Investigation of the Effect of Substituent Species/Positions and Numbers on Removal of Toxicity from Chloro and Nitro Phenol Compounds with Fenton and Fenton-like Processes

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Summary: Phenol derivatives containing substutient are used intensely in industry and their presence in surface and waste water is a problem requiring urgent solution due to their tendency for bioaccumulation, cancerogenic effects, high toxicity and weak biodegradability. In this study, the degradability and toxicity of chlorinated phenols 2-CP, 2,4-DCP and 4-CP and nitrated phenols 2-NP, 2,4-DNP and 4-NP were investigated. These phenols are included on the priority toxic pollutant list within the scope of clean water regulations according to both the US EPA-2014 and the European Union (2455/2001/CE) and form serious threats to public health and aqueous ecosystems.
The degradability of chloro and nitro phenols was researched by applying the Fenton/Fenton-like processes (under optimal conditions) and measuring the model pollutant concentrations, COD and TOC parameters. The effects of substituent type/position and number were determined with toxicity measurements using *Vibrio fischeri* bacteria (DIN/EN/ISO 11348-2). Statistical analysis was performed in detail for both Fenton/Fenton-like processes (T test) and toxicology measurement results (One-Way ANOVA) for the model pollutants (**P<0.05**).
In the first stage of the study, model pollutant removal of 95-100%, COD removal of 64-85% / 60-77% and TOC removal efficiency of 52-65% and 40-61% were achieved respectively with Fenton and Fenton-like processes.
In the second stage of the study, the results of toxicity measurements of the pollutants performed before processing found EC_{50}(mg/L) and toxic unit values (TU) were 8.10-12.34 for 2-CP, 2.24-44.67 for 2,4-DCP, 1.20-83.33 for 4-CP, 13.43-7.44 for 2-NP, 8.92-11.21 for 2,4-DNP, and 4.77-20.9 for 4-NP, respectively. After processing, the EC_{50}/EC_{20} and TU values were determined to fall to unobservable levels.
According to the order obtained with toxicity measurements of 4-CP > 2,4-DCP > 4-NP > 2-CP > 2,4-DNP > 2-NP, the chlor substituent had higher toxic effect compared to nitro. As the substituent numbers increase the toxicity increased; however, para position was identified to be more toxic compared to other positions. The reason for the 4 (para) position being more toxic than the 2,4 (ortho-para) position is thought to be due to the chlor or nitro linked to the 2 or ortho position binding to the OH group of phenol with a 5- and 6-member H-bridge in cis position forming a ring, which leads to inactivity.

Keywords: Advanced Oxidation Processes; Chlorophenols; Nitrophenols; *Vibrio fischeri*; Acute Toxicity; Fenton/Fenton-Like.

Introduction

Though industrial and technologic developments have eased life conditions in the last 50 years compared to the past, there is a dangerous rise in environmental pollution and risk to human health. Living organisms and environments are exposed to many organic and inorganic toxic chemicals with each passing day as a result of industrial, agricultural and domestic activities. This problem is increased progressively by the effect of accidents or uncontrolled use.

Representing the majority of toxic chemicals, phenol and substituent phenol species (SPS) have entered nearly all areas of life. The majority of surface and groundwater pollution is agriculturally sourced. One of the areas where SPS are mostly used is in industries producing pesticides (herbicides, fungicides, insecticides) for use with agricultural aims. Additionally, SPS are commonly used as a raw material in many industries producing plastics, polycarbonate, resin, wood preservers, medications, paint, petrol, petrochemicals, steel, textiles, in the organic synthesis sector, for disinfectant, wastewater chlorination, bleaching of paper pulp and antiseptics. They are abundantly released into the ecosystem in waste water from these industries. Additionally, the formation of substituent phenol species during chlorination of drinking and waste water and bleaching processes is unavoidable. This situation involves problems with taste and odor in drinking water [1-4]. Additionally, in addition to use as solvents, nitrophenols (NP) are used as intermediate material in synthesis of organophosphate pesticides, nitro paints and some

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medical products [5-6]. As listed above, these chemicals with very broad area of use in a variety of industries and daily life involve millions of tons of production and release into the environment annually, and are known to form an ecologic risk for aquatic organisms in surface and groundwater. From the surface and ground water included in the natural water cycle, SPS with cancerogenic and endocrine-disrupting characteristics mix with the food chain. Research into their removal is unavoidable due to causing serious problems in the ecosystem and for health. In the literature, as mentioned, CPs and NPs form an important problem in terms of public health due to estrogenic (teratogenic), mutagenic and carcinogenic effects (chronic toxicity) [7-12].

As is known, disinfection of drinking water with chlorine may create CP if phenolic compounds are present in water [7, 12-14]. At very low concentrations, formation of CP has negative effects on odor, color and taste. CPs are also known for harmful effects on living organisms. CPs firstly show corrosive effects on skin, eyes and respiratory tract, are rapidly absorbed when taken into the body by oral or respiratory routes and are known to mainly accumulate in liver, kidney, muscle and brain [15-20].

The toxicity of CPs and NPs and their continuous intensive use in many industries has led to environmental research about preserving their current importance for humans and the ecosystem by avoiding further risk and reclaiming polluted areas.

Included on the list of priority toxic pollutants by the Environmental Protection Agency (EPA) [21], removal of SPS, in the class of micropollutants, is not appropriate or economic with conventional treatment processes [22-25].

As SPS, which have highly toxic effects and are resistant to degradation (refractory), disrupt enzyme activity in microorganisms by inhibition and prevent efficient operation of most biological treatment plants, the majority are discharged without treatment. Advanced treatment methods can contribute to biologically treatment of these materials by removing toxic effects. Advanced treatment methods like advanced oxidation, adsorption-biosorption, solvent extraction and reverse osmosis are methods used for treatment of CP and NP [26]. As a result, in the present study advanced oxidation methods with appropriate cost of the Fenton and Fenton-like processes were used, along with studies about the removal of ecotoxic effects with the *Vibrio fischeri* bacteria based on photobioluminescence methods (ISO11348-2 standard). Toxicological tests are appropriate tests to obtain current and significant results ecologically, but it is important to choose the appropriate test organism [27]. In this context bioindicator species provide significant measurements with more rapid and sensitive response to environmental pollution and variations [28]. As is known, toxicity tests are used with basic aims like determining hot spots in groundwater or surface water, identifying toxicity in waste water and changes in toxicity linked to time, and determining toxicity of chemicals alone or mixed [29-31]. As one of the main targets in our study is determination of toxicity shown by toxic chemicals alone (before and after processing), we chose one of the rapid and standard tests of the luminescence inhibition test. Microorganisms are often chosen in direct toxicity research due to both cost and having a broad range in terms of speed and effort [32-37]. One of the most commonly chosen rapid toxicity tests due to test duration, sample volume, appropriate cost, result sensitivity and lack of ethical problems is the microtox acute toxicity test, performed with bioluminescent bacteria and widely used especially in European countries [38]. The only bacteria species isolated with bioluminescence capability is *Vibrio fischeri*, a gram-negative heterotrophic bacterium belonging to the Vibrionaceae family which live in saline aqueous environments at decomposer trophic level, one of the tree basic trophic levels. *Vibrio fischeri* (also known as Photobacterium fischeri named after Bernhard Fischer, a German bacteriologist) is a gram-negative, rod-shaped, flagellated, non-pathogenic bacterium, ubiquitously distributed in sub-tropical and temperate marine environments [39]. The test developed with vibrio strains later took the name microtox. As the concentration of toxic pollutants in the sample increases, there is a reduction in the light intensity radiated by the bacteria and this reduction is measured luminometrically [40].

Our study on one hand investigated conditions for advanced preliminary treatment to increase biological treatment efficiency and on the other researched the effect of species, position and number of the substituent species on toxicological removal efficiency of SPS.

In this study, the substituent phenol species (SPS) used as input material in many industries and with high toxic effects of 2-CP, 2,4-DCP, 4-CP and 2-NP, 2,4-DNP, 4-NP were used. Their physicochemical characteristics are listed in the table below (Table-1).

Table-1: Physical and chemical properties of substituent phenol species [1, 41, 42].

Compound	CAS No	Formula	Melting point (°C)	Boiling point (°C)	Density (g /cm³)	Solubility (g/L)	Log Kow	Kh (L.liq/L.gaz)	Kb (L/mg.hr)	pKa
2-Chlorophenol	95-57-8	C_6H_5ClO	9.3	174.9	1.26	28.5	2.17	2.29E-05	0.00023	8.49
2,4-Dichlorophenol	120-83-2	$C_6H_4Cl_2O$	45	210	1.38	4.5	3.2	1.96E-04	0.0033	7.68
4-Chlorophenol	106-48-9	C_6H_5ClO	43.2–43.7	220	1.22	27.1	2.4	4.50E+00	0.001182	8.85
2-Nitrophenol	88-75-5	$C_6H_5NO_3$	45	216	1.49	2.5	1.85 [43- 45]	4.09E+00	0.000649	7.23
2,4-Dinitrophenol	51-28-5	$C_6H_4N_2O_5$	114.8	312	1.68	1.97	1.54 [43- 45]	2.09E-04	0.00062	4.09
4-Nitrophenol	100-02-7	$C_6H_5NO_3$	113.8	279	1.5	7.51	1.91 [43- 45]	1.35E-06	0.00036	7.15

Bioaccumulation coefficient (K_{ow}), acidity constant (pKa), aerobic biodegradation rate (K_b), Henry coefficient (K_h)

The advanced treatment and toxicity removal efficiencies of substituents were investigated, with the effects of substituent species (chloro and nitro), substituent number in mono and disubstituent species (2-CP, 2,4-DCP, 4-CP, 2-NP, 2,4-DNP, 4-NP) and substituent position (ortho, para) in species with different positions (2-CP, 4-CP, 2-NP, 4-NP) examined. The most commonly used classic advanced treatment techniques of Fenton and Fenton-like processes were researched in detail for their effect on removal efficiency (pollutant, COD, TOC, toxic effect).

Experimental

Materials

In our study, SPS of 2-CP, 2,4-DCP, 4-CP and 2-NP, 2,4-DNP, 4-NP (Merck) were used by preparing synthetic samples. Firstly, optimal conditions for both the Fenton process and Fenton-Like process were investigated. Identification of optimal conditions used a six-paddle, Phipps-Bird controlled mixer and optimized H_2O_2, Fe^{2+}, Fe^{3+}, pH and temperature parameters in the first stage. Necessary readings were made with a MERCK Spectroquant-Prove 300 UV/VIS brand spectrophotometer using previously created standard calibration curves.

Methods

COD analysis was performed with the phthalate method stated in standard methods [46] at 148 °C with a Spectroquant TR420 thermoreactor and the spectrophotometer mentioned above. Analysis of model pollutants used the 4-aminoantipyrine method (direct photometric method) mentioned in standard methods [46-47] spectrophotometrically, with TOC analysis performed with a Schimadzu TOC-L brand device. Hydrogen peroxide analysis [48] was performed by reading with a spectrophotometer at 464 nm according to the tri iodine method.

Toxicologic analyses were completed with a Dr. Lange LUMIStox 300 luminometer with Hack, LCK482 *Vibrio fischeri* kits according to the DIN/EN/ISO 11348-2 method [49]. Experimental flow schema is presented in Fig. 1.

For determination of optimal conditions according to the order in the manuscript, the pollutants underwent Fenton and Fenton-like processes. The formation of hydroxyl radicals produced actively and at high concentration and the reaction stages for advanced oxidation processes are given for both Fenton and Fenton-Like processes, respectively.

Fenton Oxidation

The Fenton process is based on the reaction of the Fe^{+2} ion with hydrogen peroxide under acidic conditions. As a result of this reaction, hydroxyl radicals are formed [50-54].

$$Fe^{+2} + H_2O_2 \rightarrow Fe^{+3} + OH^{\bullet} + OH^{-}$$

The iron ion begins by separating H_2O_2, it is catalyzed and forms hydroxyl radicals. Radical formation occurs in a complex reaction chain in aqueous solutions.

$$OH^{\bullet} + Fe^{+2} \rightarrow OH^{-} + Fe^{+3}$$

The formed ferric ions catalyze hydrogen peroxide separating it into water and oxygen. Iron ions and radicals form in the reactions [50-54].

$$Fe^{+3} + H_2O_2 \rightarrow Fe\text{-}OOH^{+2} + H^{+}$$
$$Fe\text{-}OOH^{+2} \rightarrow HO_2^{\bullet} + Fe^{+2}$$
$$Fe^{+2} + HO_2^{\bullet} \rightarrow Fe^{+2} + O_2 + H^{+}$$
$$OH^{\bullet} + H_2O_2 \rightarrow H_2O + HO_2^{\bullet}$$
$$Fe^{3+} + HO^{2\bullet} \rightarrow Fe^{2+} + O_2 + H^{+}$$
$$RH + HO^{\bullet} \rightarrow R^{\bullet} + H_2O$$
$$R^{\bullet} + Fe^{3+} \rightarrow Fe^{2+} + \text{product}$$

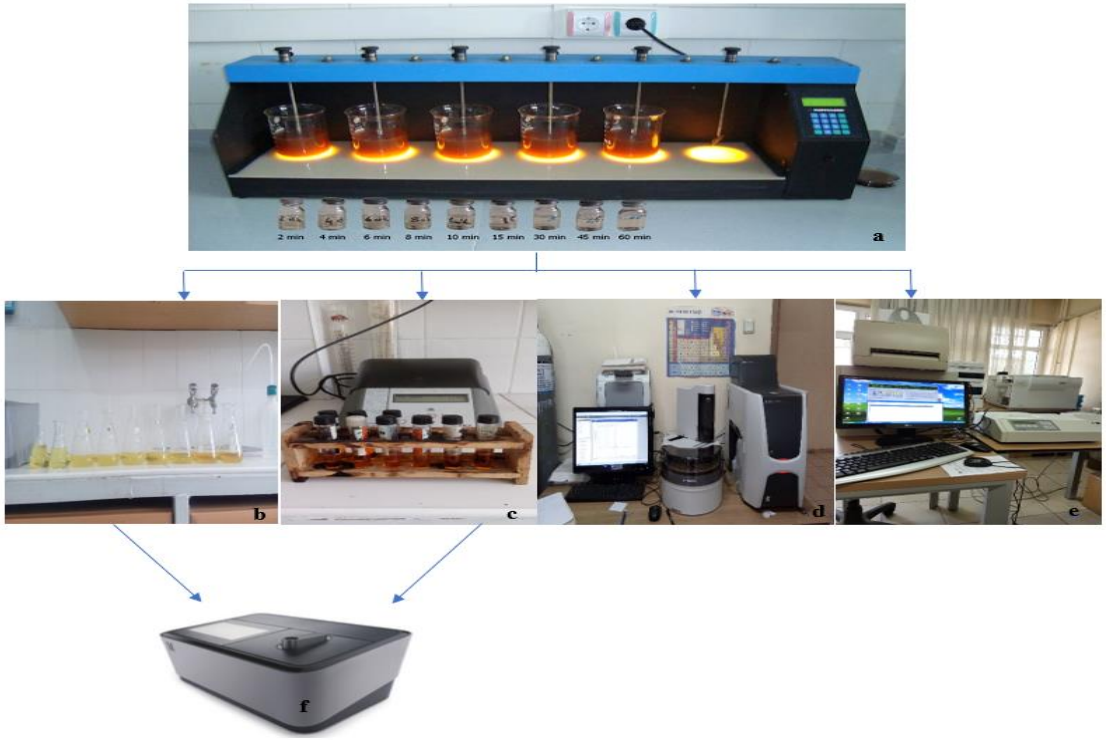


Fig. 1: Experimental flow schema.
a. Samples taken from pollutants at certain time intervals in Phipps-Bird controlled mixer
b. Model pollutant analysis (4-aminoantipyrine method)
c. COD analysis (Spectroquant TR420 thermoreactor)
d. TOC analysis (Schimadzu TOC-L)
e. Toxicology analysis (Dr. Lange LUMIStox 300 luminometer)
f. Identification of pollutant and COD removal efficiencies (MERCK Spectroquant-Prove 300 UV/VIS)

Removal Efficiency for 2-CP, 2,4-DCP, 4-CP and 2-NP, 2,4-DNP, 4-NP with Fenton Oxidation

For Fenton oxidation of each pollutant (6 different species), initially 200 mg/L concentration was taken from stock solutions to determine the optimum conditions for H_2O_2, Fe^{2+}, pH, and temperature parameters. After determining optimum conditions, the results for pollutant, COD and TOC removal efficiencies (from three replicate) were identified and are presented in Table-2 and Fig. 2 and 3.

Table-2: Removal efficiency of substituent species with the Fenton process.

Process	Species of Substituent	Pollutant Removal Efficiency (%)	COD Removal Efficiency (%)	TOC Removal Efficiency (%)
Fenton Process	2-CP	95.5	85.5	66
	2,4-DCP	96	72.8	54
	4-CP	97	75	60.9
	2-NP	96	70	56
	2,4-DNP	95	64	52
	4-NP	98	74	64

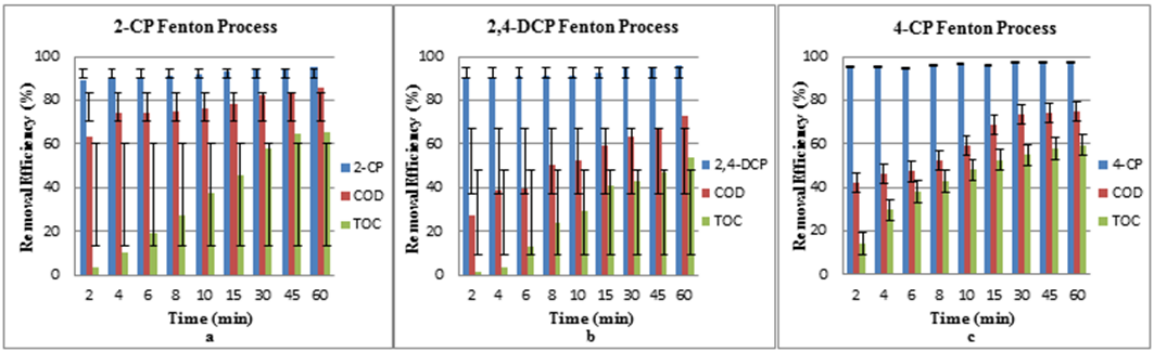


Fig. 2: Removal efficiency with the Fenton process (optimum conditions).
a. For 2-CP (C_0=200 mg/L, $[H_2O_2]$=500 mg/L, $[Fe^{+2}]$=50 mg/L, pH=2.5, t=60 min, 20±1^{0}C)
b. For 2,4-DCP (C_0=200 mg/L, $[H_2O_2]$=350 mg/L, $[Fe^{+2}]$=35 mg/L, pH=2.7, t=60 min, 20±1°C)
c. For 4-CP (C_0=200 mg/L, $[H_2O_2]$=600 mg/L, $[Fe^{+2}]$=70 mg/L, pH=3, t=60 min, 20±1°C)

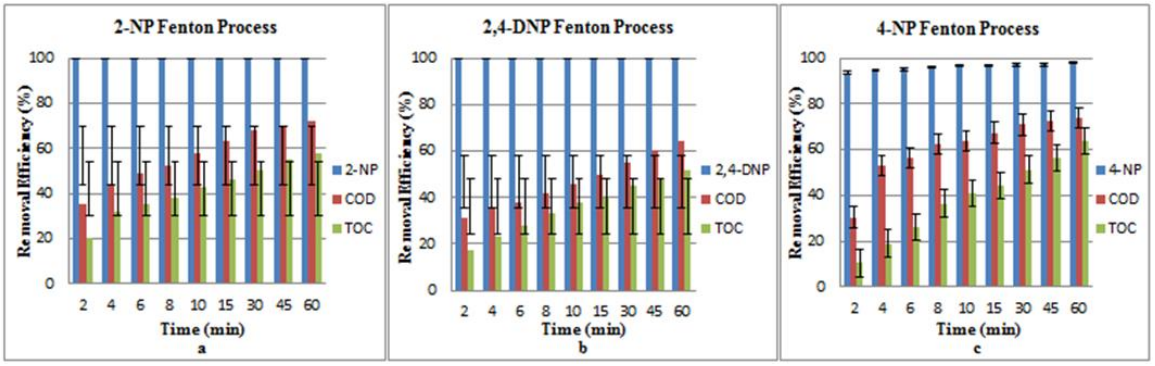


Fig. 3: Removal efficiencies for Fenton process (optimum conditions)
a. For 2-NP (C_0=200 mg/L, $[H_2O_2]$=500 mg/L, $[Fe^{+2}]$=50 mg/L, pH=2.59, t=60 min, 20±1°C)
b. For 2,4-DNP (C_0=200 mg/L, $[H_2O_2]$=200 mg/L, $[Fe^{+2}]$=30 mg/L, pH=2.89, t=60 min, 20±1°C)
c. For 4-NP (C_0=200 mg/L, $[H_2O_2]$=600 mg/L, $[Fe^{+2}]$=50 mg/L, pH=2.5, t=60 min, 20±1°C)

Fenton-Like Oxidation

The Fenton-like process is based on the reaction of the Fe^{+3} ion with H_2O_2 under acidic conditions. As a result of this reaction, again hydroxyl radicals form, as shown below [50-54].

$Fe^{+3} + H_2O_2 \rightarrow Fe(HO_2)^{+2} + H^+$
$FeOH^{+2} + H_2O_2 \rightarrow Fe(OH)(HO_2)^+ + H^+$
$Fe(HO_2)^{+2} \rightarrow Fe^{+2} + HO_2^\bullet$
$Fe(OH)(HO_2)^+ \rightarrow Fe^{+2} + \mathbf{HO^\bullet} + OH^-$
$H_2O_2 + Fe^{2+} \rightarrow OH^\bullet + OH^- + Fe^{+3}$
$OH^\bullet + RH \rightarrow R^\bullet + H_2O$

Removal Efficiencies for 2-CP, 2,4-DCP, 4-CP and 2-NP, 2,4-DNP, 4-NP with Fenton-Like Oxidation

For Fenton-like oxidation of each pollutant (6 different species), initially 200 mg/L concentration was taken from stock solutions to determine the optimum conditions for H_2O_2, Fe^{3+}, pH, and temperature parameters. After determining optimum conditions, the results for pollutant, COD and TOC removal efficiencies (three replicate) were identified and are presented in Table-3 and Fig. 4 and 5.

Table-3: Removal efficiencies for substituent species with Fenton-Like processes.

Process	Species of Substituent	Pollutant Removal Efficiency (%)	COD Removal Efficiency (%)	TOC Removal Efficiency (%)
	2-CP	92	77.1	61.3
	2,4-DCP	97	70	52.5
Fenton–Like	4-CP	97.5	75	61
Process	2-NP	95	65.8	45
	2,4-DNP	94	60	40
	4-NP	99.5	71.4	60.1

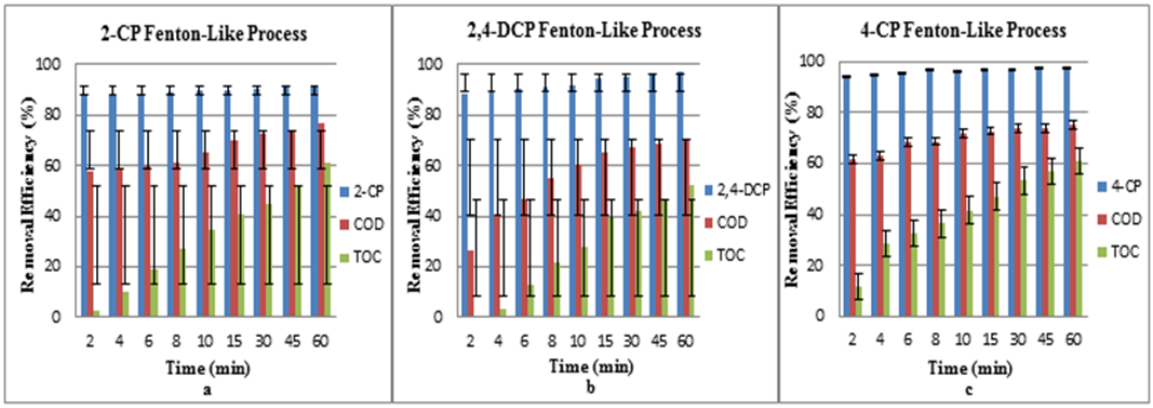


Fig. 4: Removal efficiencies with Fenton-Like process (optimum conditions).
a. For 2-CP (C_0=200 mg/L, [H_2O_2]=500 mg/L, [Fe^{+3}]=50 mg/L, pH=4, t=60 min, 20±1°C)
b. For 2,4-DCP (C_0=200 mg/L, [H_2O_2]=350 mg/L, [Fe^{+3}]=50mg/L, pH=3, t=60 min, 20±1°C)
c. For 4-CP (C_0=200 mg/L, [H_2O_2]=500 mg/L, [Fe^{+3}]=60 mg/L, pH=3, t=60 min, 20±1°C)

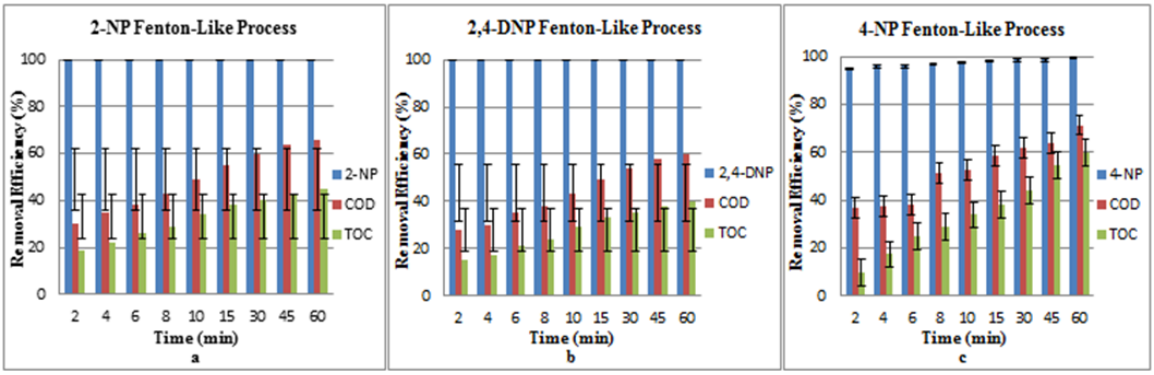


Fig. 5: Removal efficiencies with Fenton-Like process (optimum conditions).
a. For 2-NP (C_0=200 mg/L, [H_2O_2]=500 mg/L, [Fe^{+3}]=65 mg/L, pH=4, t=60 min, 20±1°C)
b. For 2,4-DNP (C_0=200 mg/L, [H_2O_2]=200 mg/L, [Fe^{+3}]=30 mg/L, pH=3.44, t=60 min, 20±1°C)
c. For 4-NP (C_0=200 mg/L, [H_2O_2]=600 mg/L, [Fe^{+3}]=40 mg/L, pH=2.5, t=60 min, 20±1°C)

Bioluminescent Toxicity Measurements

The ***Vibrio fischeri*** luminescent bacteria test was completed according to DIN/EN/ISO 11348-2 [49]. The properties of the *Vibrio fischeri* toxicity test are stated in Table-4. The *Vibrio fischeri* luminescent bacteria stored in a deep freeze must be activated before the test. For this, firstly 12 ml reactivation solution is left at 15 °C for 30 minutes in the LUMIStox device. Bacteria removed from the freezer are left in a water bath for 2 minutes to ensure they reach room temperature. Reactivation solution is poured onto the bacteria and they are left for 15 min at 15 °C. Thus, bacteria are activated for use in the test. Toxicity is assessed by measuring inhibition of luminescent bacteria after 30 minutes incubation at 15 °C with EC_{50} / EC_{20} values and accuracy data calculated according to DIN/EN/ISO 11348-2 [40].

While pollutants underwent the *Vibrio fischeri* toxicity test before and after processing, firstly $K_2Cr_2O_7$ was used as reference to ensure standardization and the toxicity on the test organism was measured as 4.1 mg/L. This value was determined to be within the limits stated by the ISO method.

Toxicity test results are stated as EC_{50}. The EC_{50} value is the concentration of chloro/nitro phenol compound that causes 50% inhibition/death. Data defined in EC_{50} were given as toxic units (TU) using the following equation [55].

$$TU = [1/E(L)C_{50}]*100$$

Table-4: Properties of the selected ecotoxicological test.

Test	Trophic Level	Group of Organisms	Type of Test	Test Duration	Test Criterion	Test Principles
Microtox (*Vibrio fischeri*)	**Decomposer**	**Bacteria**	**Acute**	**30 min.**	**Inhibition of Luminescence**	**Measurement of Luminescence reduction with luminometer**

Toxicity Measurements of 2-CP, 2,4-DCP, 4-CP and 2-NP, 2,4-DNP, 4-NP before/after Fenton and Fenton-Like processes

At the initial concentration of 200 mg/L pollutant, no significant EC_{50} value was reached, so each pollutant was analyzed at values equivalent to 20 mg/L (by diluting 10 times).

Based on 20 mg/L initial concentration of 6 different pollutants, the EC_{50} and calculated toxic unit (TU) obtained as a result of *Vibrio fischeri* toxicity tests repeated 3 times for 30 minutes before and after both processes are given collectively in Table-5, with TU graphs given below (Fig. 6-8).

Statistical Analysis

Statistical Analysis of Fenton/Fenton-Like Processes for Model Pollutants

Our model pollutants (2-CP, 2,4-DCP, 4-CP and 2-NP, 2,4-DNP, 4-NP) had the T test performed to compare the removal efficiencies (pollutant/COD/TOC) of 3 repeated Fenton and Fenton-Like processes. Test results are given in Table-6 and Table-7.

Statistical Analysis for Toxicity Tests of Model Pollutants

In our toxicology studies there were two dependent and two independent variables.

Dependent variables	Independent variables
1. Inhibition (%)	**1. Time (min)**
2. EC_{50} (mg/L)	**2. Toxic material concentration (mg/L)**

According to *Vibrio fischeri* toxicity tests, initially all pollutants had falling EC_{50} values and increasing % inhibition values as the exposure duration to toxic material and concentration of toxic material increased (Tables 8-9; Fig. 9-10).

Results and Discussion

When we tested the pollutant, COD and TOC removal efficiencies for the Fenton and Fenton-like processes, the Fenton process had higher removal efficiencies compared to the Fenton-like process, especially in terms of COD and TOC. The Fenton-like reaction is slower than the Fenton reaction and allows formation of Fe^{+2} and HOO* (hydroperoxyl radicals) in an effective circular mechanism. The hydroperoxyl radicals have slower reactions with organic pollutants compared to hydroxyl radicals [56]. Finally, the main target is to ensure mineralization and minimize toxicity of pollutants via the Fenton and Fenton-like processes [57-60]. The removal efficiencies after both processes are presented below.

For both the Fenton and Fenton-like processes, the reaction conditions should always be acidic (pH: 2-4). The reason is that Fe^{+2} may precipitate as Fe^{+3} salts in neutral or basic pH. As a result, the pH limitation is always present when working with Fe salts. Another situation is that to stop activation at the end of the reaction, the medium pH should be increased to 9-10 as H_2O_2 is an oxide. During this process, Fe immediately precipitates as a brown sludge. In order to prevent this naturally occurring turbidity affecting measurement results, it is necessary to remove the turbidity by one hundred percent filtration. The greatest handicaps for the Fenton and Fenton-like reactions are the factors just listed (acidic pH limitation, interactive effects due to the oxidizing power of H_2O_2 with Fe sludge forming at large volumes and turbidity) [61].

Based on experimental data as seen in Tables 2 and 3 and Figures 2-5, removal efficiency with the Fenton process is higher than the removal efficiency of the Fenton-like process. When previous studies related to this topic are investigated, the reaction of the main oxidizing material of H_2O_2 with water when Fe^{+2} salts are used as catalyst in the Fenton process is given by [62-63];

$$Fe^{+2} + HO\text{-}OH \rightarrow Fe^{+3} + HO^{\bullet} + OH^{-}$$

As seen from the reaction, H_2O_2 is degraded and forms the main oxidizing agent in AOP of HO● (hydroxyl radical). The hydroxyl radical forming at high concentrations and high rates has redox potential of 2.8 volts and has no selectivity. In the reaction medium, it forms in vivo and enters reactions degrading organic pollutants at the same rate [64].

With the Fenton-like process, Fe^{+3} salts are chosen as catalyst instead of Fe^{+2} salts. The Fe^{+3} salts enter reactions with the main oxidizing material of H_2O_2 in water as follows [62-63];

$Fe^{+3} + HO\text{-}OH \rightarrow Fe^{+2} + HOO^{\bullet} + H^{+}$

As seen from the reaction, when H_2O_2 is degraded, this time the $HOO^{\bullet}$ (hydroperoxyl radical) forms instead of the hydroxyl radical. Forming with slower rate compared to hydroxyl radical formation, the hydroperoxyl radical has redox potential of 1.78 volts with lower power and rate for organic matter degradation compared to the potential of hydroxyl radicals. Additionally, as emphasized in articles, the $HO^{\bullet}$ radical degrades organic matter without any selectivity, while the $HOO^{\bullet}$ radical acts more selectively in degrading organic matter. It is considered the efficiency difference between the Fenton and Fenton-like processes for the same pollutant species is due to the reasons explained above.

The *Vibrio fischeri* bacteria used in our ecotoxicology tests was chosen due to advantages such as ability to obtain rapid results, high repeatability, lack of ethical problems and cheap costs [38]. The properties of these bacteria are presented in Table-4.

The toxicity test results for both processes (before and after the processes) are tabulated in the Table-5.

Table-5: *Vibrio fischeri* toxicity test results measured for 30 min before and after both processes.

Species of Substituent	Before Process			After Process			
				Fenton		Fenton-Like	
	EC_{50} (mg/L)	References	TU	EC_{50} (mg/L)	TU	EC_{50} (mg/L)	TU
2-Chlorophenol	**8.10**	**8.05-34.75** [65-67, 71]	**12.34**	**No Detected ***	**NO**	**No Detected ***	**NO**
2,4-Dichlorophenol	**2.24**	**0.62-3.03** [66-67,71]	**44.67**	**No Detected ***	**NO**	**No Detected ***	**NO**
4- Chlorophenol	**1.20**	**0.91-1.35** [66,67]	**83.33**	**No Detected ***	**NO**	**No Detected ***	**NO**
2-Nitrophenol	**13.43**	**10.18-16.68** [68,69]	**7.44**	**No Detected ***	**NO**	**No Detected ***	**NO**
2,4-Dinitrophenol	**8.92**	**4.80-8.93**[70]	**11.21**	**No Detected ***	**NO**	**No Detected ***	**NO**
4-Nitrophenol	**4.77**	**4.01-5.54** [66]	**20.96**	**No Detected ***	**NO**	**No Detected ***	**NO**

*: Co concentration did not reach 50% inhibition
NO: **EC_{50}** and **EC_{20}** values not identified

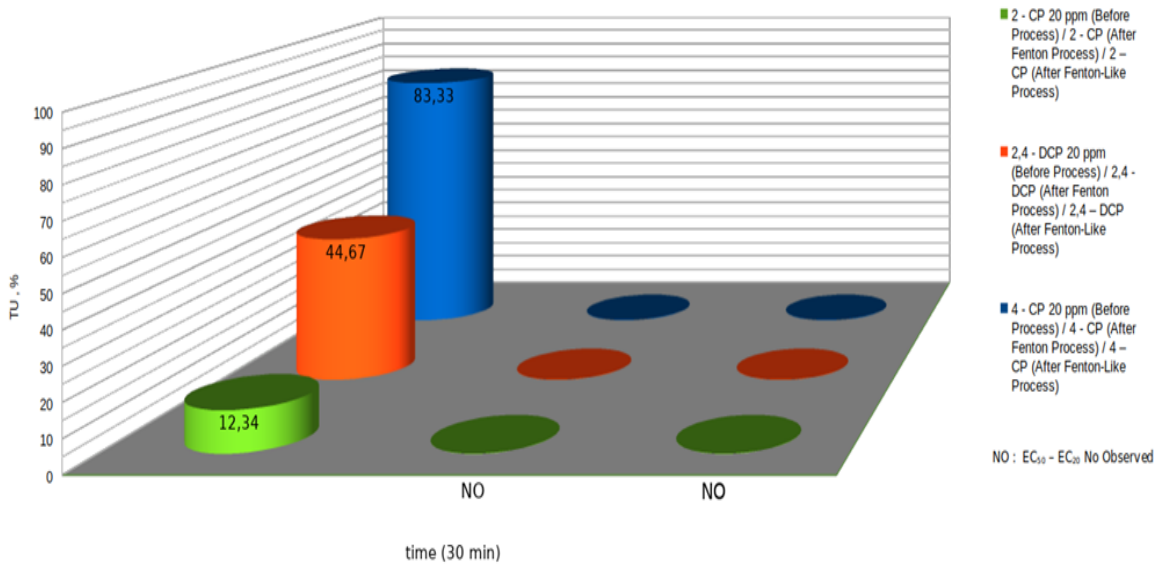


Fig. 6: *Vibrio fischeri* toxicity test results measured for 30 min before and after both processes for CPs.

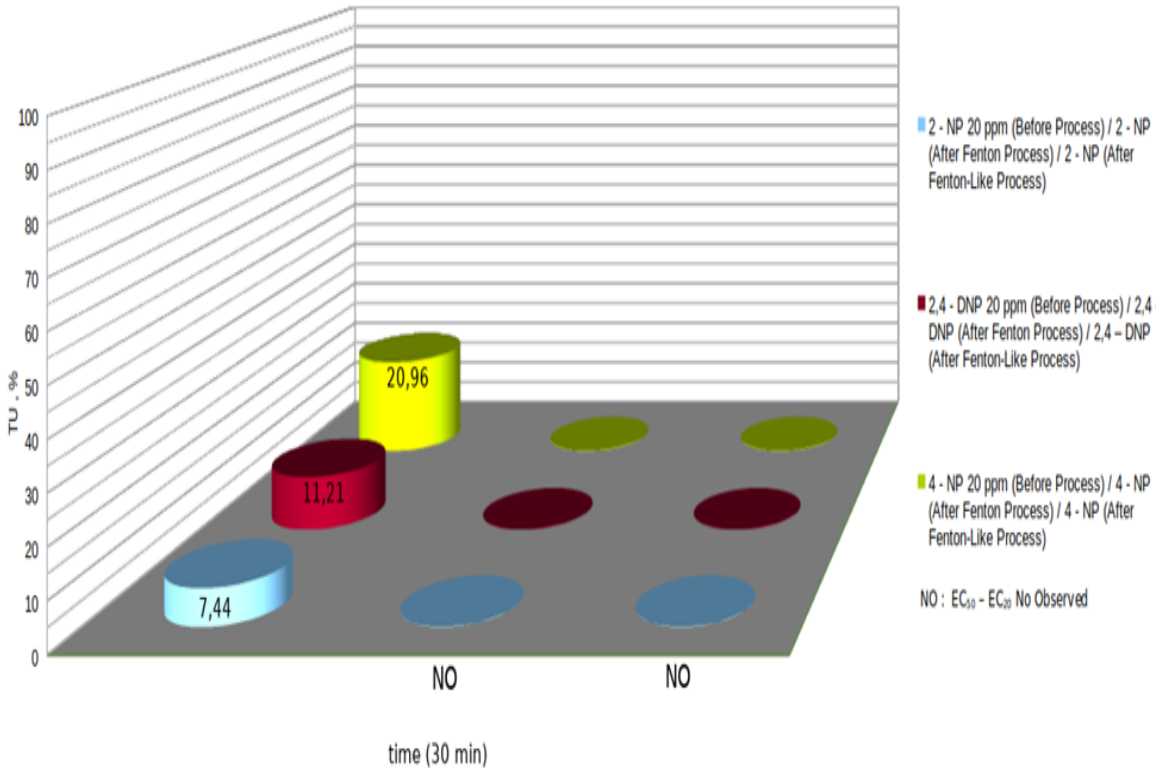


Fig. 7: *Vibrio fischeri* toxicity test results measured for 30 min before and after both processes for NPs.

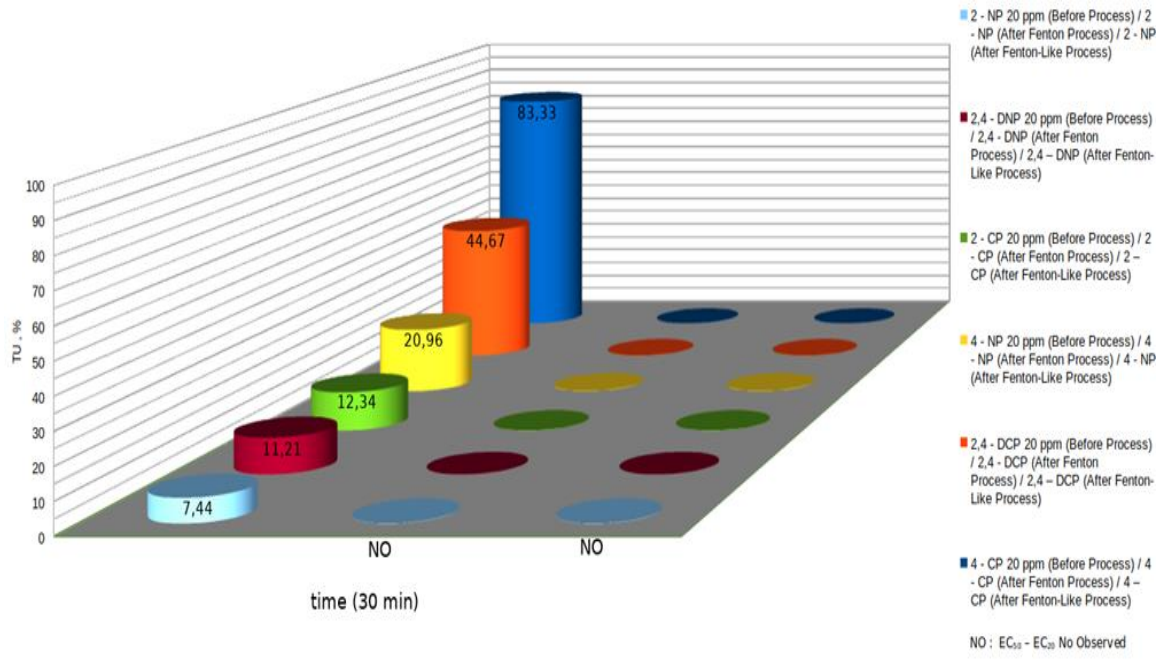


Fig. 8: *Vibrio fischeri* toxicity test results measured for 30 min before and after both processes for CPs and NPs.

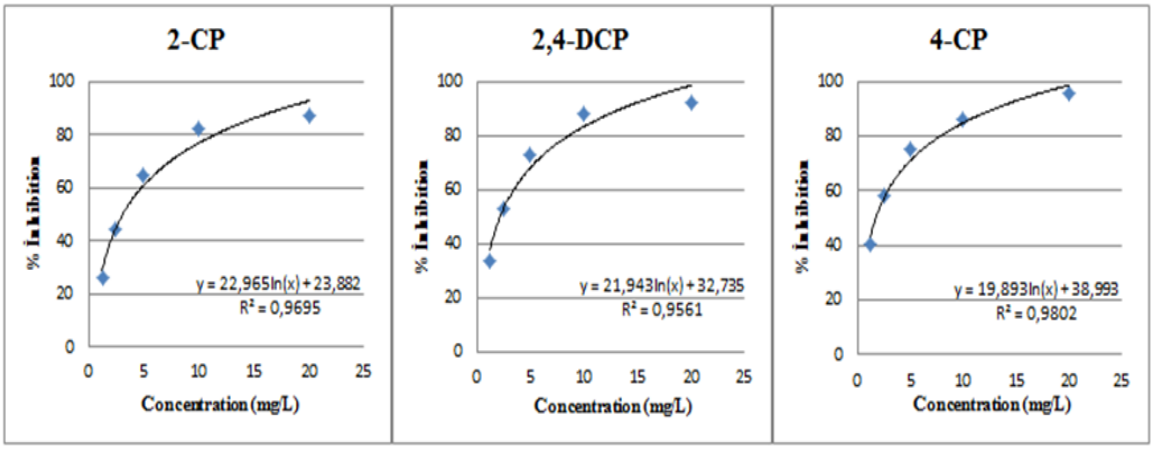


Fig. 9: % inhibition values against increasing CP concentration.

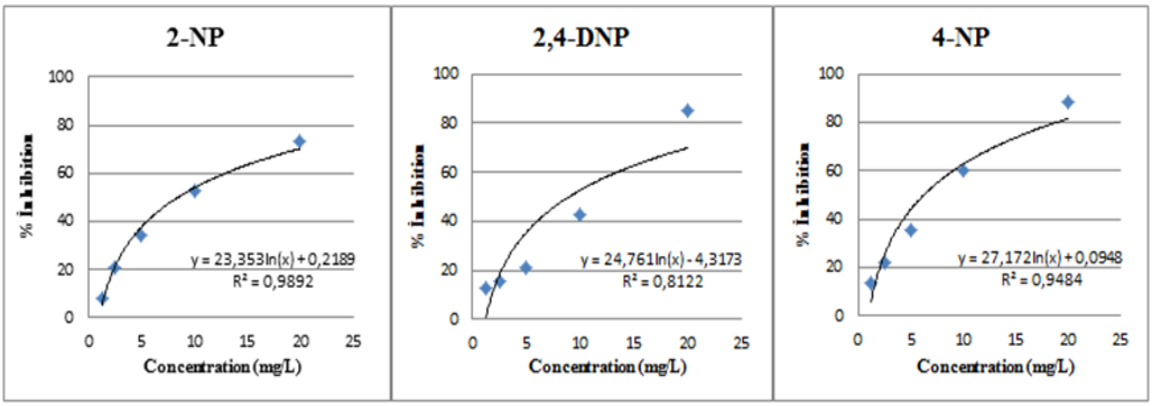


Fig. 10: % inhibition value against increasing NP concentration.

As shown by Person et al., TU results can be classified based on the acute toxicity classification method. The classification is as follows [55]:

Class I (no acute toxicity, TU<0.4)
Class II (mild acute toxicity, 0.4<TU<1)
Class III (acute toxicity, 1<TU<10)
Class IV (high acute toxicity, 10<TU<100)
Class V (very high acute toxicity, TU≥100)

The TU values before and after processing for each pollutant in the research are summarized in Table-5. As seen in Table-5, for the 6 different pollutants, 4-CP has highest toxicity while 2-NP has lowest toxicity. The general toxicity classification of pollutants before Fenton and Fenton-like processes are in the order;

4-CP (Class IV) > 2,4-DCP (Class IV) > 4-NP (Class IV) > 2-CP (Class IV) > 2,4-DNP (Class IV) > 2-NP (Class III).

Five pollutants have different levels of high acute toxicity, while only 2-NP appears to have normal acute toxicity.

Statistical analysis in toxicology studies was performed with the One-Way ANOVA test. Statistical analysis firstly investigated the correlation between toxic matter concentration and EC_{50} value. As a result of this investigation it was determined that as toxic matter concentration increased, the EC_{50} value decreased and the %inhibition value increased. Our analysis was completed at α=0.05 significance level. The effect of the increase in pollutant concentration on EC_{50} value was determined in the 5^{th}, 15^{th} and 30^{th} minutes. Later the One-Way ANOVA test was used to determine whether there were differences in EC_{50} values between the different time periods (5^{th}, 15^{th} and 30^{th} minutes) and whether there were differences in pollutant concentration at these time periods. The differences in time and concentrations for EC_{50} values belonging to each pollutant were identified with the Tukey multiple

comparison test. Results of the statistical analysis are tabulated in the Table-6.

As seen in Table-6, the COD, TOC and pollutant removal efficiency for the pollutants were higher for the Fenton process compared to the Fenton-Like process.

There were significant differences identified for pollutant, COD and TOC removal efficiencies for 2-CP **(P<0.05)** (Table 7). While there were no significant differences between the pollutant and TOC removal efficiencies for 2,4-DCP and 4-CP, there was a significant difference identified for the COD removal efficiency (**P<0.05).** While there were no significant differences observed between the pollutant and COD removal efficiencies for 2-NP, there was a significant difference identified for the TOC removal efficiency (**P<0.05).** There was no significant difference observed for 2,4-DNP pollutant removal efficiency, while there were significant differences identified for COD and TOC removal efficiencies (**P<0.05).** There were no significant differences for pollutant and COD removal efficiencies for 4-NP, while there was a significant difference identified for TOC removal efficiency (**P<0.05).**

Based on the data in Tables 8-9, the details of our statistical analyses are presented below.

Table-6: T test results for removal efficiencies of model pollutants with the processes.

Substituent species	Process		N	% Efficiency
2-CP	Pollutant	Fenton	3	94
		Fenton-Like	3	91.5
	COD	Fenton	3	84.3
		Fenton-Like	3	75.6
	TOC	Fenton	3	64
		Fenton-Like	3	60.1
2,4-DCP	Pollutant	Fenton	3	95.1
		Fenton-Like	3	96
	COD	Fenton	3	71.4
		Fenton-Like	3	69.1
	TOC	Fenton	3	52.6
		Fenton-Like	3	51.4
4-CP	Pollutant	Fenton	3	96.1
		Fenton-Like	3	97.5
	COD	Fenton	3	74.5
		Fenton-Like	3	72.6
	TOC	Fenton	3	60
		Fenton-Like	3	60.4
2-NP	Pollutant	Fenton	3	95.5
		Fenton-Like	3	94
	COD	Fenton	3	68
		Fenton-Like	3	64.8
	TOC	Fenton	3	54.3
		Fenton-Like	3	43.6
2,4-DNP	Pollutant	Fenton	3	94
		Fenton-Like	3	93.1
	COD	Fenton	3	63.1
		Fenton-Like	3	59
	TOC	Fenton	3	51.1
		Fenton-Like	3	39
4-NP	Pollutant	Fenton	3	97.5
		Fenton-Like	3	98.8
	COD	Fenton	3	72.6
		Fenton-Like	3	71
	TOC	Fenton	3	63.5
		Fenton-Like	3	59.9

N: Repeat number

Table-7: T test results for model pollutants.

		t	DOF	P
	Pollutant	**3.873**	**4**	**0.018**
2-CP	**COD**	**7.839**	**4**	**0.001**
	TOC	**2.945**	**4**	**0.042**
	Pollutant	**-1.147**	**4**	**0.315**
2,4-DCP	**COD**	**3.481**	**4**	**0.025**
	TOC	**1.292**	**4**	**0.266**
	Pollutant	**-2.53**	**4**	**0.065**
4-CP	**COD**	**4.158**	**4**	**0.014**
	TOC	**-0.722**	**4**	**0.51**
	Pollutant	**2.324**	**4**	**0.081**
2-NP	**COD**	**2.562**	**4**	**0.063**
	TOC	**7.155**	**4**	**0.002**
	Pollutant	**1.147**	**4**	**0.315**
2,4-DNP	**COD**	**5.568**	**4**	**0.005**
	TOC	**16.344**	**4**	**0**
	Pollutant	**-2.53**	**4**	**0.065**
4-NP	**COD**	**1.581**	**4**	**0.189**
	TOC	**8.227**	**4**	**0.001**

t: calculation value **DOF:** degree of freedom **P:** significance

Table-8: For 30 min duration, % inhibition values against increasing CP concentration.

Concentration (mg/L)	2-CP Inhibition (%)	2,4-DCP Inhibition (%)	4-CP Inhibition (%)
1.25	**26.39**	**33.93**	**40.43**
2.5	**44.14**	**52.68**	**58.07**
5	**64.68**	**73.13**	**75.13**
10	**81.9**	**88.4**	**86.02**
20	**87.1**	**92.12**	**95.4**

Table-9: For 30 min duration, % inhibition values against increasing NP concentration.

Concentration (mg/L)	2-NP Inhibition (%)	2,4-DNP Inhibition (%)	4-NP Inhibition (%)
1.25	**8.1**	**12.81**	**13.51**
2.5	**20.65**	**15.75**	**21.76**
5	**34.65**	**21.31**	**35.14**
10	**52.52**	**42.6**	**60.32**
20	**73.1**	**85.2**	**88.4**

The results of correlation analysis for all pollutant species (6 different species), as seen in Table-10 and 11, showed there were linear negative correlations identified between both increasing pollutant concentration and EC_{50} values for *Vibrio fischeri* bacteria in the 5th, 15th and 30th minutes and between time and EC_{50} values for *Vibrio fischeri* bacteria in the 5th, 15th and 30th minutes.

Our analyses were completed at α=0.05 significance level, with the effect of increasing pollutant concentration on EC_{50} values revealed in the 5th, 15th and 30th minutes.

Table-10: Concentration-EC_{50} correlation coefficients for each pollutant species.

Pollutant type	Correlation Coefficient
2-CP	**R= -0.737**
2,4-DCP	**R= -0.657**
4-CP	**R= -0.598**
2-NP	**R= -0.747**
2,4-DNP	**R= -0.742**
4-NP	**R= -0.718**

Table-11: Time-EC_{50} correlation coefficients for each pollutant species.

Pollutant type	Correlation Coefficient
2-CP	**R= -0.823**
2,4-DCP	**R= -0.764**
4-CP	**R= -0.713**
2-NP	**R= -0.827**
2,4-DNP	**R= -0.824**
4-NP	**R= -0.811**

In addition, the One-Way ANOVA test was used to determine whether there were differences in EC_{50} values between the time periods (5th, 15th and 30th min) and whether there were differences in pollutant concentration values between the time periods (5th, 15th and 30th min) (Table-12 and 13). Analyses were again completed at α=0.05 significance level with the results of the One-Way ANOVA test identifying significant differences below 0.05 in terms of EC_{50} values at all time values (5th, 15th, 30th min) ($P<0.05$). Similarly, for all pollutant species, there were again significant differences lower than 0.05 identified in terms of the determined EC_{50} values with increasing pollutant concentrations (two times) ($P<0.05$).

To determine the differences between the groups, the differences in EC_{50} values according to time and concentration for each pollutant were identified with the Tukey test, as shown in Table-14 and 15, respectively.

According to Tukey test results, the differences between the groups for EC_{50} values belonging to each pollutant based on time (5th, 15th and 30th min) are summarized in Table-14, while the differences between the groups based on concentration are summarized in Table-15 (a, b, c, d constants).

Table-12: Time-EC_{50} One Way Anova test results.

		TS	DOF	MS	F	P
2-CP	between groups	34427.248	2	17213.624	23.734	.000
	in groups	8703.196	12	725.266		
	Total	43130.444	14			
2,4-DCP	between groups	5789.648	2	2894.824	14.104	.001
	in groups	2463.045	12	205.254		
	Total	8252.693	14			
4-CP	between groups	3366.177	2	1683.089	9.813	.003
	in groups	2058.12	12	171.51		
	Total	5424.297	14			
2-NP	between groups	81177.961	2	40588.98	24.093	.000
	in groups	20216.262	12	1684.688		
	Total	101394.223	14			
2,4-DNP	between groups	36083.083	2	18041.542	22.893	.000
	in groups	9456.953	12	788.079		
	Total	45540.037	14			
4-NP	between groups	14016.738	2	7008.369	20.865	.000
	in groups	4030.661	12	335.888		
	Total	18047.399	14			

TS: Total squares **MS:** mean squares
P: significance level **DOF:** degree of freedom

Table-13: Concentration-EC_{50} One Way Anova test results.

		TS	DOF	MS	F	P
2-CP	between groups	41242.571	4	10310.643	54.615	.000
	in groups	1887.873	10	188.787		
	Total	43130.444	14			
2,4-DCP	between groups	6266.345	4	1566.586	7.887	0.004
	in groups	1986.348	10	198.635		
	Total	8252.693	14			
4-CP	between groups	3416.944	4	854.236	4.256	0.029
	in groups	2007.353	10	200.735		
	Total	5424.297	14			
2-NP	between groups	99460.321	4	24865.08	128.575	.000
	in groups	1933.902	10	193.39		
	Total	101394.223	14			
2,4-DNP	between groups	44105.927	4	11026.482	76.887	.000
	in groups	1434.11	10	143.411		
	Total	45540.037	14			
4-NP	between groups	16375.385	4	4093.846	24.485	.000
	in groups	1672.014	10	167.201		
	Total	18047.399	14			

TS: Total squares **MS:** mean squares
P: significance level **DOF:** degree of freedom

Table-14: Tukey test results for EC_{50} values of pollutants according to time.

Pollutant type	5 min	15 min	30 min
2-CP	111.05±34.23[a]	40.40±16.088[b]	12.72±4.52[b]
2,4-DCP	40.56±11.69[a]	14.82±5.38[b]	4.50±1.79[b]
4-CP	29±10.94[a]	10.40±4.94[b]	3.14±1.60[b]
2-NP	174.82±58.00[a]	64.01±27.22[b]	20.16±7.32[b]
2,4-DNP	114.68±36.75[a]	42.76±17.99[b]	13.25±4.65[b]
4-NP	68.28±19.19[a]	25.28±9.24[b]	7.78±2.67[b]

[a, b]: Differences between groups

Table-15: Tukey test results for EC_{50} values of pollutants according to concentration.

Pollutant type	1.25 mg/L	2.5 mg/L	5 mg/L	10 mg/L	20 mg/L
2-CP	153.66±26.64[a]	76.80±13.36[b]	38.40±6.68[c]	18.93±2.91[c]	9.60±1.67[c]
2,4-DCP	59.95±27.30[a]	29.97±13.65[a]	14.99±6.83[b]	7.49±3.41[b]	3.75±1.71[b]
4-CP	44.26±27.45[a]	22.13±13.72[a]	11.06±6.86[a]	5.53±3.43[b]	2.76±1.71[b]
2-NP	238.82±26.94[a]	119.41±13.47[b]	59.70±6.73[c]	29.85±3.36[c]	14.92±1.68[d]
2,4-DNP	159.04±23.20[a]	79.52±11.60[b]	39.76±5.80[c]	19.88±2.90[c]	9.94±1.45[c]
4-NP	96.90±25.05[a]	48.45±12.52[b]	24.22±6.26[b]	12.11±3.13[c]	6.05±1.56[c]

[a, b, c, d]: Differences between groups

As can be seen from the superscript **a and b** constants in Table-14, there was no effect on pollutant EC_{50} value according to time for 15th and 30th minutes (**b**), but there was a difference observed for the 5th minute **(a**).

As can be seen from the superscript **a, b, c and d** constants in Table-15, there were differences according to increased concentration on pollutant EC_{50} values observed for four different species.

The next stage of our statistical analysis was to determine the concentration and time models

equivalent to the EC_{50} values for each pollutant, as summarized in Table-16.

Table-16: Models for EC_{50} value of each pollutant against concentration and time.

Pollutant type	Concentration (x)	Time (x')
2-CP	y=104.394-5.795x	y=131.048-4.294x'
2,4-DCP	y=40.745-2.260x	y=52.296-1.744x'
4-CP	y=30.087-1.669x	y=39.146-1.320x'
2-NP	y=162.328-9.004x	y=202.796-6.615x'
2,4-DNP	y=108.098-5.996x	y=135.208-4.415x'
4-NP	y=65.866-3.654x	y=83.176-2.737x'

y: EC_{50}

In the equations, **Y** is the **EC_{50}** dependent variable, with the mathematical models including the independent variables of concentration **(X)** and time (**X'**). As both concentration (**X**) and time (**X'**) increase, the value of the dependent variable **Y:EC_{50}** falls and, as emphasized initially, the negative linear correlation is proven again by these models.

Finally, mathematical models determined by regression analysis 100% support the correlations initially identified.

As emphasized in the introduction, the comparison of toxicity removal with Fenton and Fenton-like processes according to substituent species/position/number are summarized in detail in Table 17-19.

The effect of substituent species is as follows: 4-CP is nearly 4 times more toxic than 4-NP (3.97) and 2,4-DCP is nearly 4 times more toxic than 2,4-DNP (3.98), while 2-CP is nearly 2 times more toxic than 2-NP (1.65) (Table-17). The result of Fenton/Fenton-like processes is that the toxic effect (EC_{50}-EC_{20}) reduced to unobservable levels.

Table-17: Toxicity removal effect of substituent species.

			Species of Substituent		Species of Substituent		Species of Substituent	
			4-CP	4-NP	2-CP	2-NP	2,4-DCP	2,4-DNP
Before Process		EC50	1.2	4.77	8.1	13.43	2.24	8.92
		TU	83.33	20.96	12.34	7.44	44.67	11.21
After Process	Fenton	EC50	No Detected	No Detected	No Detected	No Detected	No Detected	No Detected
		TU	NO	NO	NO	NO	NO	NO
	Fenton-Like	EC50	No Detected	No Detected	No Detected	No Detected	No Detected	No Detected
		TU	NO	NO	NO	NO	NO	NO

Table-18: Toxicity removal effect of substituent position.

			Position of Substituent		Position of Substituent	
			4-CP	2-CP	4-NP	2-NP
Before Process		EC50	1.2	8.1	4.77	13.43
		TU	83.33	12.34	20.96	7.44
After Process	Fenton	EC50	No Detected	No Detected	No Detected	No Detected
		TU	NO	NO	NO	NO
	Fenton-Like	EC50	No Detected	No Detected	No Detected	No Detected
		TU	NO	NO	NO	NO

Table-19: Toxicity removal effect of substituent number.

			Number of Substituent			Number of Substituent		
			4-CP	2-CP	2,4-DCP	4-NP	2-NP	2,4-DNP
Before Process		EC50	1,2	8,1	2,24	4,77	13,43	8,92
		TU	83,33	12,34	44,67	20,96	7,44	11,21
After Process	Fenton	EC50	No Detected	No Detected	No Detected	No Detected	No Detected	No Detected
		TU	NO	NO	NO	NO	NO	NO
	Fenton-Like	EC50	No Detected	No Detected	No Detected	No Detected	No Detected	No Detected
		TU	NO	NO	NO	NO	NO	NO

As can be seen from Table-18, the effect of the substituent position is as follows: Cl in the para position, or 4-CP, is nearly 7 times more toxic compared to Cl in the ortho position, or 2-CP (6.75). NO_2 in the para position, or 4-NP, is nearly 3 times more toxic compared to NO_2 in the ortho position, or 2-NP (2.81). We link this situation to the probable formation of intramolecular hydrogen bridge bonds in the ortho position.

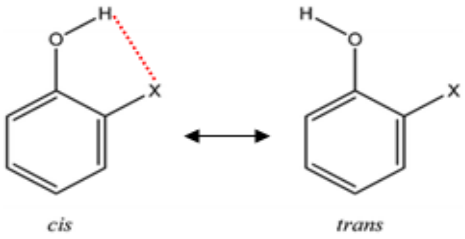
As seen in Table-19, the effect of substituent number on toxicity is as follows: 4-CP is 2 times more toxic than 2,4-DCP, while 2,4-DCP is 4 times more toxic than 2-CP (3.61). 4-NP is nearly 2 times more toxic than 2,4-DNP (1,87), while 2,4-DNP is 1.5 times more toxic than 2-NP. Here, again the comparative figures occur linked to the effect of possible intramolecular hydrogen bridge bonds in the ortho position.

Conclusions

When we compare Fenton and Fenton-like processes in terms of pollutant, COD and TOC removal efficiencies, the Fenton process has higher efficiency, especially for COD and TOC, compared to the Fenton-like process. Additionally, after both processes, the degradation products (quantitatively close to pollutant removal efficiency) were observed to have toxicity low enough to be unobservable in

terms of both EC_{50} and EC_{20} as revealed by our measurements.

If we compare the pollutants in terms of substituent **species/position/number**; we can easily say that in terms of substituent species, chloro species are more toxic compared to nitro species (according to *Vibrio fischeri* toxicity test results). This is an expected result as 2-CP is less toxic than 4-NP as explained by the molecular structure. As shown in the molecular structures below, the Cl substituent OH linked to the 2- or ortho position is inactivated to a certain level by forming an intramolecular hydrogen bridge bond in the cis-position. In fact, 5-member rings are stable, with short duration of formation probability [72]. Though the C-O bond linking the aromatic ring is rigid, the O-H bond may rotate freely. For cis and trans situations forming the two isomeric structures caused by this rotation, the cis position is an appropriate position to form a 5-member ring. In the trans isomer situation, the substituent of CI may actively affect toxicity, while the toxicity effect is relatively reduced due to the possibility of ring formation in the cis situation.



cis *trans*

In fact, the same explanation may be the cause of greater toxicity of 4-CP compared to 2,4-DCP. Hence, the possibility of a 5-member intramolecular hydrogen bridge bond forming with Cl (cis-form) in the 2-(ortho) position of 2,4-DCP is high.

For 2-NP, 2,4-DNP and 4-NP species, the possibility of a 6-member intramolecular H-bridge bond which is relatively more stable compared to a 5-member ring, is again present between the H of OH in the cis position with oxygen in the $-NO_2$ group linked to the 2-ortho position. As a result, 4-NP may display greater toxic effect compared to 2,4-DNP, while 2-NP has the lowest toxic effect. The 6-member cyclic lactone formed by the NO_2 group in the 2 position with the OH group in phenol is more stable compared to the 5-member cyclic lactone formed by Cl in the 2 position and the OH group in phenol, which lowers the toxicity of 2-NP. This is because the Cl in the cis position forms a cyclic lactone and becomes inactive.

It is a known reality that Cl- substituents have more contribution to toxicity compared to NO_2 - substituents. In fact, the inductive and mesomeric properties of substituents linked to the aromatic ring have a dominant effect on the nucleophilic character of the aromatic ring.

Cl substituent has –I / +M effect.

NO_2 substituent has -I / - M effect.

In other words, Cl inductively pulls e- from the ring and mesomerically donates e- to the ring. As is known, the mesomeric effect is always dominant over the inductive effect. Hence, the nucleophilic character of the aromatic ring increases which increases stability and linked to this, toxicity. Contrary to this, the NO_2 group pulls e- from the ring both inductively and mesomerically, which may reduce the stability of the aromatic ring and in parallel with this relatively reduce the toxicity. The effect of substituents linked to the aromatic ring inductively and mesomerically affects the aromatic stability (nucleophilic) of the ring and directly affects toxicity. Our toxicity measurements results support this view. The effects of substituent species, position and number on toxicity are summarized in detail in Tables 17-19 in the Results and Discussion section. The effects of substituent species, position and number tested according to two different substituent groups were not encountered in the literature.

Toxicology studies performed with *Vibrio fischeri* bacteria using bare phenol without chloro or nitro identified TU as 2.42 [73-74]. However, according to the results of this research in addition to the presence of substituent linked to an aromatic ring, it is noted again that species and position are very effective parameters on toxicity. The effects of substituent positions on toxicity were investigated in detail in terms of inductive, mesomeric and steric effects.

Another important element that requires noting is that according to toxicity results revealed in our study and in the literature, 4- (para) CP with higher toxicity compared to other CP species as presented in Table 5 was not included on the priority toxic chemical list of the US EPA-2014.

Our studies continue to reveal the effect of substituent number (2,3,4) and substituent position (ortho, meta, para) on one hand and mixed

substituents on the other, on toxicity removal using different AOP.

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