus* (OECD, 2002, 2003).

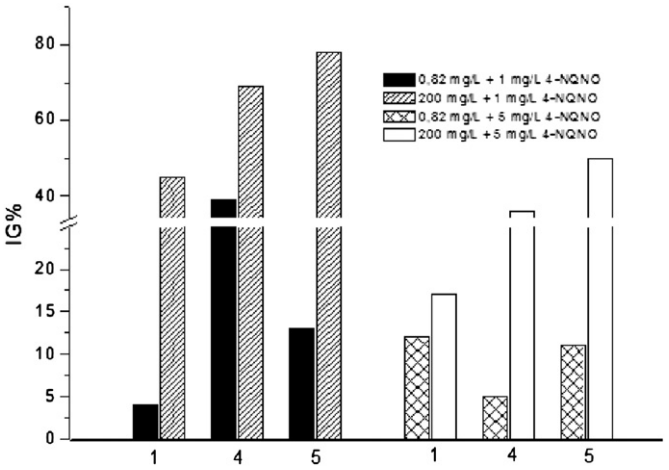


Fig. 3. Antigenotoxic effect (genotoxicity inhibition percentage) of 1, 4 and 5 compounds against 4-NQNO in *E. coli* PQ37.

As expected, only the chlorinated derivative **2** was a mutagen. This result might be explained by the study of Traczewska et al. (2009) who found a positive correlation between the presence of chlorinated derivatives in disinfected waters and mutagenic activity in the Ames Test conducted without metabolic activation. The negative results found for caffeine (**1**) and *N*-*N*′-dimethylurea (**7**) confirm previous studies (OECD, 2002, 2003; Kirkland et al., 2011).

Recent studies demonstrated the anticarcinogenic and antigenotoxic activities of coffee (Abraham and Stopper, 2004; Woziwodzka et al., 2011), hence we studied the possible protective effects of caffeine and its derivatives co-incubating them with the standard genotoxin, 4-nitroquinoline 1-oxide, using the SOS Chromotest. As reported in the results, caffeine (**1**) and derivatives **4** and **5** could act as protective factors for genotoxicity at concentrations starting from 0.82 mg/L (Table 2). The inhibition percentage of the antigenotoxic compounds is shown in Fig. 3. As reported by Woziwodzka et al. (2011), this effect could be due to the interaction of caffeine and the two derivatives with aromatic compounds.

5. Conclusion

The simulated chlorination process of caffeine determines the formation of six by-products among which only one is chlorinated. The acute toxicity of caffeine and its derivatives was found at very high concentrations to be of no environmental concern considering caffeine occurrence in surface water, in the order of μg/L.

Mutagenic activity was observed only for the chlorine derivative. Interestingly, the present study has demonstrated that caffeine along with two by-products, *N*,*N*′-dimethyloxamide (**4**) and *N*,*N*′-dimethylparabanic acid (**5**), could play a possible protective role acting as antigenotoxic compounds. This protective effect could be of interest from an environmental point of view for the continuous discharge of caffeine in sewage treatment plants.

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