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Chemical characterisation of a new estuarine pollutant (2,4-Dichloro-6-Nitrophenol) and assessment of the acute toxicity of its quinoid form for *Artemia salina*.

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Abstract

It is known that the compound 2,4-dichloro-6-nitrophenol (2,4DC6NP) is formed upon nitration of 2,4-dichlorophenol, which in turn is a transformation intermediate of the herbicide dichlorprop. However, chemical and spectroscopic characteristics of 2,4DC6NP, as well as its toxicity, are poorly known.

This work shows that 2,4DC6NP behaves as a diprotic acid in aqueous solutions, with pK_a values of 3.0±0.9 and 4.9±0.5. At pH<3, 2,4DC6NP would undergo protonation. The absorption spectra suggest that anionic 2,4DC6NP, which prevails at pH>5 would have an ortho-quinoid structure that is responsible for the absorption peak centred at 428 nm. Considering that 2,4DC6NP has been detected in the brackish lagoons of the Rhône delta (Southern France), where its levels are comparable to those of the parent herbicide, it is necessary to use suitable organisms to examine possible effects of 2,4DC6NP on the species living in that environment. For this reason, the acute toxicity of the anionic form of 2,4DC6NP was assessed for the brine shrimp *Artemia salina*, a zooplankton species that lives both in brackish and in saline aquatic environments. The toxicity test yielded a LC_{20} value of 8±2 $mg \cdot L^{-1}$ and a LC_{50} value of 18.7±0.8 $mg \cdot L^{-1}$. Such values are safely higher than the maximum detected concentration of 2,4DC6NP in the Rhône delta lagoons, which allows exclusion of acute toxicity effects for brackish-water crustaceans. Further studies should be concentrated on the long-term effects of 2,4DC6NP, and in particular on its potential genotoxicity.

Keywords: Artemia salina, 2,4-dichloro-6-nitrophenol, acute toxicity, nitroaromatic compounds, saltwater crustaceans, pesticides.

1. Introduction

Polychlorophenols are a widespread category of pollutants with considerable impacts on human health and the environment. These compounds have been detected by means of several techniques [1-3] in many environmental matrices, in particular water and soil [4-6]. They showed relevant toxicological effects on aquatic species and humans, most notably upon alteration of the activity of antioxidant enzymes, which can result into disorder in antioxidant defences and in lipid peroxidation [7-10]. It has been found that chlorophenolic compounds are readily transformed in flooded paddy fields and that the transformation process gives a high yield of the corresponding nitroderivatives, which are more persistent than the parent compounds [6,11]. There is potential concern for the occurrence of a range of chloronitrophenols, and in particular of 2,4-dichloro-6-nitrophenol (2,4DC6NP) in the brackish lagoons of the Rhône river delta

(Southern France), which is an important area for nature conservation. Such an occurrence is a result of paddy-field water drainage into the lagoons [5]. Furthermore, the presence of the nitro group strongly influences the electronic distribution over the aromatic ring, with consequences on the reactivity of the molecule (*e.g.* decrease of pK_a, occurrence of additional intra- and inter-molecular interactions such as hydrogen bonding, possibly enhanced bioavailability because of higher hydrophobicity) [12,13].Therefore, the nitro group could considerably modify the toxicity of 2,4DC6NP compared to the parent compound 2,4-dichlorophenol (2,4DCP), and long-term effects are also possible in the case of 2,4DC6NP [14,15]. Experimental model systems and bioassays are largely used in ecotoxicology and environmental toxicology, to provide information for risk assessment, to register new chemicals as well as to investigate on their effects and mechanisms of action [16-19].

Artemia sp. (Crustacea, Anostraca), also called *brine shrimp Artemia*, is suggested for both field and laboratory testing. Tolerance of *Artemia sp.* specimens makes this genus adaptable to a great variety of testing conditions in estuarine, marine or hypersaline environments, from 2 to 204 $g \cdot L^{-1}$ of salt, thus responding to the actual demands of standardised tests for brackish and saline ecosystems [20-25].

An internationally recognised toxicity test with *Artemia sp*. is the APAT / IRSA-CNR [26,27]. Because this test employs an organism living in saline water, it can be very significant to the assessment of the acute toxicity of 2,4DC6NP for zooplankton species in the brackish lagoons of the Rhône delta, where the compound has been detected.

Due the importance of 2,4DC6NP in aquatic ecosystems, it is surprising that toxicity tests on zooplankton species are at the moment not available. The aim of the present work is the evaluation of the acute toxicity of 2,4DC6NP for the crustacean *Artemia salina*, with the purpose of assessing the short-term exposure risk for organisms living in the brackish waters of the Rhône delta lagoons.
It should be further noted that 2,4DC6NP is involved in acid-base equilibriums in aqueous solution. Depending on pH, nitrophenols can be transformed into the corresponding quinoid forms [28], which usually show an elevated toxicity [29]. Therefore, a preliminary assessment of the 2,4DC6NP forms occurring in water was carried out by means of acid-base titration and spectrophotometric characterisation.

2. Materials and methods

2.1. Chemical characterisation

The substance analysed in this work is the 2,4–dichloro–6–nitrophenol (2,4DC6NP, Purity ≥ 98.0%, Fluka). Nitrated phenols show an acid-base equilibrium that is influenced by a different electronic distribution over the conjugated ring compared to the corresponding non-nitrated compounds, due to the presence of the nitro group. The spectral characteristics of 2,4DC6NP were evaluated by UV-Vis Spectroscopy. UV-Vis absorption spectra between 300 and 600 nm were assessed for four aqueous solutions of 2,4DC6NP ($2.40 \cdot 10^{-4}$ M) at pH 3.0, 5.0, 7.4 and 9.0 (UV-Vis Lambda 25, Perkin Elmer Inc.). By fixing the pH value to 7.5, it was investigated the effect of the concentration of 2,4DC6NP on the shape of its UV-Vis absorption spectra between 300 nm and 600 nm. The solubility limit for 2,4DC6NP at pH 7.5 was also spectrophotometrically determined. After checking for the linearity of the absorbance at 428 nm versus the

concentration of 2,4DC6NP, a calibration curve was obtained in the concentration range $1.00 \cdot 10^{-9}$ M - $2.50 \cdot 10^{-4}$ M.

The acidic dissociation constants of 2,4DC6NP were determined by back-titration of the $2.40 \cdot 10^{-4}$ M solution of 2,4DC6NP, in the presence of $1.0 \cdot 10^{-3}$ M HCl, with $1.1 \cdot 10^{-3}$ M NaOH. The standardisation of the NaOH solution was performed in triplicate by titration of different amounts of potassium hydrogen phthalate (KHP, $KHC_8O_4H_4$, Purity ≥99.5%, Fluka).

2.2. Acute toxicity test

Immobilisation of brine shrimp was assayed using APAT procedures [27].The biological system used was *Artemia salina* (AAA Premium cysts, Great Salt Lake, USA). Artemia cysts (1 g) were added to a 35 $g \cdot L^{-1}$ saline solution (Instant Ocean, Aquarium Systems Inc., France) and incubated for 48h. During incubation, cysts were exposed for 1 hour to artificial light, provided by an Osram® L58W20 lamp [30]. The distance between lamp and cysts culture was adjusted to measure an irradiance value of 15±1 $W \cdot m^{-2}$. Irradiance measurements were carried out with a broadband radiometer (SKE510, Skye Inst. Ltd., calibrated in 2010) able to measure irradiance between 400-700 nm with a sensitivity curve of about 1 [31]. After light exposure, the culture remained in a dark environment until collection. Temperature was maintained at 25±2 ℃ by a thermal bath. Using a commercial air pump, a low flow of ultrapure air was generated and injected in the culture using a ceramic diffuser. The eventuality that ceramic diffuser and air pump released soluble compounds was checked, to avoid the presence of undesired potentially toxic substances in solution during incubation. For this reason, two identical incubators were filled with salt water and one of them was equipped with the ceramic diffuser and connected to the pump. After 24, 48 and 72 hours, samples of water from the two systems were collected and the absorbance was measured, as previously described [32,33]. No statistical differences were detected between 200 and 800 nm.

After incubation for 48 h, nauplii (instar II-III) were collected with a sterile Pasteur pipette and kept for testing. 20 nauplii were transferred to each 15 ml well of polystyrene plates (6 multiwell sterile plates with lids – BD Falcon, USA), containing the test solutions with 2,4DCNP (10 ml total volume). Negative controls (test solutions without 2,4DCNP) were included in each experiment. Experiments were conducted in triplicate for each tested concentration of 2,4DC6NP, as is reported in Table 1. After one day (24±1 h), the number of survivors was counted and recorded.

Test solutions were prepared with ultrapure water (Milli-Q, Millipore - USA) spiked with different amounts of 2,4DC6NP, in the concentration range 0.00– $2.40 \cdot \cdot 10^{-4}$ M. Salinity of 35 $g \cdot L^{-1}$ was obtained by adding pre-mixed marine salts (Instant Ocean®, Aquarium Systems Inc., France). Prior to analysis, the pH value was adjusted to 7.5 with NaOH (1M) or HCl (1M). The control of pH is of primary importance for both the validity of the test (the accepted pH range is between 6.5 and 8.5 pH units) and the molecular characteristics of 2,4DC6NP, which undergoes acid-base equilibriums. Test solutions were kept at 25±2 ℃ during the whole test duration by using a thermal bath.

The Normalised Mortality (fraction of immobilised individuals) for each concentration value of 2,4DC6NP was obtained by dividing the total number of died individuals in the triplicate tests for the total number of tested

organisms. Moreover, the “non effect” dilution was determined as the highest concentration value that yielded a Normalised Mortality lower than 0.2.

The Median Lethal Concentration LC_{50} was determined by fitting the experimental data points with a logistic function (equation 1). The LC_{50} value (x_c) is the concentration of 2,4DC6NP that yields a normalised mortality equal to the 50% of the maximum (a). The relative error was calculated following equation 2.

$$\text{Norm.Mort.} = \frac{a}{1 + e^{(-k \times ([2,4DC6NP] - x_c))}} \qquad \textit{(eq.1)}$$

$$\Delta[2,4DC6NP] = \left[\Delta x_C + \frac{\ln\left(\frac{a}{y} - 1\right) \times \Delta k - \frac{1}{\left(\frac{a}{y} - 1\right)} \times k \times \Delta a}{k^2} \right] \qquad \textit{(eq.2)}$$

The upper limit of the “no effect” dilutions (LC_{20}) was calculated using equations 1 and 2, by fixing the value of Normalised Mortality to 0.2.

3. Results

3.1. 2,4DC6NP spectrophotometric characterisation

The spectral characteristics of 2,4DC6NP were determined in aqueous solution as a function of pH. Figure 1 reports the absorption spectra (300-600 nm) of $2.40 \cdot 10^{-4}$ M 2,4DC6NP at different pH values (pH 3.0, 5.0, 7.4 and 9.0). The solution at pH 3.0 has an absorption maximum at 368 nm, while a shift of the maximum from 368 to 428 nm was observed at the higher pH values. An isosbestic point is present at 380 nm.

Considering that most surface waters have pH > 7, the effect of the concentration of 2,4DC6NP on the shape of its UV-Vis absorption spectra was investigated at pH 7.5. Figure 2 reports the UV-Vis spectra of 2,4DC6NP solutions at pH 7.5, between 300 and 600 nm. The spectra shape was not modified in the concentration range $1.00 \cdot 10^{-9}$ - $2.50 \cdot 10^{-4}$ M, yielding a linear trend of the maximum absorbance (428 nm) *vs.* the concentration of 2,4DC6NP. The linear trend excluded the presence of association phenomena for 2,4DC6NP at the tested concentration values, and allowed the construction of a spectrophotometric calibration curve.

Titration showed that 2,4DC6NP is a diprotic acid, with pK_{a1} and pK_{a2} values determined as 3.0±0.9 and 4.9±0.5, respectively. During the titration, at the first equivalence point, it was observed a colour change of the solution from transparent / pale yellowish to intense yellow. The 2,4DC6NP forms existing in the aqueous solution are thus the protonated one at pH < 3, the neutral one between pH 3 and 5, and the anionic one at pH > 5. The latter form is the one prevailing at the pH values of the tests of toxicity.

3.2. Acute toxicity test with Artemia salina

Before the acute toxicity tests, the concentration of each test solution was spectrophotometrically checked by comparing the absorbance at 428 nm with the calibration curve previously determined. Table 1 reports the tested concentrations of 2,4DC6NP, the number of replicates, and the relative Normalised Mortality values and standard deviations. Data between $1.0 \cdot 10^{-6}$ M and $2.40 \cdot 10^{-4}$ M (Normalised Mortality vs. Concentration of 2,4DC6NP) are shown in the plot of Figure 3. By using the determined fit parameters in eq.1 and eq.2, it was possible to determine the LC_{50} (18.7 ± 0.8 $mg \cdot L^{-1}$) and LC_{20} (8 ± 2 mg·L-1) values of 2,4DC6NP for *Artemia salina*.

4. Discussion

4.1. 2,4DC6NP spectrophotometric characterisation

The pH trend of the 2,4DC6NP spectra and the titration results are consistent with an acid-base equilibrium involving the presence of different species in solution, with different electronic distributions. The two absorption maxima at 368 nm and 428 nm can be associated to a π→ π* transition of different classes of chromophores. The energy gaps between the HOMO and the LUMO molecular orbitals (considering E= hc/λ and hc=1239.8424 eV·nm) would be 3.37 eV and 2.90 eV for the first (368 nm) and the second (428 nm) class of chromophores, respectively. The shift might be attributed to the presence of a tautomeric form of 2,4DC6NP, characterised by a conjugated structure involving the C-N bond of the nitro group. The protonation of nitro groups in substituted aromatic compounds has been demonstrated [34,35], together with the formation of stable quinone-like structures when the protonated group is in *para* or *ortho* position relative to the phenolic one [36,37]. For these reasons we hypothesise that 2,4DC6NP behaves like a diprotic acid in solution, able to generate three species (*a*, *b* and *c*), as described in figure 4. The anionic and the neutral forms of 2,4DC6NP (*a*, *b*) should be characterised by an *ortho*-quinoid structure, responsible for the absorption peak centred at 428 nm. The same structure is not hypothesised for the cationic form (*c*), where both the nitro group and the phenyl one would be protonated. The less extensive delocalisation of the π electrons of the aromatic ring for the *c* species could explain the shift of the absorption maximum towards lower wavelengths (368 nm).The presence of two dissociation constants as detected by titration and the colour change observed at the first equivalence point confirm our hypothesis. Moreover, the measured pK_{a2} value (4.9±0.5) is in good agreement with the pK_a value of 4.8 reported by Tehan et al. [12]. In moderately alkaline environments that can be found in brackish and saline waters, the anionic *ortho*-quinoid species (*a*) is predominant.

The chromophoric characteristics of a and b suggest a direct influence on the visible light attenuation coefficients within the water column, causing possible light limitations for aquatic species [38].

4.2. Acute toxicity test with Artemia salina

Apart from *Artemia salina*, toxicity data of 2,4DC6NP are only available with the ciliate *Tetrahymena pyriformis*. In this case the IGC_{50} value (50% inhibition growth concentration) for 2,4DC6NP is $1.8 \cdot 10^{-5}$ M [39], to be compared with $(9.0 \pm 0.4) \cdot 10^{-5}$ M for *Artemia salina* obtained in this work. It appears that the toxic effects of 2,4DC6NP can be detected in the 1-10 µM range in both protozoan and invertebrate tests,

although *Artemia salina* is slightly more resistant. Interestingly, the toxicity of quinones is comparable with that of non-phenolic nitroaromatic compounds [29], thus the quinone moiety and the nitro group could both contribute to the observed toxicity of 2,4DC6NP.

Considering the occurrence of 2,4DC6NP in the Rhône delta, in which a maximum value of 2.5±0.2 $\mu g \cdot L^{-1}$ and an average value (over all the sampling stations) of 0.5±0.6 $\mu g \cdot L^{-1}$ was determined, we can remark that the measured environmental concentrations are about 10^4 times lower than the LC_{20} of 2,4DC6NP determined for *Artemia salina* (8±2 $mg \cdot L^{-1}$, this work). Therefore the risk of mortality of invertebrates, related to acute toxicity phenomena for the presence of 2,4DC6NP in solution, is negligible for that environment. Also note that in the Rhône delta, 2,4DC6NP derives from 2,4DCP that is in turn a transformation intermediate of the herbicide dichlorprop. The maximum observed concentrations of 2,4DC6NP were not very far from those of dichlorprop, which suggests that the transformation yield of the herbicide into 2,4DC6NP was fairly high [5]. Therefore, there is little room for 2,4DC6NP in the delta lagoons to reach much higher concentration values than those observed, even with changing environmental conditions that might further increase the transformation yield of dichlorprop into 2,4DC6NP.

Deposition of 2,4DCP and/or 2,4DC6NP in the sediment [4], ligand-macromolecule interaction with other toxicants in water [40] and the bioaccumulation in higher organisms of the trophic web (*e,g.* plants, fishes or birds, with flamingos being a particularly significant species in the Rhône delta) [41-43] are possibilities that need to be investigated. It should also be noted that the genotoxicity of 2,4DC6NP, as shown by gene mutations and chromosomal aberrations [15], could constitute a long-term threat even in the absence of acute toxicity effects. The persistence of 2,4DCNP is of just a few weeks in the dissolved phase of the Rhône delta lagoons [44], thus any long-term effect could involve sediment, particles and trophic web accumulation.

4. Conclusions

The 2,4DC6NP behaves like a diprotic acid in solution and can generate *ortho*-quinoid species stabilised by the protonation of the nitro group. These species show a yellow colour in solution, due to delocalisation of the π electrons of the aromatic ring on the C-N bond of the nitro group. In moderately alkaline environments that can be found in brackish and saline waters, the anionic *ortho*-quinoid species is predominant.

The toxicity test of 2,4DC6NP for the brine shrimp *Artemia salina* at pH 7.5 yielded a LC_{20} value of 8±2 $mg \cdot L^{-1}$ and a LC_{50} value of 18.7±0.8 $mg \cdot L^{-1}$. Interestingly, these values are of the same order of magnitude as the IGC_{50} observed for 2,4DC6NP with the ciliate *Tetrahymena pyriformis* [39]. The LC_{20} and LC_{50} values determined in this study are safely higher than the maximum detected concentration of 2,4DC6NP in the Rhône delta lagoons, where acute toxicity effects toward brackish-water crustaceans can be excluded. Further studies should be concentrated into the long-term effects of 2,4DC6NP, and in particular on its genotoxicity. In addition, molecular dynamics calculations as well as interactions mechanisms of 2,4DC6NP with other molecules in solution will be investigated.

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TABLES

Table 1. Tested 2,4DC6NP concentrations (M) and number of replicates, associated with the determined Normalised Mortality values and its standard deviation (SD).

CONCENTRATION OF 2,4DC6NP TEST SOLUTIONS (M)	NUMBER OF REPLICATES	NORMALISED MORTALITY (FRACTION OF IMMOBILIZED INDIVIDUALS)
0	9	0.06 ± 0.06
$1.00 \cdot 10^{-8}$	3	0.10 ± 0.03
$1.00 \cdot 10^{-7}$	3	0.05 ± 0.03
$1.00 \cdot 10^{-6}$	3	0.10 ± 0.03
$1.00 \cdot 10^{-5}$	3	0.05 ± 0.05
$2.31 \cdot 10^{-5}$	3	0.08 ± 0.03
$4.50 \cdot 10^{-5}$	3	0.33 ± 0.06
$6.92 \cdot 10^{-5}$	3	0.33 ± 0.06
$9.00 \cdot 10^{-5}$	3	0.63 ± 0.06
$1.23 \cdot 10^{-4}$	3	0.65 ± 0.05
$1.50 \cdot 10^{-4}$	3	0.88 ± 0.03
$2.40 \cdot 10^{-4}$	3	1.00 ± 0.03

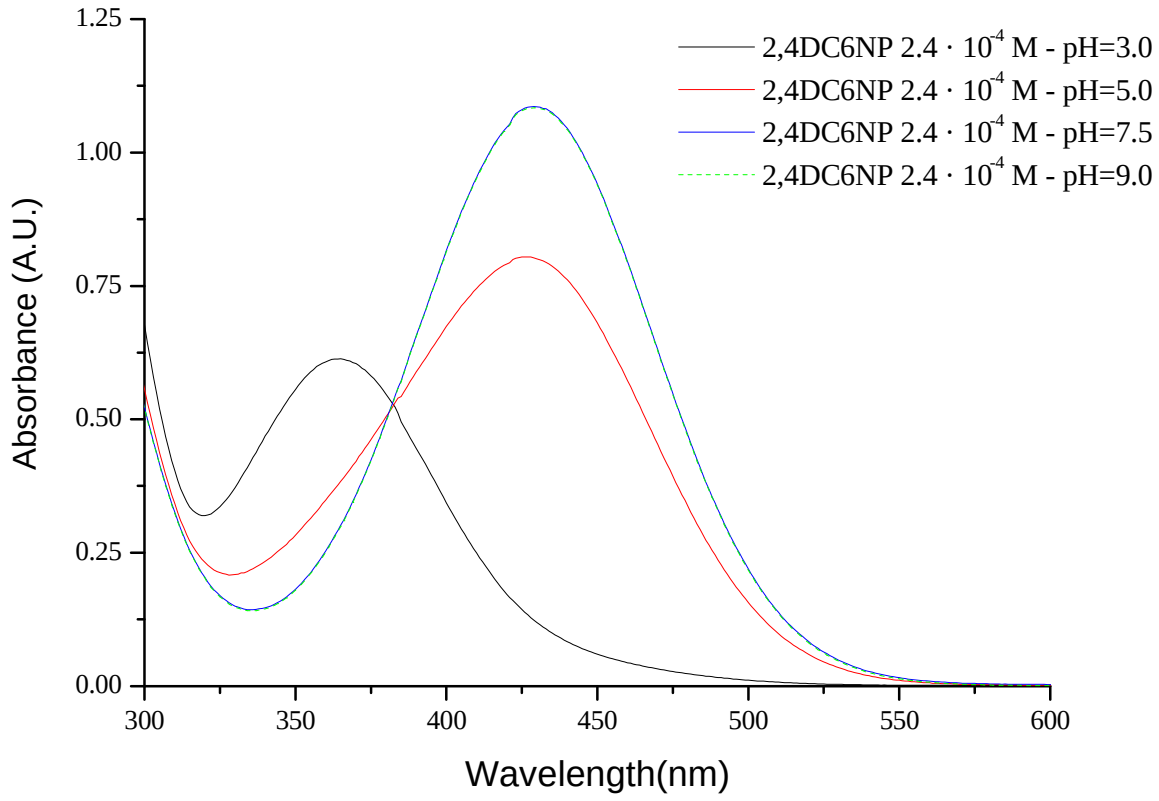

Figure 1. UV-Vis absorption spectra, between 300 and 600 nm, of four aqueous solutions of $2.40 \cdot 10^{-4}$ M 2,4DC6NP at different pH values (pH 3.0, ∗; pH 5.0 •; pH 7.5, △; and pH 9.0, ▽). The presence of different absorption maxima (368 nm and 428 nm) at different pH values is clearly shown.

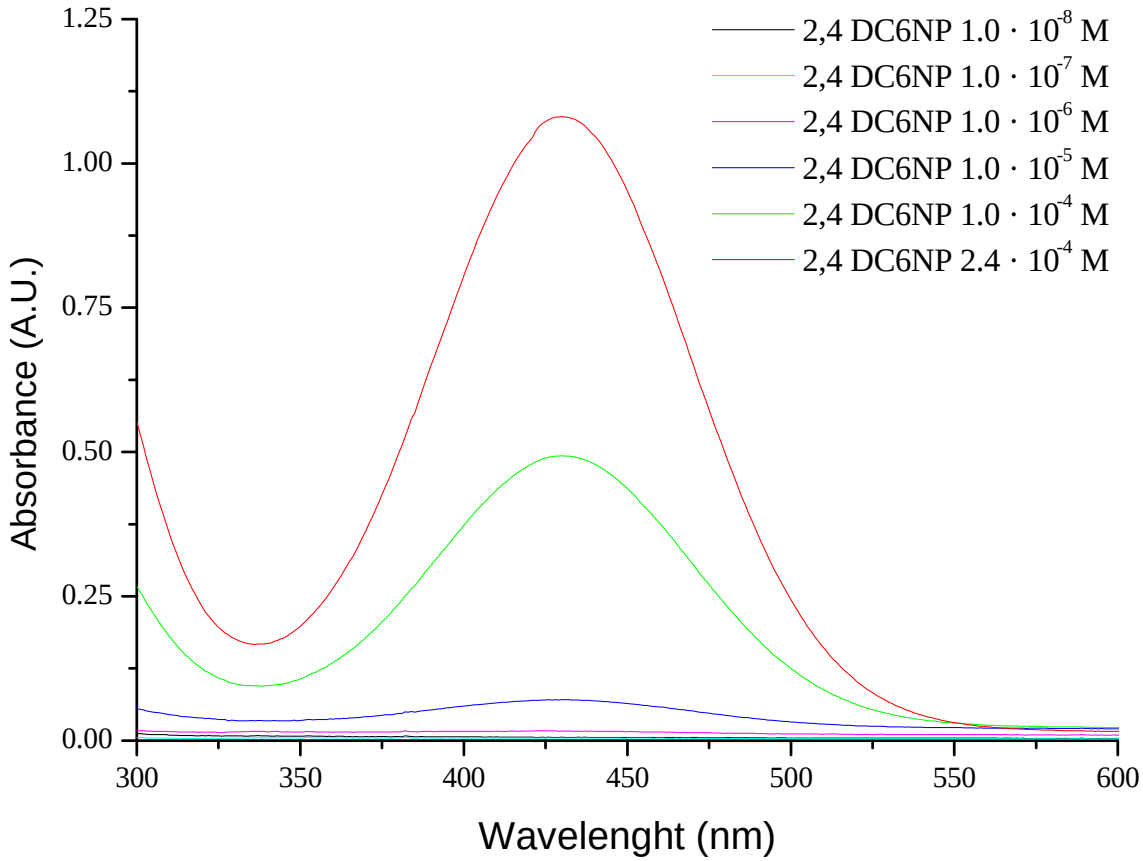

Figure 2. UV-Vis Spectra, between 300 and 600 nm, of the tested solutions of 2,4DC6NP in the concentration range $1.0 \cdot 10^{-8}$ M - $2.4 \cdot 10^{-4}$ M.

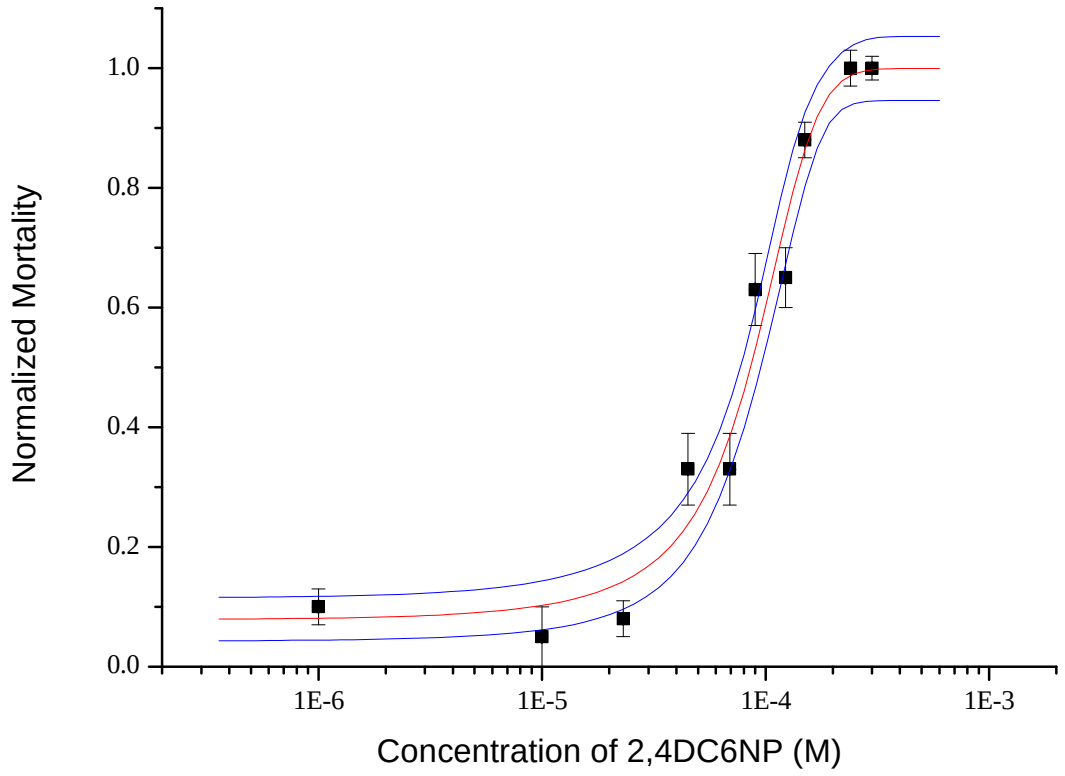

Figure 3. Plot of Normalised Mortality vs. Concentration of 2,4DC6NP. The fitting curve (solid) and the confidence (dash to dot) bands, with a confidence level of 0.95, are reported. The fitting curve was calculated with eq.1, with the hypothesis of a logistic distribution of experimental points. The best fitting parameters are: $a = 9.9 \cdot 10^{-1} \pm 0.2 \cdot 10^{-1}$; $x_c = 9.0 \cdot 10^{-5} \pm 0.5 \cdot 10^{-5}$; $k = 2.9 \cdot 10^{4} \pm 0.3 \cdot 10^{4}$; $Chi^2 = 0.003$; $R^2 = 0.985$. Concentrations below $1.0 \cdot 10^{-7}$ M are not shown.

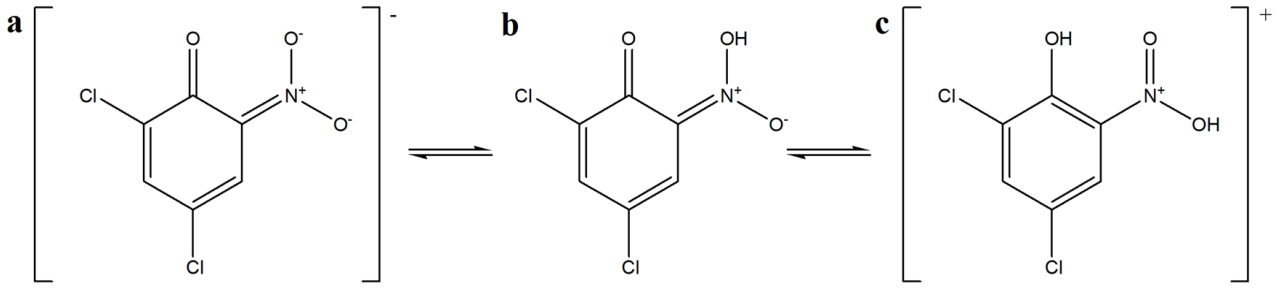

Figure 4. The chemical structure of the three tautomeric species of 2,4DC6NP are shown. The anionic (a) and the neutral (b) forms are characterised by an *ortho*-quinoid structure. This latter structure is not hypothesised for the cationic form (*c*).