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Oxidation of diclofenac in water by sodium hypochlorite: Identification of new degradation by-products and their ecotoxicological evaluation

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ABSTRACT

Diclofenac (DCF) is the most widely prescribed non-steroidal anti-inflammatory drug in the world and it has been detected in drinking and surface waters. In this paper, the effect of chlorination process on DCF in aqueous solutions was investigated and the structures of 14 isolated degradation by-products (DPs), of which nine are new, have been determined from combining mass spectrometry and nuclear magnetic resonance data and justified by a proposed mechanism of formation beginning from the parent drug. Some degradation by-products show only one phenyl, others are dimers or trimers of the parental compound, which has undergone oxidative decarboxylation of the side chain and/or chlorination of this or one or both aromatic rings. Ecotoxicological bioassays evidenced the following sensitivities *D. magna* < R. subcapitata < A. fischeri. The isolated DPs (DP1–8, except for DP9) exhibited effects ≥ 50 % in the exposed microalgae and crustaceans showing toxicities mainly ranked from slight to acute.

1. Introduction

Diclofenac (DCF) is the most widely prescribed non-steroidal anti-inflammatory drug (NSAID) in the world [1,2] and it is used in the treatment of both musculoskeletal and systemic inflammatory states. Since its introduction in 1973 [3], numerous new drugs containing DCF [4] have been approved and are available in numerous pharmaceutical formulations, suitable for different routes of administration and with analgesic, antipyretic and inflammatory actions.

It's used in the veterinary field too, but it was banned in several South Asian countries [5] because its traces in cattle carcasses are lethal to vultures and eagles that eat them [6,7].

DCF ranks 13th among the best-selling generic drugs (Voltaren; Torsilax; Diclofenac). It is included in the list of emergency drugs in over 74 countries, had a total production of over 877 t in 2007 [8], and has a total estimated revenue of more than 1.60 billion dollars and an annual sales growth rate of more than 15 % [9]. Yearly DCF consumption has been reported to vary between 195 and 940 mg per inhabitant in different countries [10–12]. It was detected up to 10 ng L^{-1} in drinking water [8,13], while in surface waters ranged between 100 and 500 ng L^{-1} with values up to 1200 ng L^{-1} in some German rivers and up to 8500 ng L^{-1} in some Pakistani rivers [14,15]. In groundwater, the detected limits are considerably lower and are below the limits of instrumental detectability, although there are certainly exceptions such as 380 ng L^{-1} measured in the underground waters of Barcelona or some German locations [16]. In municipal wastewaters, concentrations between 0.44 and 7.1 μg L^{-1} were detected, with mean values between 0.11 and 2.3 μg L^{-1}. The highest concentrations were measured in hospital (6.88 μg L^{-1}) and pharmaceutical manufacturers (> 203 μg L^{-1}) wastewater. In municipal wastewater treatment plants (WWTP) effluents, DCF is among the most frequently detected drugs with concentrations between 800 and 1600 ng L^{-1} [8]. Its percentage of

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removal oscillates between 0% is absorbed by the ecosystem and the rest ends up in the oceans.

In this paper, the pathway of DCF was investigated after chlorination, one of the processes normally used in WWTP for disinfection [17–19], carrying out two different experiments, one at a concentration similar to that at which the DCF was detected in the environment and one at least 100 times higher concentration in order to isolate and identify the degradation by-products (DPs).

Normally the disinfection processes both in laboratory-scale studies and in full-scale applications are studied only in relation to the abatement of the bacterial load and the amount of emerging pollutant considered. The risk is that the pollutant present is not mineralized but only transformed into a series of products that are in turn more recalcitrant to degradation and even more toxic than the pollutant from which they derive. In this frame, it is interesting identify the degradation by-products formed during the chlorination process and outline their eco-toxicological profile.

Indeed, the structures of 14 isolated DPs, of which nine are new, have been determined from combining mass spectrometry (MS) and nuclear magnetic resonance (NMR) data and justified by a proposed mechanism of formation starting from the parent drug. Ecotoxicological bioassays with *Daphnia magna* were used to provide information about the potential residual toxicity effects and to compare degradation by-products to the parent compound.

2. Material and methods

2.1. Drug and reagents

DCF (99.3 %) was purchased from Sigma Aldrich (Milan, Italy). All the other chemicals and solvents were purchased from Fluka (Saint-Quentin Fallavier, France) at HPLC grade and were used as received. The reagents and other required test solutions (including dilution water and reconstitution water) for ecotoxicity assays were purchased from Ecotox LDS s.r.l. (Milan, Italy).

2.2. Analytical measurements

HPLC was performed on a Shimadzu LC-10AD by using UV–vis detector Shimadzu RID-10A. A semipreparative HPLC was performed using an 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). ^{1}H and ^{13}C NMR spectra were recorded on an NMR spectrometer operating at 400 MHz (Bruker DRX, Bruker Avance, MA, USA), referenced in ppm to residual solvent signals ($CDCl_3$, at δ_H 7.27 and δ_C 77.0 ppm) at 25 °C. Proton-detected heteronuclear correlations were measured using a gradient heteronuclear single-quantum coherence (HSQC), optimized for $^{1}J_{HC}$ = 155 Hz, and a gradient heteronuclear multiple bond coherence (HMBC), optimized for $^{n}J_{HC}$ = 8 Hz. The samples were analysed by LC–MS on a 6520 Accurate-Mass Q-TOF LC/MS System (Agilent Technologies, Palo Alto, CA, USA) equipped with a 1200 HPLC System and a chip cube (Agilent Technologies). After loading, the sample solution was first concentrated and then washed on a 40 nL enrichment column (Agilent Technologies chip) with 0.1 % formic acid in 2 % acetonitrile as the eluent. The sample was then fractionated on a C18 reverse-phase capillary column (Agilent Technologies chip) at a flow rate of 400 nL min^{-1}, with a linear gradient of eluent B (0.1 % formic acid in 95 % acetonitrile) in A (0.1 % formic acid in 2 % acetonitrile) from 3 % to 80 % in 30 min. Mass spectral acquisition was selected from 50 to 1000 Da. Each LC—MS analysis was preceded and followed by blank runs to avoid carryover contamination. UV/Vis spectra were recorded on a PerkinElmer Lambda 7 spectrophotometer.

2.3. Chlorination reaction

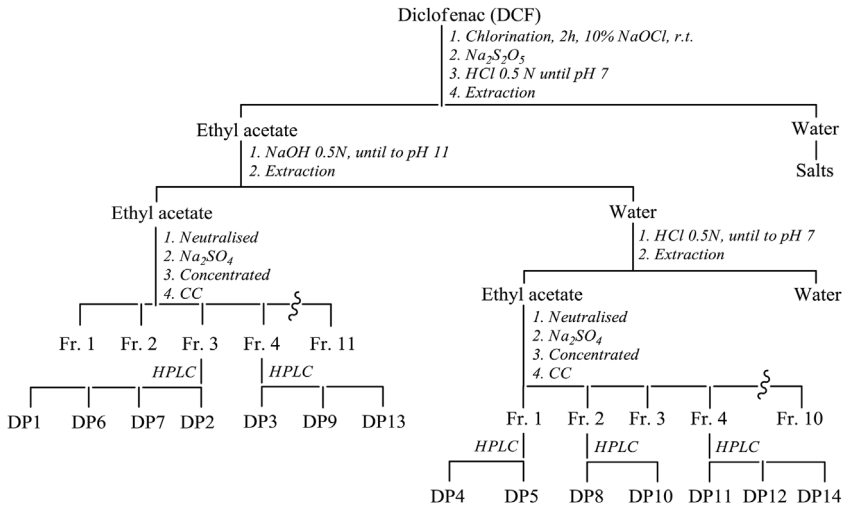
2.3.1. Chlorination experiments

A 10^{-5} M solution of DCF was treated for 2 h with 10 % hypochlorite (molar ratio DCF:hypochlorite 1:1. The chlorine concentration was spectroscopically determined λ_{max} 292 nm, ε 350 dm^3 mol^{-1} cm) at room temperature [20,21], simulating a chlorination process. The pH of the solution, measured by a pH meter at 10 min intervals, rose from the initial pH 8.5–9.0 after 10 min, and it remained at this value through the duration of the reaction. An aliquot of the solution was taken every 10 min, quenched by sodium sulfite excess: $Na_2SO_3 + Cl_2 \rightarrow 2NaCl + SO_3^{2-}$, filtered and fractionated into acidic and neutral fractions. The course of the reaction was monitored by HPLC. The main degradation by-products (DP1 – DP3, DP6 – DP7, DP9 and DP13 for the neutral fraction and DP4 – DP5, DP8, DP10 – DP12 and DP14 for the acidic fraction; Scheme 1) were identified by comparing their retention times with those of standard compounds commercially available or compounds isolated for the first time (Fig. 1). The latter were obtained by performing preparative experiments with a solution of DCF at a concentration higher than 10^{-3} M treated with 12 % hypochlorite at room temperature. The degradation by-products obtained were isolated by column chromatography and HPLC and completely characterized using NMR and MS analysis. DP1 – DP14 were isolated in relative % of 1.68, 4.39, 5.74, 5.74, 3.71, 1.01, 3.38, 1.35, 2.36, 1.35, 1.68, 2.36, 1.68 and 2.36, respectively. The proposed mechanism of their formation from DCF is shown in Figs. 3 and 4.

2.3.2. Isolation of degradation by-products

The 10^{-3} M solution of DCF after chlorination was quenched with excess sodium sulfite, neutralized with HCl 0.5 N and extracted with ethyl acetate (Scheme 1). The organic fraction (1067 mg) was fractionated into acidic and neutral fractions with aqueous NaOH 0.5 N. The organic alkaline layer was neutralized, dried with Na_2SO_4 and concentrated under vacuum. The crude neutral residue (750 mg) was chromatographed on silica gel with a gradient of dichloromethane:methanol (99:1 to 80:20), to give 11 fractions. The 3rd fraction (75 mg), eluted with dichloromethane:methanol (99:1), was purified by HPLC using a reversed phase column and eluting with a gradient of CH_3COONH_4, pH 4, 10 mM, and CH_3OH (15:85 for 5 min; 15:85 to 0:100 in 15 min; 0:100 for 10 min) to give DP1 (5 mg), DP2 (13 mg), DP6 (3 mg) and DP7 (7 mg). The 4th fraction (83 mg), eluted with dichloromethane:methanol (99:1), was purified by HPLC using a reversed phase column and eluting with a gradient of CH_3COONH_4, pH 4, 10 mM, and CH_3OH (15:85 for 5 min; 15:85 to 0:100 in 15 min; 0:100 for 10 min) to give DP3 (17 mg), DP9 (4 mg) and DP13 (5 mg).

The aqueous layer was neutralized with HCl 0.5 N and extracted with ethyl acetate. It was dried with Na_2SO_4, concentrated under vacuum (287 mg), and chromatographed on silica gel with a gradient of chloroform: methanol (99:1 to 80:20) to give 10 fractions. The 1st fraction (36 mg), eluted with chloroform:methanol (97:3), was purified by HPLC using a reversed phase column and eluting with a gradient of CH_3COONH_4, pH 4, 10 mM, and CH_3OH (10:90 for 5 min; 10:90 to 0:100 in 20 min; 0:100 for 10 min) to give DP4 (17 mg) and DP5 (11 mg). The 2nd fraction (24 mg), eluted with dichloromethane:methanol (80:20), was purified by HPLC using a reversed phase column and eluting with a gradient of CH_3COONH_4, pH 4, 10 mM, and CH_3OH (25:75 for 5 min; 25:75 to 0:100 in 30 min; 0:100 for 10 min) to give DP8 (10 mg) and DP10 (4 mg). The 4th fraction (41 mg), eluted with dichloromethane:methanol (80:20), was purified by HPLC using a reversed phase column and eluting with a gradient of CH_3COONH_4, pH 4, 10 mM, and CH_3OH (15:85 for 5 min; 15:85 to 0:100 in 15 min; 0:100 for 10 min) to give DP11



Scheme 1. Isolation of 14 identified degradation by-products.

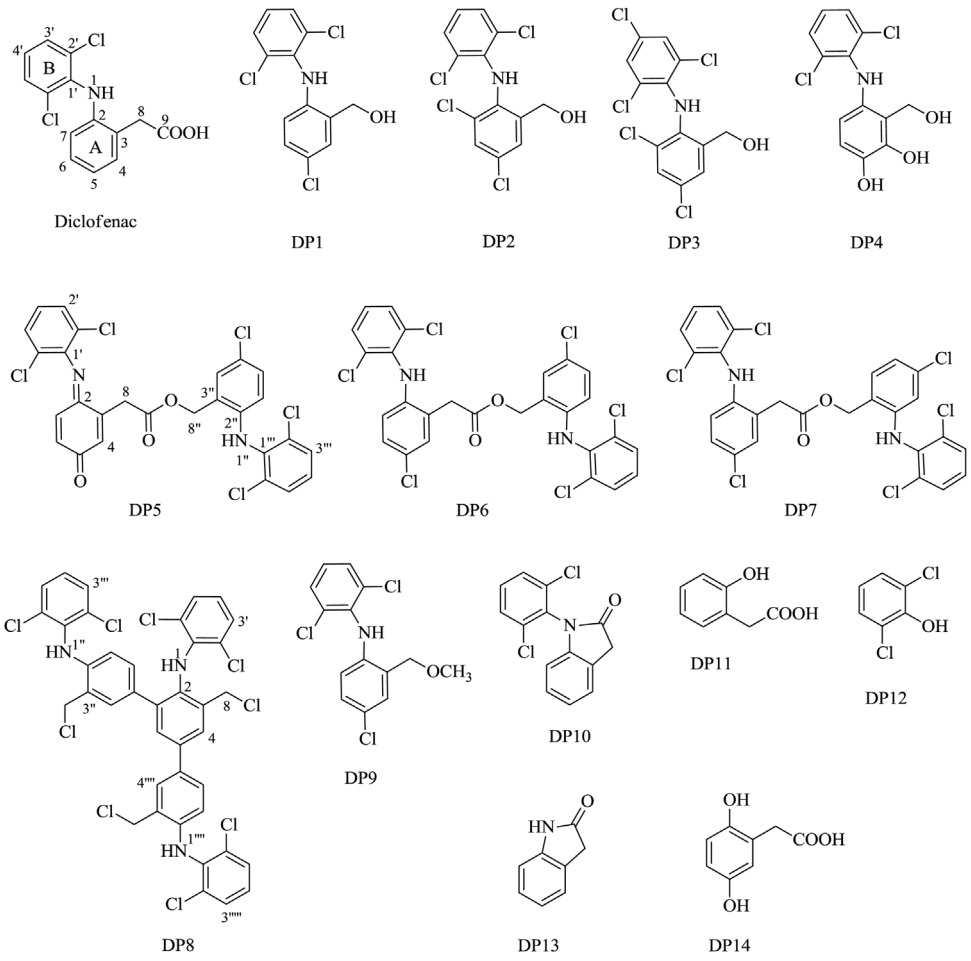


Fig. 1. DCF and its transformation by-products by chlorination.

(5 mg), DP12 (7 mg) and DP14 (5 mg). Structures of all compounds are shown in Fig. 1.

2.4. Ecotoxicity assays

The acute bioluminescence assay was conducted in accordance with the standard protocol ISO 11348-3 [22]. The experiments used *Aliivibrio fischeri* (NRRLB-11177) bacteria that were liquid dried and frozen at −20 °C. Toxicity tests are carried out on *A. fischeri* being a consolidated biological model that in included in most regulation for wastewater assessment on an end-of-pipe basis. The battery of toxicity tests proposed in the paper is of widespread use also for drugs detection. *A. fischeri* bioluminescence inhibition observed in the presence of pharmaceuticals was measured after different treatment durations (5, 15 and 30 min). All tests were performed in triplicate. To provide the relevant osmotic pressure for test organ-

isms, the salinity concentration of the stock solution was adjusted by 2 % for NaCl. The temperature during exposure was 15 °C according to the Microtox standard procedure. For the final analysis, only the data from the 30-min exposure were reported due to the negligible difference in toxicity between results from different exposure durations. The toxic effect values reflect the ratio of the decrease in bacterial light production to the remaining light.

An algal growth-inhibition test was performed according to the European standard EN ISO 8692 [23] using *Raphidocelis subcapitata*, formerly known as *Selenastrum capricornutum* or *Pseudokirchneriella subcapitata*.

The following salts were used for the preparation of algal test medium: $CaCl_2{\cdot}2H_2O$ (18 mg L^{-1}), $MgSO_4{\cdot}7H_2O$ (15 mg L^{-1}), NH_4Cl (15 mg L^{-1}), $MgCl_2{\cdot}6H_2O$ (12 mg L^{-1}), KH_2PO_4 (1.6 mg L^{-1}), $FeCl_3{\cdot}6H_2O$ (0.08 mg L^{-1}), $Na_2EDTA{\cdot}2H_2O$ (0.1 mg L^{-1}), H_3BO_3 (0.185 mg L^{-1}), $MnCl_2{\cdot}4H_2O$ (0.415 mg L^{-1}), $ZnCl_2$ (0.003 mg L^{-1}), $CoCl_2{\cdot}6H_2O$ (0.0015 mg L^{-1}), $Na_2MoO_4{\cdot}2H_2O$ (0.007 mg L^{-1}), $CuCl_2{\cdot}2H_2O$ (0.00001 mg L^{-1}). ISO artificial freshwater (ISO, 2012; ISO, 2013), containing $CaCl_2{\cdot}2H_2O$ (294 mg L^{-1}), $MgSO_4{\cdot}7H_2O$ (123.25 mg L^{-1}), $NaHCO_3$ (64.75 mg L^{-1}), and KCl (5.75 mg L^{-1}), was used for the preparation of cladoceran tests and for the control medium.

The growth of algae exposed to the sample was compared with the growth of algae in a negative control. For each sample, six replicates were inoculated with 10^7 algal cells L^{-1} in well plates and incubated for 72 h at 23 ± 2 °C under continuous illumination (in an irradiance range of 120–60 μein m^{-2} s^{-1}). The specific growth rate (μ) of *R. subcapitata* in each replicate was calculated from the logarithmic increase in cell density in the interval from 0 to 72 h as follows:

$$\mu = \frac{\ln N_i - \ln N_0}{t_i - t_0}$$

where N_0 and N_i represent the cell concentration at times t_0 and t_i, respectively. The results were expressed as the mean (±standard deviation) of the percentage inhibition of cell growth compared to the negative control.

R. subcapitata density was determined by an indirect procedure using a spectrophotometer (Hach Lange DR5000) and a 1 cm cuvette. The acute toxicity bioassay at 48 h with *D. magna* was conducted according to ISO 6341 [24]. *D. magna* were selected from laboratory stock cultures, were moved to 2.0 L glass beakers maintained at 24 ± 10 °C and were fed on *R. subcapitata*. Newly hatched neonates (less than 24 h old) obtained from the continuous laboratory culture were used (20 animals for each tested concentration and control). The total duration of the exposure was 48 h. Immobilized organisms were counted, and the results were expressed as the percentage of control. The test was considered valid if the immobilization in the control did not exceed 10 %. *D. magna* viability and mobility were observed with a stereomicroscope (LEICA EZ4-HD).

Ecotoxicity data were expressed as the EC_{50} (median effect concentration) values, and its 95 % confidence intervals were calculated by non-linear regression. After verification of normality (Shapiro-Wilk test) and homogeneity of variance (F-test), the significance of differences between mean values of experimental treatments and controls was assessed by Student's *t*-test and analysis of variance (ANOVA) with a 0.05 significance level. When ANOVA revealed significant differences among treatments, post-hoc analyses were carried out with Dunnett's method and Tukey's test. Statistical analyses were performed using GraphPad Prism software. Toxicity data have been integrated according to Persoone et al. and Lofrano et al. [25,26]. The hazard classification system based on the percentage of effect and the integrated class weight score (CWS) was determined by averaging the values corresponding to each biotest class.

3. Results and discussion

3.1. Chlorination experiments

Chlorination of DCF produced degradation by-products DP1 — DP14, in relative % of 2.53, 2.78, 2.24, 2.01, 0.85, 0.89, 0.79, 0.29, 2.11, 4.20, 3.35, 5.78, 4.10 and 7.01, respectively, that were isolated by chromatographic processes and identified on the basis of their spectroscopic data (Scheme 1).

3.2. Structure elucidation of degradation by-products DP1–DP14

The first four degradation by-products are DCF chloro-derivatives which have undergone oxidative decarboxylation of the side chain.

DP1/5-Chloro-2-[(2,6-dichlorophenyl)aminophenyl]methanol: the MS-TOF analysis showed a molecular ion peak at *m/z* 301.98 corresponding to molecular formula $C_{13}H_{10}Cl_3NO$. In the ^1H NMR spectrum, six signals were present, of which five related to aromatic protons at 7.40, 7.22, 7.13, 7.09 and 6.39 ppm and one to the methylene CH_2-8 at 4.83 ppm. These signals correlated to the carbons in the HSQC spectrum at 128.91, 128.48, 128.59, 125.29, 116.63 and 64.18 ppm, respectively. In addition to the signals of protonated carbons, the ^{13}C NMR spectrum showed five quaternary carbon signals at 141.78, 128.80, 124.94, 136.53 and 130.92 ppm. HMBC experiments allowed the assignment of the first three signals to the carbons C-2, C-3 and C-5, respectively, of the ring A 1,2,4-trisubstituted, while the last two signals were attributed to the carbons C-1′ and C-2′/C-6′ of the ring B 1,2,3-trisubstituted.

DP2/3,5-Dichloro-2-[(2,6-dichlorophenyl)amino]phenyl) methanol: the MS-TOF analysis showed a molecular ion peak at *m/z* 337.95 corresponding to molecular formula $C_{13}H_9Cl_4NO$. In the ^1H NMR spectrum, only five signals were present. Four were related to aromatic protons at 7.33, 7.30, 7.27 and 6.90 ppm and one to the methylene CH_2-8 at 4.64 ppm, and they correlated to the carbons in the HSQC spectrum at 129.05, 129.79, 128.67, 122.67 and 63.07 ppm, respectively.

In addition to the signals of the protonated carbons, the ^{13}C NMR spectrum showed six quaternary carbon signals at 136.85, 136.56, 126.49, 129.06, 137.56 and 126.81 ppm. HMBC experiments related the first four signals to the carbons C-2, C-3, C-5 and C-7, respectively, of the ring A 1,2,3,5-tetrasubstituted, while the last two signals were attributed to the carbons C-1′ and C-2′/C-6′ of the ring B 1,2,3-trisubstituted.

DP3/3,5-Dichloro-2-[(2,4,6-trichlorophenyl)amino)phenyl] methanol: the MS-TOF analysis showed a molecular ion peak at *m/z* 371.91 corresponding to molecular formula $C_{13}H_8Cl_5NO$. In the ^1H NMR spectrum, only three signals were present. Two were related to aromatic protons at 7.32 and 7.29 ppm, and one to the methylene CH_2-8 at 4.66 ppm. The first signal in the HSQC spectrum was correlated to the carbon at 129.21 ppm, the second, of area three, was correlated to the carbons a 128.41 (x2) and 126.76 ppm, and the last one to the carbon a 63.23 ppm. In addition to the signals of the protonated carbons, the ^{13}C NMR spectrum showed seven quaternary carbon signals at 136.53, 136.32, 128.94, 128.96, 136.65, 126.93 and 126.76 ppm. HMBC experiments allowed the assignment of the first four signals to the carbons C-2, C-3, C-5 and C-7, respectively, of the ring A 1,2,3,5-tetrasubstituted, while the last three were attributed to the carbons C-1′, C-2′/C-6′ and C-4′ of the ring B 1,2,3,5-tetrasubstituted.

DP4/3-Hydroxymethyl-4-[(2,4,6-trichlorophenyl)amino] benzene-1,2-diol: the MS-TOF analysis showed a molecular ion peak at *m/z* 334.96 corresponding to molecular formula $C_{13}H_{10}Cl_3NO_3$. Only four signals were present in the ^1H NMR spectrum; three of them were related to aromatic protons at 7.27, 6.79 and 6.51 ppm, and one at the methylene CH_2-8 at 5.09 ppm. They

correlated to the carbons in the HSQC spectrum at 128.90, 115.60, 120.70 and 60.50 ppm, respectively. In addition to the signals of the protonated carbons, the ^{13}C NMR spectrum showed six quaternary carbon signals at 133.83, 116.74, 136.98, 152.43, 137.54 and 129.25 ppm.

HMBC experiments allowed the assignment of the first four signals at ring A 1,2,3,4-tetrasubstituted which were respectively identified as the carbons C-2, C-3, C-4 and C-5; the last two signals were attributed to the carbons C-1′ and C-2′/C-6′ of the ring B 1,2,3-trisubstituted.

DP5/(*E*)-5-chloro-2-((2,6-dichlorophenyl)amino)benzyl 2-(6-((2,6-dichlorophenyl)imino)-3-oxocyclo hexa-1,4-dien-1-yl)acetate: the MS-TOF analysis showed a molecular ion peak at *m/z* 594.89 corresponding to molecular formula $C_{27}H_{17}Cl_5N_2O_3$. In the ^{1}H NMR spectrum, 12 signals were present, 10 of which were related to aromatic protons at 7.31, 7.30, 7.22, 7.10, 7.01, 6.93, 6.72, 6.63, 6.49 and 6.28 ppm. The other two were related to methylenes CH_2-8 and CH_2-8″, of which the second linked to an oxygen at 3.82 and 5.28 ppm. These signals were correlated to the carbons in the HSQC spectrum at 128.92, 130.63, 128.18, 129.33, 125.36, 125.84, 133.80, 128.81, 133.49, 117.06, 37.80 and 65.48 ppm, respectively. In addition to the signals of the protonated carbons, the ^{13}C NMR spectrum showed 11 quaternary carbon signals at 160.18, 144.45, 186.75, 143.55, 124.01 (x2), 141.98, 125.43, 124.36, 136.34, 129.33 (x2) and 169.63 ppm. HMBC experiments allowed for identification and definition of four aromatic rings, including two 1,2,3-trisubstituted and two 1,2,4-trisubstituted. One of the latter two was oxidized to *p*-quinone and an ester in which the acidic part was a residue of DCF oxidized at C-4 and the corresponding alcoholic part a residue of DCF chlorinated at C-4 and decarboxylated on the side chain.

DP6/5-chloro-2-((2,6-dichlorophenyl)amino)benzyl 2-(5-chloro-2-((2,6-dichlorophenyl)amino)phenyl) acetate: the MS-TOF analysis showed a molecular ion peak at *m/z* 615.61 corresponding to molecular formula $C_{27}H_{18}Cl_6N_2O_2$. In the ^{1}H NMR spectrum, 12 signals were present, 10 of which related to aromatic protons at 7.37, 7.35, 7.33, 7.23, 7.15, 7.10, 7.07, 7.04, 6.48 and 6.41 ppm. The other two related to methylenes CH_2-8 and CH_2-8″, of which the second linked to an oxygen, at 3.87 and 5.32 ppm. These signals were correlated to the carbons in the HSQC spectrum at 128.96, 128.12, 128.89, 129.52, 130.53, 130.95, 117.91, 119.70, 124.42, 125.31, 38.38 and 53.41 ppm, respectively. In addition to the signals of the protonated carbons, the ^{13}C NMR spectrum showed 11 quaternary carbon signals at 130.70, 126.94, 125.98, 136.38, 137.37, 141.49, 141.59, 125.55, 136.38, 137.37 and 170.01 ppm. HMBC experiments allowed for identification and definition of four aromatic rings, including two 1,2,3-trisubstituted and two 1,2,4-trisubstituted. There was also an ester function in which the acidic part was a residue of DCF chlorinated at C-4 and the corresponding alcoholic part a residue of DCF which was always chlorinated at C-4 but decarboxylated on the side chain.

DP7/4-chloro-2-((2,6-dichlorophenyl)amino)benzyl 2-(5-chloro-2-((2,6-dichlorophenyl)amino)phenyl) acetate: the MS-TOF analysis showed a molecular ion peak at *m/z* 615.48 corresponding to molecular formula $C_{27}H_{18}Cl_6N_2O_2$. In the ^{1}H NMR spectrum, 12 signals were present, 10 of which were related to aromatic protons at 7.37, 7.33, 7.30, 7.19, 7.15, 7.09, 7.04, 6.76, 6.49 and 6.40 ppm. The other two were related to methylenes CH_2-8 and CH_2-8″, of which the second linked to an oxygen, at 3.88 and 5.28 ppm. These signals were correlated to the carbons in the HSQC spectrum at 129.12, 131.01, 130.59, 128.50, 129.55, 125.49, 125.51, 121.50, 119.70, 117.75, 39.06 and 64.79 ppm, respectively. In addition to the signals of the protonated carbons, the ^{13}C NMR spectrum showed 11 quaternary carbon signals at 131.24, 125.82, 124.37, 171.51, 138.57, 136.33, 138.52, 141.57, 131.77, 136.33 and 137.65 ppm. HMBC experiments allowed for identification and definition of four aromatic rings, including two 1,2,3-trisubstituted and two 1,2,4-trisubstituted. There was also an ester function in which the acidic part was a residue of DCF chlorinated at C-5 and the corresponding alcoholic part a residue of DCF chlorinated at C-6 but decarboxylated on the side chain.

DP8/3,3″,5′-tris(chloromethyl)-N4,N4′,N4″-tris(2,6-dichlorophenyl)-[1,1′:3′,1″-terphenyl]-4,4′,4″-triamine: the MS-TOF analysis showed a molecular ion peak at *m/z* 856.29 corresponding to molecular formula $C_{39}H_{26}Cl_9N_3$. The ^{1}H NMR spectrum showed the presence of 14 signals, which, through the COSY spectra, were attributed to six different aromatic rings, including three 1,2,3-trisubstituted, two 1,3,4-trisubstituted and one 1,2,3,5-tetrasubstituted. The ^{1}H NMR spectrum also shows the presence of three signals relating to three chloromethylene functions. The presence of the three 1,2,3-trisubstituted rings indicated the presence of three residues of DCF, which also underwent an oxidative decarboxylation of the side chain and subsequent chlorination of the hydroxymethyl function. The connection of these residues was determined on the basis of ^{1}H-^{1}H COSY and HMBC correlations, as shown in Fig. 2.

DP9/4-chloro-*N*-(2,6-dichlorophenyl)-2-(methoxymethyl)aniline: the MS-TOF analysis showed a molecular ion peak at *m/z* 316.01 corresponding to molecular formula $C_{14}H_{12}Cl_3NO$. In the ^{1}H NMR spectrum, seven signals were present, of which five were related to aromatic protons at 7.41, 7.20, 7.13, 7.08 and 6.73 ppm. These correlated to the carbons in the HSQC spectrum at 128.88, 129.62, 128.44, 125.25 and 115.99 ppm. The ^{1}H NMR spectrum also revealed two signals related to a methoxyl and a methylene function at 3.44 e 4.62 ppm, which correlated to the carbons in the HSQC experiments at 57.55 and 73.31 ppm, respectively. In addition to the signals of the protonated carbons, the ^{13}C NMR spectrum showed five quaternary carbon signals at 141.79, 125.73, 124.55, 136.45 and 130.94 ppm. HMBC experiments assigned the first three to the aromatic ring A 1,2,4-trisubstituted, which were identified as the C-2, C-3 and C-4 carbons, respectively. The last two were attributed to the carbons C-1′ and C-2′/C-6′ of the ring B 1,2,3-trisubstituted.

DP10-DP14 were identified by the comparison of their spectroscopic data (EI mass spectrum, ^{1}H- and ^{13}C NMR spectra) with those of authentic standards.

Miyamoto et al. [27] reported that oxidation of DCF by HOCl resulted in formation of the monochloroderivatives to the positions 5 and 7 of ring A and of a generic dichloroderivative. From the hypochlorination of DCF, the formation of a generic monochloroderivative of the decarboxy-DCF was reported [28]. The latter was obtained by oxidative decarboxylation [29,30]. We isolated and determined the structure of mono-, di- and tri-chloroderivatives of decarboxy-DCF as well as three ester derivatives that contain DCF, a carboxy-DCF derivative, and a product with three residues of DCF. Soufan et al. [31] reported the UV data and masses of a generic decarboxy-DCF monochloride, that we have now isolated and structurally determined, and a plausible mechanism for its formation. We enlarged and presented this mechanism in Figs. 3 and 4, in the light of the isolated degradation by-products. In particular, the DCF could undergo an intramolecular cyclization with the formation of the DP10 lactone, which could then undergo saponification with the formation of the by-products DP12 and DP13 and the latter then lead to the by-product DP11. DCF could undergo chlorination on the amino function to obtain the intermediate *I1*, which through a carbocation intermediate could lead to the formation of its structural isomer *I4*, which by oxidative decarboxylation of the side chain would give the by-product DP1. The latter could evolve to its derivative DP9, or first undergo chlorination at ring A to obtain the derivative DP2 and then also to ring B to have its derivative DP3. The coupling reaction of the DP1 with the intermediate *I5* would explain the obtainment of the product

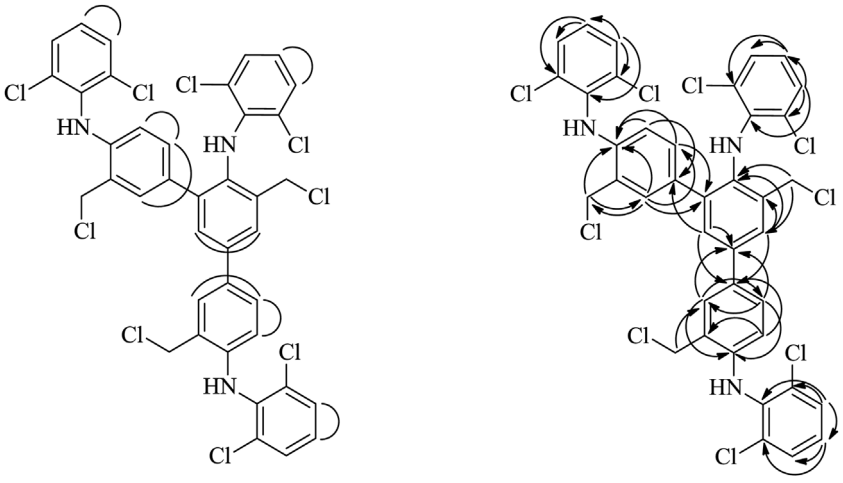


Fig. 2. Selected $^{1}H–^{1}H$ COSY (left) and HMBC (right) interactions of compound DP8.

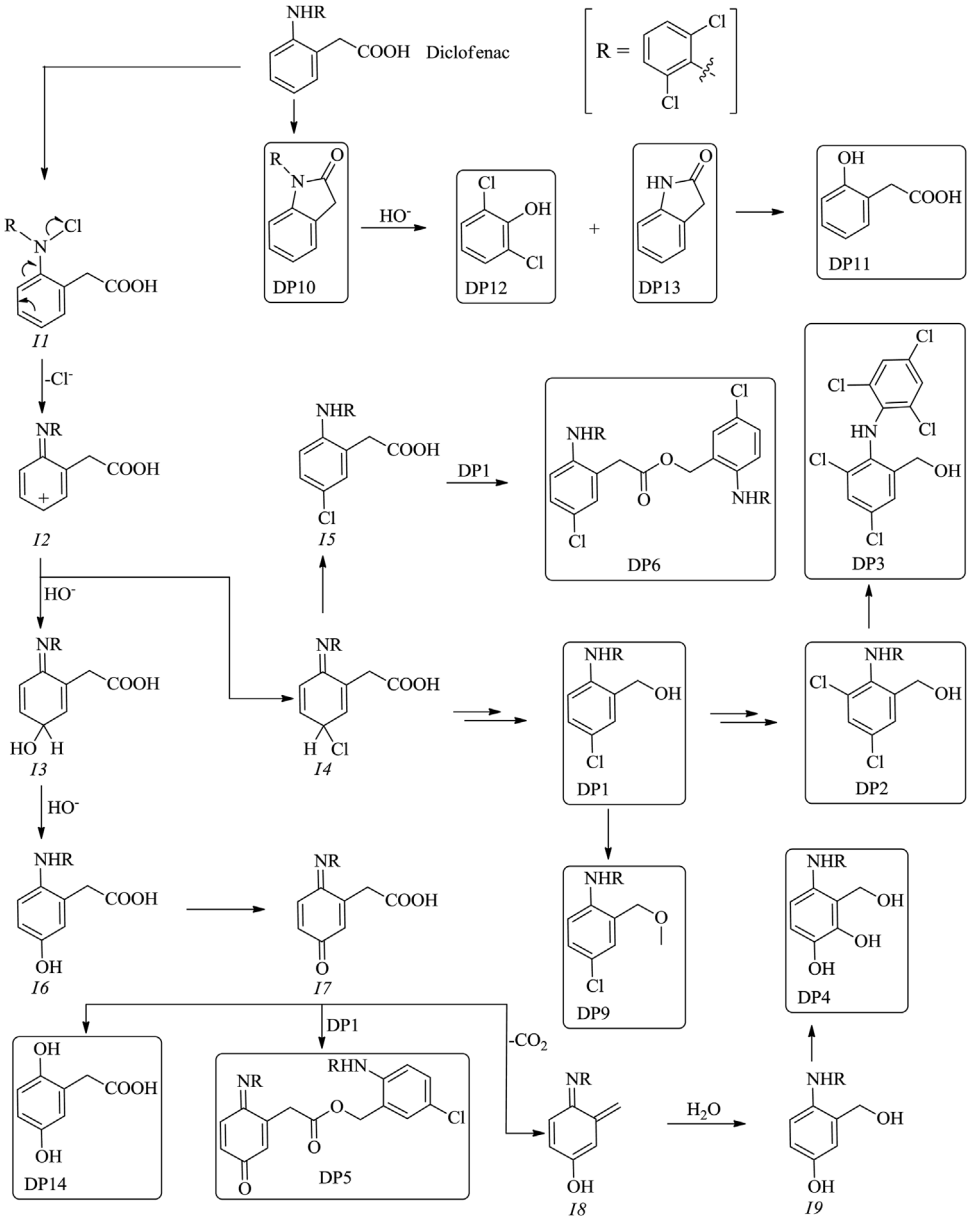


Fig. 3. Proposed mechanism for the formation of DP1 – DP6 and DP9 – DP14. Boxed structures represent isolated by-products.

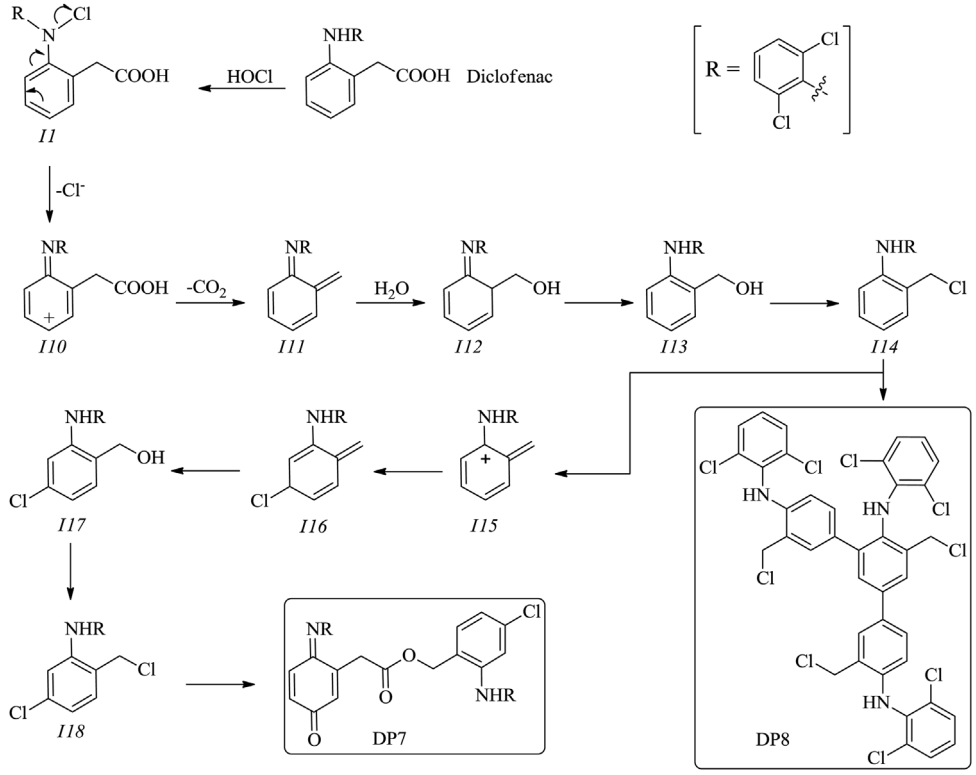


Fig. 4. Proposed mechanism for the formation of DP7 and DP8. Boxed structures represent isolated by-products.

Table 1
Toxicity effect of DCF on *A. fischeri*, *R. subcapitata* and *D. magna* presented as effective concentration able to promote 50 % effect (EC_{50}) and its confidence interval (95 % CI).

Species	EC_{50} mg L^{-1}	95 % IC mg L^{-1}
A. fischeri	14.09	10.94–20.28
R. subcapitata	19.05	15.58–23.30
D. magna	49.29	40.30–57.86

DP6. The intermediate *I1*, first by loss of the chloride ion and then by oxidation, could lead to the formation of the intermediate *I7*. The latter could be hydrolyzed to DP14, react with DP1 to form DP5, or undergo an oxidative decarboxylation of the side chain and subsequent oxidation of the ring A to give DP4. The intermediate *I1* could also undergo decarboxylation and subsequent chlorination to the side chain to obtain the intermediate *I14*, which could give its DP8 trimer or provide its derivative chlorine *I18*, which by reaction with *I7* leads to the formation of DP7.

3.3. Ecotoxicity data

Although not entirely clear for most ecological models, DCF is considered a compound that negatively affects non-target organisms belonging to different biological levels [32].

The effects of DCF on A. fischeri, R. subcapitata and D. magna in terms of EC_{50} are reported in Table 1. Species presented a great range of sensitivities with the following order of magnitude: *D. magna* < R. subcapitata < *A. fischeri*. In particular, the EC_{50} of DCF was estimated as 14.09 mg L^{-1} (10.94 mg L^{-1} to 20.28 mg L^{-1}), 19.05 mg L^{-1} (15.58 mg L^{-1} to 23.30 mg L^{-1}) and 49.29 mg L^{-1} (40.30 mg L^{-1} to 57.86 mg L^{-1}) for *A. fischeri*, *R. subcapitata* and *D. magna*, respectively. Despite a slight discrepancy which was expected due to biological variation, our results generally correlate well with those previously reported. Luminescent bacteria were the most sensitive biological model, and the EC_{50} values, measured here, are very close to those stated by Ferrer et al. [33](13.3 mg L^{-1} and 13.7 mg L^{-1}) and Zhang et al. [8] (13.8 mg L^{-1}).

DCF EC_{50} for *R. subcapitata* was similar to that reported by Ferrari et al. [34] (16 mg L^{-1}); DCF EC_{50} for *D. magna* was in accordance with that reported by Cleuvers [35] (68 mg L^{-1}) but was approximately two orders of magnitude below that published by de Oliveira et al. [32].

According to the results and the present EU-Directives 93/67/ECC on Risk Assessment for Existing Substances [36], DCF can be classified as "Harmful to aquatic organisms and may cause long term adverse effects in the aquatic environment".

Information about DCF by-product samples and toxicity data are displayed for single species effects in Fig. 5; the results were integrated according to Persoone et al. [25] in Fig. 6. Generally, the results from *A. fischeri* (Fig. 5) showed that six of nine DPs did not significantly influence the luminescence with an average residual toxicity of 0–16 %. Nevertheless, when bacteria were exposed to DP1, DP4 and DP5 samples, the luminescence inhibition greatly increased with an average residual toxicity of 56–91 %. Interestingly, compared to parent compound, toxicity in *A. fischeri* exposed to DP1 remained unchanged ($p > 0.05$).

To the best of our knowledge, scientific data on the aquatic toxicity of the DPs tested here is rather scarce. Nevertheless, there are several reports on DCF by-products that showed an increased toxicity compared to the parent compound in *A. fischeri*. However, the toxicity decreased following degradation of the intermediates [37,38].

Data from *R. subcapitata* showed that DP1 and DP5 can be more toxic than DCF at the same concentration. However, average residual toxicity was always > 42 %, suggesting that continuous exposure to DPs may lead to more adverse effects in algae compared to DCF. Daphnids evidenced the same increasing toxicity trend of *R. subcapitata* with DP1 and DP5 deemed most toxic. In this context, the results agree with Schmitt-Jansen et al. [39] findings of an increase

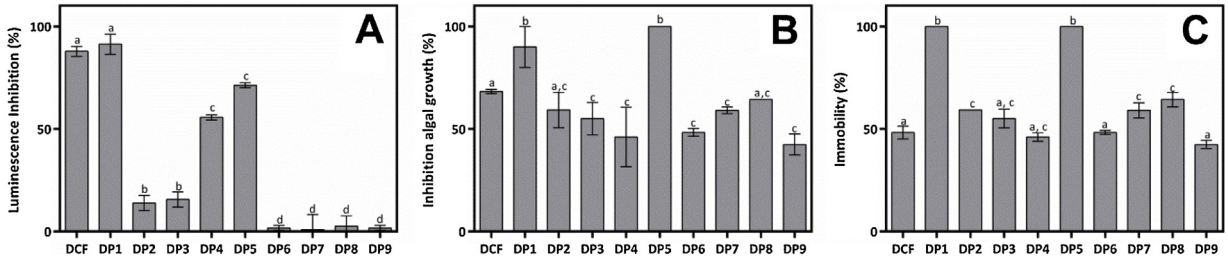


Fig. 5. Toxicity results of freshly prepared DCF solution (50 mg L^{-1}; initial concentration) and its DP (50 mg L^{-1}; initial concentration) including *A. fischeri* luminescence inhibition after 30 min contact time (A), *R. subcapitata* inhibition of algal growth (B) and *D. magna* immobility after 48 h contact time (C); data with different letters (a–c) are significantly different (Tukey's, $p < 0.05$); error bars represent standard deviation (n = 3).

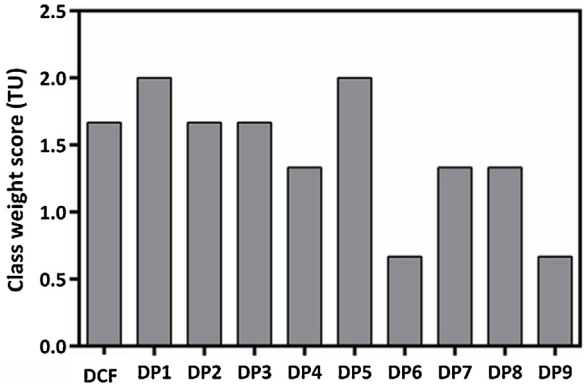


Fig. 6. Class weight score according to Persoone et al. (2003) and hazard classification for DCF and its DPs: no acute toxicity (TU < 0.4); slight acute toxicity (0.4 ≤ TU < 1); acute toxicity (1 ≤ TU < 10).

in the toxicity of DCF transformation products compared to the parent substance using *Scenedesmus vacuolatus*.

In regard to data integration [25] (Fig. 6), the results confirmed DP1 and DP5 as the most toxic compounds (TU 2); DP2 and DP3 had the same toxicity as DIC, while the other by-products had reduced effects. However, data supported the general issue related to the presence of residual slight acute hazard in DCF and DPs with score > 0.4 TU.

4. Conclusions

This study investigated the fate of DCF following disinfection treatment by chlorination. The reaction was carried out by simulating a chlorination process. After chlorination treatment, chromatographic techniques were used to isolate the 14 disinfection by-products, including nine new compounds, which were fully characterized by MS and NMR analyses. Just over 40 % of DCF underwent complete mineralization, while ~20 % was recovered and unchanged and almost 39 % was transformed into at least 14 disinfection by-products.

All nine compounds isolated for the first time are by-products chlorinated at the aromatic ring (DP1 — DP7, DP9) or in the side chain (DP8). Three of these appear to contain two (DP5 — DP7) or three (DP8) units of DCF. A mechanism explaining the achievement of isolated products has also been suggested. Considering the biological effect of DPs, DP1 and DP5 presented the highest potential to generate significant adverse effects on aquatic organisms, sometimes greater than DCF. Effects are strictly concentration-dependent, thus effects in real wastewater could be of limited impact, but gaps into the knowledge still remained about their potential interactive adverse effects not yet explored.

Author contributions

G.L. performed chlorination experiments; A.A. performed the mass spectrometry analysis; M.G., A.S., G.L. and L.S. performed acute and chronic toxicity tests; L.P. supervision; A.Z. designed the research and wrote the paper; A.Z. and G.D.F. review & editing.

Conflict of interest and authorship conformation form

Please check the following as appropriate:

√ All authors have participated in (a) conception and design, or analysis and interpretation of the data; (b) drafting the article or revising it critically for important intellectual content; and (c) approval of the final version.
√ This manuscript has not been submitted to, nor is under review at, another journal or other publishing venue.
√ The authors have no affiliation with any organization with a direct or indirect financial interest in the subject matter discussed in the manuscript

CRediT authorship contribution statement

Lucio Previtera: Supervision. **Giovanni Di Fabio:** Writing - review & editing. **Armando Zarrelli:** Writing - review & editing.

Declaration of Competing Interest

The authors have no conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jpba.2020.113762.

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