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APPLICATION OF COMBINED CHEMICAL COAGULATION AND ELECTRO-COAGULATION PROCESS FOR CARWASH WASTEWATER TREATMENT

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ABSTRACT

The composition of the wastewater generated from washing vehicles is complex since it may contain detergents used in the washing process, high levels of oils, grease, gasoline residues, metals, organic matter and particles, such as dust, carbon and salt, and, therefore, its treatment is difficult but also necessary for protection of the environment. The purpose of this work was to investigate the feasibility of treating carwash wastewater by combined chemical coagulation (using polyaluminum chloride (PACl) as coagulant) and electro-coagulation process (using aluminum electrodes) to achieve the required standards. The influence of the operating variables, such as coagulant dose, electrical potential and reaction time, on the removal efficiencies of major pollutants was determined. The rate of removal of pollutants linearly increased with increasing doses of PACl and applied voltage. COD, BOD₅, TSS and MBAS removal rates of 96.87, 94.0, 98.43 and 98.62 % were obtained by adding 100 mg L⁻¹ PACl and applying a voltage of 40. Also, an increase in applied voltage from 10 to 40 V causes an increase in energy consumption from 0.01 to 0.069 kWh L⁻¹, and from 0.014 to 0.095 kWh L⁻¹ for 25 and 100 mg L⁻¹ of PACl, respectively. The experiments demonstrated the effectiveness of chemical and electrochemical techniques for the treatment of carwash wastewaters. Consequently, combined processes are inferred to be superior to electro-coagulation alone, for the removal of both organic and inorganic compounds from carwash wastewater.

KEYWORDS: Carwash wastewater; Coagulation; Electrocoagulation, Poly aluminum chloride

1 INTRODUCTION

Water is one of the abundantly available resources in nature and essential for animal and plant life [1]. The continuous population growth and the increased agricultural and industrial activities raise the demand for water and motivate to look for alternative water sources, such as the reuse of purified wastewater [2].

Carwashing stations are among the activities that consume large capacities of fresh water on daily basis and can benefit from recycling programs. Also, car washing stations play a great role in our modern daily; people often take their automobiles to be washed on a regular basis [3]. The car wash industry appears today to be more conscious of the need for wastewater treatment and water reclamation [4]. A carwash is defined as a non-domestic installation for external cleaning of cars. Three types of carwashes occur: a roll-over (in which the washing installation moves over the car), an automatic carwash (in which the car is pulled through the washing installation) and a self-carwash [5].

Carwash wastewaters can be harmful to humans and environment if released untreated to surface water-bodies because they contain many pollutants, such as detergents that can be poisonous to fish, oil, grease, chemicals and solvent-based solutions, road grime, heavy metals, carbon, asphalt, salts, surfactants, organic matter etc [6, 7].

Various methods such as nanofiltration [5], flocculation [4], sand filtration, adsorption, biological treatment [5], chemical oxidation [8], cellulose acetate ultrafiltration membrane aided by flocculation and activated carbon treatments [7] have been used for treatment of carwash wastewaters.

Recently, electrochemical methods (electrooxidation [9], electrocoagulation) have been widely used as attractive and suitable method for the treatment of water and wastewater containing oil wastes [10, 11], colloids, surfactants [12, 13], color, heavy metals and pesticides, by virtue of various benefits including environmental com-
Colloidal particles. These flocs polymerize as follows:

\[ \text{Al(OH)}_3 \text{ according to complex precipitation kinetics} \ [22]. \]

Hydroxide flocs normally act as adsorbents and/or traps for strong oxidants [23, 24]. On the other hand, the aluminum ions generated during H₂ evolution at high pH [21]:

\[ 2\text{Al} + 6\text{H}_2\text{O} + 2\text{OH}^- \rightarrow 2\text{Al(OH)}_3^+ + 3\text{H}_2 \quad (3) \]

They are easily removed from aqueous medium by sedimentation and H₂ flotation. Secondary anodic reactions occur also during electrocoagulation process; for example, in neutral and acidic chloride solutions, native and free chlorine and hypochlorite are formed which are strong oxidants [23, 24]. On the other hand, the aluminium hydroxide flocs normally act as adsorbents and/or traps for pollutants eliminating them from the solution [17, 20].

The main purpose of this paper is to explore the possibility of treating and upgrading car-washing water to an acceptable level that can be recycled and reused for the same application. Hence, the outcome would render several benefits to car-washing stations, including water conservation and abating water pollution.

## 2 Materials and Methods

### 2.1 Carwash wastewater

The wastewater samples were collected from the top of the settling tank in an automatic carwash installation situated in Zahedan, Iran. The characteristics of the raw carwash wastewater are presented in Table 1. The effluent used throughout this study was taken from a local traditional carwash installation (washing by hand with a hose) with 25 (mean value) cars per day capacity, located in Zahedan City in the province of Sistan and Baluchestan (Iran), producing approximately 7500-8000 L of wastewater daily. Samples were collected in polypropylene bottles, shipped cold, and kept at 4 °C before use. The length of the storage before starting experiments varied from one day to six weeks. The effluent has been sampled at different times during this study, and the initial characteristics varied with time (Table 1). This effluent initially contained high concentrations of soluble and suspended materials (856.01±217.11 mg COD L⁻¹, 239.38±70.52 mg BOD L⁻¹, 193.55±71.53 mg TSS L⁻¹).

### 2.2 Chemical treatment (coagulation) of carwash wastewater

All the chemicals used in the study were of analytical reagent (AR) grade. Poly-aluminum chloride (PACl) Al₁₂Cl₁₄(OH)₂₄ was chosen for this study because it has been extensively at water and wastewater treatment plants to remove solids, and may function as an effective and less expensive coagulant. PACl was used in this study up to 100 mg L⁻¹ (25, 50, 75 and 100 mg L⁻¹). A six-beaker jar test (flocculator) was set up at room temperature for each trial. Each of the beakers contained 2 L of settled wastewater. The coagulants were added into the beakers, and the pH values were immediately adjusted to the preset values (7±0.1) using NaOH or H₂SO₄ for pH-controlled experiments. Rapid stirring at 150 rpm for 2 min was followed by gentle mixing at 50 rpm for 20 min, and the solids formed were left to settle for 30 min. Samples were taken from the water surface (supernatant) and filtered through a 0.45-μm membrane. After chemical coagulation, electro-coagulation process with aluminum electrodes was performed on the supernatant.

### 2.3 Electrochemical treatment of carwash wastewater

In each run, wastewater (supernatant) after chemical coagulation (first stage of treatment) was poured into the electro-coagulation cell. All experiments were performed in a bipolar batch reactor (Fig. 1), with four aluminum electrodes connected in parallel. Only the outer electrodes were connected to the power source, and anodic and cathodic reactions occurred on each surface of the inner electrode when the current passed through the electrodes. The internal size of the cell was 15 × 15 × 25 cm (width × length × depth) with an effective volume of 2000 cm³. The volume (V) of the solution of each batch was 2 L. The active area of each electrode (plate) was 14×20 cm, with a total area of 280 cm². The distance between electrodes was 1.5 cm. A power supply having an input of 220 V and variable output of 0–40 V (10, 20, 30 and 40 V) with maximum current of 5 amperes was used as direct current source. The temperature of each system was maintained at 25 ± 1 °C. Different samples of 100 ml were taken at 15 min intervals for up to 1 h and filtered before being analyzed to determine BOD₅, COD, TSS and other parameters. During
the runs, the reactor unit was stirred at 150 rpm by a magnetic stirrer to allow the chemical precipitate to grow large enough for removal. During electro-coagulation, an oxide film was formed at the anode. In order to overcome electrode passivation at the anode, the electrodes were rinsed with diluted HCl solution (5%, v/v) and tap water after each experiment, and finally weighted. Also the electrodes were re-weighted to calculate sacrificial electrode consumptions. These weights are used in the calculations of the total operating costs. In addition, the electrical energy consumed per unit volume of treated wastewater has been calculated for different experimental conditions. All analyses were conducted in duplicate for reproducibility of the experimental results, and all of the data in Figs. and Tables were average values.

2.4 Analytical methods

COD, BOD, total suspended solids (TSS), anionic surfactants, turbidity, lead (Pb), iron (Fe), zinc (Zn), conductivity and pH determinations were determined according to the standard methods [24]. COD was measured using a COD reactor and a direct-reading spectrophotometer (DR/5000, HACH, USA). Five-day biological oxygen demand (BOD₅) was determined by the manometric method with a respirometric OxiTop system (WTW). Anionic surfactants were determined spectrometrically (T80 UV/VIS spectrometer) as methylene blue active substances (MBAS). The pH and conductivity were adjusted to a desirable value using NaOH or H₂SO₄ and NaCl, and measured using a pH-meter model UB-10 (Ultra Basic, U.S.) and a conductivity meter model Cond 3110 (WTW), respectively. Also iron, lead and zinc concentrations were measured using an Analyst 700 Atomic absorption spectrometer according to Standard Methods [25].

3 RESULTS AND DISCUSSION

3.1 Wastewater characterization

Table 1 presents the carwash wastewater characteristics prior to any treatment, after 24 h settling time, and also the guidelines from Iran for effluent discharge in the sewage urban works. The values of the pollution parameters were lowered after 24 h of preliminary settling time. Also, the comparison of these values shows that COD, BOD₅, TSS and grease were extremely higher (5-14 times) than those recommended by Iran. Consequently, the carwash effluent needs to be treated before discharge.

3.2 Effect of preliminary settling time

Preliminary settling process is a natural treatment method that requires no chemical addition. Although some workers realized the importance of the natural settling process, there is little information available in the literature on the effect of the preliminary settling time on pollutants removal capacity.

In this study, the raw carwash wastewater was allowed to settle in a preliminary settling tank before the addition of a coagulant. The process had an effect on BOD₅, COD, TSS, surfactants and turbidity removals during the first 24 h. TSS was reduced from 291 to 193 mg L⁻¹ (resulting in a 33.68% TSS removal efficiency), COD was reduced in the wastewater from 954 to 856 mg L⁻¹ (resulting in a 10.27% COD removal efficiency) whereas BOD₅ was reduced in the wastewater from 266 to 239 mg L⁻¹ (resulting in a 10.15% BOD₅ removal efficiency).

3.3 Effect of coagulation process (first step)

To obtain dischargeable effluents, PACl was intended to be used as the coagulant. Therefore, PACl was added to
### TABLE 1 - Characteristics of the raw carwash wastewater used for this study.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Raw wastewater</th>
<th>24 h-settled wastewater</th>
<th>Permissive levels (Iran Standard)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of samples</td>
<td>32</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>Total COD (mg L⁻¹)</td>
<td>924.17± 167.43</td>
<td>856.01± 217.11</td>
<td>60</td>
</tr>
<tr>
<td>Total BOD₅ (mg L⁻¹)</td>
<td>266.31± 78.86</td>
<td>239.38± 70.52</td>
<td>30</td>
</tr>
<tr>
<td>Total Suspended Solids (mg L⁻¹)</td>
<td>291.35± 46.14</td>
<td>193.55± 71.53</td>
<td>40</td>
</tr>
<tr>
<td>Foaming agents (MBAS) (mg L⁻¹)</td>
<td>34.17± 14.21</td>
<td>31.22± 18.75</td>
<td>1.5</td>
</tr>
<tr>
<td>Lead (mg L⁻¹)</td>
<td>-</td>
<td>0.79± 0.72</td>
<td>1</td>
</tr>
<tr>
<td>Iron (mg L⁻¹)</td>
<td>-</td>
<td>14.26± 7.18</td>
<td>3</td>
</tr>
<tr>
<td>Zinc (mg L⁻¹)</td>
<td>-</td>
<td>5.52± 2.13</td>
<td>2</td>
</tr>
<tr>
<td>Conductivity (mS/cm)</td>
<td>7.08± 1.4</td>
<td>7.05± 2.3</td>
<td>-</td>
</tr>
<tr>
<td>Turbidity (NTU)</td>
<td>137.2± 36.45</td>
<td>166.8± 51.72</td>
<td>50</td>
</tr>
<tr>
<td>pH</td>
<td>7.65± 0.02</td>
<td>7.31±0.12</td>
<td>6.5-8.5</td>
</tr>
</tbody>
</table>

### TABLE 2 - Influence of PACl dosage on water quality parameters of coagulated mixed liquor.

<table>
<thead>
<tr>
<th>PACI dosage (mg/L)</th>
<th>COD (mg L⁻¹)</th>
<th>BOD₅ (mg L⁻¹)</th>
<th>TSS (mg L⁻¹)</th>
<th>MBAS (mg L⁻¹)</th>
<th>Pb (mg L⁻¹)</th>
<th>Fe (mg L⁻¹)</th>
<th>Zn (mg L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>856.01</td>
<td>239.38</td>
<td>193.55</td>
<td>31.22</td>
<td>0.79</td>
<td>14.26</td>
<td>5.52</td>
</tr>
<tr>
<td>25</td>
<td>481.36</td>
<td>141.84</td>
<td>122.49</td>
<td>20.89</td>
<td>0.48</td>
<td>8.9</td>
<td>3.53</td>
</tr>
<tr>
<td>50</td>
<td>349.49</td>
<td>111.42</td>
<td>87.35</td>
<td>8.40</td>
<td>0.35</td>
<td>6.28</td>
<td>2.34</td>
</tr>
<tr>
<td>75</td>
<td>171.29</td>
<td>82.37</td>
<td>55.16</td>
<td>7.74</td>
<td>0.20</td>
<td>4.73</td>
<td>1.30</td>
</tr>
<tr>
<td>100</td>
<td>125.28</td>
<td>61.06</td>
<td>50.23</td>
<td>7.61</td>
<td>0.17</td>
<td>3.64</td>
<td>1.07</td>
</tr>
</tbody>
</table>

The curve obtained with PACI points to a considerable increase in performance from the lowest dose up to 100 mg L⁻¹. On the other hand, in chemical coagulation, as shown in Fig. 2, an increase in COD, BOD, TSS, surfactant and other pollutants’ removal percentages is noted with increasing PACI dosage, reaching nearly 74.05 – 85.36% at a dosage of 100 mg L⁻¹. Similar results were obtained in previous reports concerning the electrocoagulation of wastewater from a vegetable oil refinery (with PACI addition as coagulant) [26].

### FIGURE 2 - Effects of coagulant dose (PACI) on pollutants removal efficiency at pilot-scale coagulation process.

The wastewater to achieve particle instability and increase in particle size, consequently achieving effective removal of organic substances present as COD and BOD₅ but also other pollutants (such as TSS, turbidity, heavy metals and surfactants). The doses of PACI as coagulant were varied between 0 and 100 mg L⁻¹. The results of the tests using the PACI individually are presented in Table 2 and Fig. 2. It is shown that at lower doses of the PACI (25 mg L⁻¹), COD, BOD₅, and TSS removal efficiencies reached 43.77, 40.75 and 36.71%. As shown in Fig. 2, the efficiency of the process increased with increasing dosages of coagulant (PACI). Maximum Pb, Fe and Zn removal efficiencies (74 to 80%) were obtained by using PACI at the dosage of 100 mg L⁻¹.

3.4 Effect of electrocoagulation process (second step)

Electrocoagulation processes a direct current source between metal electrodes immersed in wastewater. The electrical current causes the dissolution of metal electrodes commonly iron and aluminum into wastewater. The dissolved metal ions, at an appropriate pH, can form wide ranges of coagulated species and metal hydroxides that destabilize and aggregate the suspended particles, or precipitate and adsorb dissolved contaminants [19, 27].

An examination of the chemical reactions occurring in the electro-coagulation process shows that the main reactions occurring at the aluminum electrodes are:

\[
\text{Al (s)} \quad \rightarrow \quad \text{Al}^{3+} \quad \text{aq} + \quad 3e^- \quad \text{(anode)} \quad (5)
\]

\[
3\text{H}_2\text{O} + \quad 3e^- \quad \rightarrow \quad 3/2 \text{H}_2 \quad \text{g} + \quad 3\text{OH}^- \quad \text{(cathode)} \quad (6)
\]

\[
\text{Al}^{3+} \quad \text{aq} + \quad 3\text{OH}^- \quad \rightarrow \quad \text{Al(OH)}_3 \quad (7)
\]

Monomeric species, such as Al(OH)²⁺, Al(OH)³⁺, Al₃(OH)⁶⁺, and Al(OH)⁴⁺ but also polymeric species, such as Al₅(OH)₁₀⁺, Al₆(OH)₁₇⁺, Al₇(OH)₂₀⁺, Al₉(OH)₂₄⁺, and Al₁₃(OH)₃₄⁺ are formed during the electrocoagulation process [27, 28]. The aluminum hydroxide flocs act as ad-
sorbents and/or traps for pollutants thus eliminating them from the solution [11, 29].

The performances by the two pretreatments, namely, preliminary settling and chemical coagulation, were not carried out efficiently enough to satisfy the national guideline of effluent qualities. Additional dosage of coagulant (PACl) and longer time are needed to keep the national guideline of the effluent qualities. Therefore, the electro-coagulation process was employed as the final treatment step in this study. In adopting the electro-coagulation process, it was intended to treat the pollutants efficiently as well as economically.

The effects of applied voltage and reaction time on electrocoagulation process of carwash wastewater treatment were determined. The results of effects of operating parameters on a pilot-scale electrocoagulation process are shown in Table 3 and Figs. 3–6.

3.5 Effect of applied voltage

One of the most important parameters influencing the performance and economy of electrocoagulation process is the voltage applied at the electrodes [30]. To understand the effect of applied voltage on the efficacy of electrocoagulation in carwash wastewater treatment, several voltages in the range of 10 to 40 V were applied between the electrodes in the electrocoagulation cell, and pollutants removal was determined at the conditions given in Table 3.

The applied voltage is expected to exhibit a strong effect on electrocoagulation, especially on the TSS and surfactants abatement: the higher the current (voltage), the shorter the treatment. The supply of current to the electrocoagulation system determines the amount of Al3+ ions released from the respective electrodes and the amount of resulting coagulant. Thus, more Al3+ ions get dissolved in the solution, and the formation rate of Al(OH)3 is increased.

### TABLE 3 - Influence of electrocoagulation process using aluminum electrodes on effluent quality parameters (after 60 min reaction time).

<table>
<thead>
<tr>
<th>PACl dosage (mg L⁻¹)</th>
<th>Applied voltage (V)</th>
<th>COD (mg L⁻¹)</th>
<th>BOD5 (mg L⁻¹)</th>
<th>TSS (mg L⁻¹)</th>
<th>MBAS (mg L⁻¹)</th>
<th>Pb (mg L⁻¹)</th>
<th>Fe (mg L⁻¹)</th>
<th>Zn (mg L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>25</td>
<td>306.45</td>
<td>92.55</td>
<td>70.64</td>
<td>11.20</td>
<td>0.269</td>
<td>4.97</td>
<td>2.02</td>
</tr>
<tr>
<td>20</td>
<td>20</td>
<td>270.32</td>
<td>73.75</td>
<td>56.74</td>
<td>8.22</td>
<td>0.224</td>
<td>3.65</td>
<td>1.6</td>
</tr>
<tr>
<td>30</td>
<td>30</td>
<td>241.90</td>
<td>72.46</td>
<td>52.22</td>
<td>7.84</td>
<td>0.217</td>
<td>3.54</td>
<td>1.42</td>
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<tr>
<td>40</td>
<td>40</td>
<td>223.41</td>
<td>71.11</td>
<td>48.37</td>
<td>6.92</td>
<td>0.201</td>
<td>3.09</td>
<td>1.29</td>
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<td>50</td>
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<td>220.93</td>
<td>69.97</td>
<td>48.32</td>
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<td>0.204</td>
<td>3.05</td>
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<td></td>
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<td>181.90</td>
<td>58.45</td>
<td>39.67</td>
<td>5.92</td>
<td>0.156</td>
<td>2.53</td>
<td>1.03</td>
</tr>
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<td>179.16</td>
<td>55.91</td>
<td>38.05</td>
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<td>75</td>
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<td>153.14</td>
<td>55.44</td>
<td>32.90</td>
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<td>1.83</td>
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<td>150.83</td>
<td>49.79</td>
<td>28.06</td>
<td>4.03</td>
<td>0.109</td>
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<td>87.57</td>
<td>37.75</td>
<td>21.38</td>
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<td>0.07</td>
<td>1.14</td>
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<td></td>
<td>40</td>
<td>73.01</td>
<td>22.14</td>
<td>11.74</td>
<td>1.82</td>
<td>0.049</td>
<td>0.638</td>
<td>0.373</td>
</tr>
<tr>
<td>100</td>
<td>10</td>
<td>118.47</td>
<td>47.43</td>
<td>24.97</td>
<td>2.27</td>
<td>0.067</td>
<td>0.818</td>
<td>0.524</td>
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<td></td>
<td>20</td>
<td>101.77</td>
<td>39.88</td>
<td>17.01</td>
<td>1.26</td>
<td>0.028</td>
<td>0.327</td>
<td>0.276</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>56.58</td>
<td>28.12</td>
<td>6.94</td>
<td>0.61</td>
<td>0.007</td>
<td>0.098</td>
<td>0.131</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>26.79</td>
<td>14.36</td>
<td>3.03</td>
<td>0.43</td>
<td>0.004</td>
<td>0.047</td>
<td>0.052</td>
</tr>
</tbody>
</table>

**FIGURE 3** - Effect of applied voltage on pollutants removal efficiency (coagulant dose: 25 mg L⁻¹, reaction time: 60 min).

**FIGURE 4** - Effect of applied voltage on pollutants removal efficiency (coagulant dose: 50 mg L⁻¹, reaction time: 60 min).
FIGURE 5 - Effect of applied voltage on pollutants removal efficiency (coagulant dose: 75 mg L⁻¹, reaction time: 60 min).

FIGURE 6 - Effect of applied voltage on pollutants removal efficiency (coagulant dose: 100 mg L⁻¹, reaction time: 60 min).

3.6 Electrical energy and electrode consumption:

Electrical energy consumption is a very important economical parameter in electrocoagulation process. Therefore, for the same operating conditions, after 60 min of electrocoagulation, consumption of energy and electrode material is also represented in Figs. 7 and 8. The electrical energy consumption was calculated using the related equations [31].

It can be seen from Figs. 7 and 8 that electrical energy and electrode consumption were found to increase with increasing the applied voltage, as would be expected in any other electrolytic process. An increase in applied voltage from 10 to 40 V causes an increase in energy consumption from 0.01 to 0.069 kWh L⁻¹ and 0.014 to 0.095 kWh L⁻¹ for PACl treatment doses of 25 and 100 mg L⁻¹, respectively. Also, an increase in applied voltage from 10 to 40 V causes an increase in electrode consumption (Fig. 8) from 0.92 to 2.37 g L⁻¹ and 1.36 to 2.62 g L⁻¹ for 25 and 100 mg L⁻¹ of PACl, respectively.

When the applied voltage was increased from 10 to 40 V, the COD and BOD₅ removal efficiency increased appreciably, from 5.44 to 78.62% and from 22.32 to 76.48%, respectively, whereas the corresponding specific energy consumption increased only slightly. Therefore, in the present study, 40 V is chosen as optimum operating voltage for electrocoagulation process. These results are according to phenolic compounds removal efficiency by aluminum electrodes reported by Adhoum et al. [32], and zinc and copper removal from aqueous solutions by aluminum electrodes [33].

FIGURE 7 - Electrical energy consumption during coagulation-electrocoagulation process (kWh L⁻¹).

FIGURE 8 - Electrode consumption during coagulation-electrocoagulation process (g).

4 CONCLUSIONS

In this study, chemical coagulation in carwash wastewater using poly-aluminum chloride (PACl) and electrocoagulation process with aluminum electrodes was investigated. The effects of the different operational parameters on the removal rates of pollutants were analyzed. The following conclusions can be drawn from the results obtained in this work:

Preliminary settling times were investigated and found to be important operational parameter for effective treatment of carwash wastewater.

A preliminary settling time of 24 h had an effect on COD, BOD₅, TSS and MBAS, with removal efficiencies up to 7.37, 10.11, 33.57 and 8.63%, respectively.
According to the results obtained from the above experiments, the removal efficiencies increased by increasing the coagulant dose and electrical potential. Moreover, the energy consumption increased by increasing the applied electrical potential.

Finally, it can be concluded that the combined chemical coagulation and electrocoagulation process has the potential to be utilized for the cost-effective removal of pollutants from carwash wastewater.

ACKNOWLEDGMENT

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DISTRIBUTION AND ASSESSMENT OF HEAVY METALS IN SURFACE WATER AND SEDIMENTS FROM NANJING CHEMICAL INDUSTRIAL PARK

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ABSTRACT

Nanjing Chemical Industry Park (NCIP) is an important industrial base with more than 300 chemical plants. In order to evaluate the extent of pollution and potential eco-toxicity of heavy metals, a survey on metal pollution in this area was conducted. The contents of metals (Cd, Cr, Pb, Cu, Zn and Ni) in surface water and sediments as well as fractionation of them in sediments were studied, and then analyzed for their environmental impact. The investigation demonstrated that among all sites, site 1 (Piehong River), which is close to the industrial area, had the highest metal concentrations. Levels of Pb and Cd in water, at most sites, exceeded the Criterion Continuous Concentration (CCC) of USEPA. Sediment pollution assessed by using the criteria recommended by the National Oceanic and Atmospheric Administration (USA) and enrichment factor also indicated that some anthropogenic inputs of Cd and Pb occurred. Furthermore, Cd was preferentially associated with the more labile fraction, while other metals were dominantly confined in the residual fraction. To sum up, Cd in some sites is likely to pose a threat to aquatic organisms.

KEYWORDS: Heavy metals, sediment, fractionation, risk assessment, industrial park.

1 INTRODUCTION

Heavy metal contamination is a worldwide problem. In recent years, the problem of urban soil contamination by metals has aroused much concern due to rapid industrialization and urbanization [1]. As a result of intensive human activities and the development of industry, the pollution of metals in some industrialized areas has increased considerably in China [2]. Heavy metals are generally indecomposable, and when their concentrations exceed a certain level, most have toxic effects on living organisms [3]. Thus, trace metal contamination of the environment can result in long-term ecological environment and health implications.

Serving as both a sink and possible non-point source in aquatic ecosystems, sediment has the potential to release sediment-bound metals to overlying water and, in turn, adversely affect aquatic organisms [4]. Therefore, providing safe and clean aquatic media is of great significance regarding human health, economy and environment [5].

The capital of Jiangsu province, Nanjing, is a rapidly developing modern city, and also one of the most active regions in economic development in China [6]. Nanjing Chemical Industrial Park (NCIP) is located in the north of Nanjing, on the northern bank of the Yangtze River. NCIP was named a State-level Chemical Hub, ranking the second largest chemical industrial park in China. Products of NCIP range over petrochemical and natural gas derivatives, polymers, new chemical materials and so on.

No relevant study on the metal pollution in NCIP has been carried out before, and the main objectives are to examine and determine the distribution, potential toxicity and ecological risk of hazardous trace metals in surface water and sediments.

2 MATERIALS AND METHODS

Site description and sampling

NCIP is located at the lower reaches of the Yangtze River. It has 22 jetties that can berth 5,000 to 30,000 ton vessels. Piehong River, Changfeng River, Zhongxin River, Macha River and Zhaoqiao River flow through NCIP (Fig. 1), mainly receiving the runoff from the surrounding industrial plants. Siliu River is the peripheral river, mostly receiving the agricultural runoff.

Water and sediment samples were collected from eight sites in March 2009. Six of the sampling sites were selected on the main rivers located in NCIP, and the other two sites were selected on the Yangtze River (Fig. 1). These sites were positioned by a global positioning system. At each site, about 1-2 kg of surface sediment was...
FIGURE 1 - Location map of sampling sites in the studied area NCIP (thin arrowheads indicate the water-flow direction).

TABLE 1 - Location of the sampling sites and physicochemical parameters of surface water and sediment samples in the studied area.

<table>
<thead>
<tr>
<th>Sampling sites</th>
<th>Description</th>
<th>Water temperature (°C)</th>
<th>TOC (%) a</th>
<th>TIC (%) b</th>
<th>Total C (%)</th>
<th>Total N (%)</th>
<th>pH Surface water</th>
<th>pH Surface sediment</th>
<th>Latitude</th>
<th>Longitude</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>Piehong River</td>
<td>14.4</td>
<td>1.85</td>
<td>0.90</td>
<td>3.26</td>
<td>0.09</td>
<td>8.07</td>
<td>7.59</td>
<td>E 118°48'32.4&quot;</td>
<td>N 32°16'55.2&quot;</td>
</tr>
<tr>
<td>S2</td>
<td>Siiliu River</td>
<td>11.7</td>
<td>2.05</td>
<td>0.59</td>
<td>1.92</td>
<td>0.14</td>
<td>8.28</td>
<td>7.48</td>
<td>E 118°50'13.9&quot;</td>
<td>N 32°17'51.2&quot;</td>
</tr>
<tr>
<td>S3</td>
<td>Zhaoqiao River</td>
<td>12.1</td>
<td>2.07</td>
<td>0.45</td>
<td>1.79</td>
<td>0.35</td>
<td>8.10</td>
<td>7.60</td>
<td>E 118°49'35.2&quot;</td>
<td>N 32°16'58.6&quot;</td>
</tr>
<tr>
<td>S4</td>
<td>Zhongxin River</td>
<td>14.0</td>
<td>3.90</td>
<td>5.50</td>
<td>10.05</td>
<td>0.48</td>
<td>7.82</td>
<td>7.90</td>
<td>E 118°49'37.9&quot;</td>
<td>N 32°16'05.8&quot;</td>
</tr>
<tr>
<td>S5</td>
<td>Macha River</td>
<td>9.8</td>
<td>1.79</td>
<td>0.62</td>
<td>1.75</td>
<td>0.31</td>
<td>7.61</td>
<td>7.87</td>
<td>E 118°47'57.4&quot;</td>
<td>N 32°14'11.7&quot;</td>
</tr>
<tr>
<td>S6</td>
<td>Yangtze water intake</td>
<td>10.0</td>
<td>1.41</td>
<td>0.73</td>
<td>2.49</td>
<td>0.21</td>
<td>7.38</td>
<td>7.26</td>
<td>E 118°49'32.0&quot;</td>
<td>N 32°14'13.9&quot;</td>
</tr>
<tr>
<td>S7</td>
<td>Yuezi River</td>
<td>13.0</td>
<td>2.12</td>
<td>1.15</td>
<td>3.19</td>
<td>0.37</td>
<td>7.70</td>
<td>7.75</td>
<td>E 118°49'06.5&quot;</td>
<td>N 32°14'31.4&quot;</td>
</tr>
<tr>
<td>S8</td>
<td>Tongjiang Ji River</td>
<td>9.0</td>
<td>1.35</td>
<td>1.45</td>
<td>2.50</td>
<td>0.15</td>
<td>7.90</td>
<td>7.87</td>
<td>E 118°51'45.8&quot;</td>
<td>N 32°12'10.9&quot;</td>
</tr>
<tr>
<td>Mean Values</td>
<td></td>
<td></td>
<td>2.07</td>
<td>1.43</td>
<td>3.37</td>
<td>0.26</td>
<td>7.86</td>
<td>7.67</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Total organic carbon (TOC) was calculated using the equation: TOC (%) = LOI_{550°C} * 12 / 30 [14].

b Total inorganic carbon (TIC) was calculated using the equation: TIC (%) = LOI_{950°C} * 12 / 44 [14].

in the dark at 4 °C. The geographical location of sampling sites, and main characteristics of surface water and sediments, are given in Table 1.

2.1 Sample preparation and analytical methods

In the laboratory, sediment samples were thoroughly mixed to create composite samples for each site, and kept frozen at -20 °C before processing. They were freeze-dried by an Advantage Freeze Dryer. The dried samples were homogenized to make representative ones. Each representative sample was ground to remove large clast, plant and animal residues. All sub-samples were then further sized to go through a 100-mesh nylon sieve [9]. These final samples were stored in cleaned amber glass bottles at 4 °C until analysis. Both water and sediment samples were digested using an Electric Heating Board.
For the determination of metal concentrations, 200 ml of each acid preservation water sample was digested with 5 ml HNO₃ under heating at 90 °C for solution evaporation. The sample solution was reduced to about 1 ml, cooled, and quantitatively transferred to a 10-ml volumetric flask [8].

A mixture of concentrated HF/HClO₄/HNO₃ acid was used to digest prepared sediment samples. After cooling, de-ionized water was added, the mixture was filtered, and the volume was made up to 25 ml with de-ionized water [9].

The three-step sequential extraction procedure recommended by the European Community Bureau of Reference (BCR) [10] was applied to analyze metal fractionation. The optimized extraction procedure is summarized in Table 2 [11, 12]. To determine the content of insoluble residual fraction (B4) in samples, the residue obtained from step (3) (Table 2) was transferred to a suitable digestion vessel, and was determined by the same process ut supra for total metal analysis.

Concentrations of metal ions in water and sediments were determined by Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) (Jarrell-Ash Corporation, America). Metal fractions were measured by a Thermo Electron Z-8100 atomic absorption spectrometer (AAS) fitted with flame or graphite furnace atomization (Japan). The operational conditions were adjusted in accordance with the manufacturer’s guidelines to yield optimal determination.

### 2.2 Sediment pH, carbon and nitrogen contents

Sediment pH was measured with a Sartorius PB-10 pH-meter by suspending 20 g of dried sample in 50 ml de-ionized water.

Total carbon (TC) and total nitrogen (TN) contents of sediments were determined with an elemental analyzer (vario MICRO, ELEMENTAR Analysensysteme GmbH, Germany). Loss-on-ignition (LOI) analysis was used to estimate the total organic matter and carbonates contents in sediments with standard methods [13, 14]. Total organic carbon (TOC) and inorganic carbon (TIC) contents were calculated with the LOI values at 550 °C (LOI₅₅₀ °C) and 950 °C (LOI₉₅₀ °C), respectively, which were transformed to TOC and TIC using the following equations [14]:

\[
\text{TOC} (%) = \frac{\text{LOI}_{550\,\text{°C}}}{30} \\
\text{TIC} (%) = \frac{\text{LOI}_{950\,\text{°C}}}{44}
\]

### 2.3 Quality assurance and quality control

All chemicals and reagents were of analytical grade. De-ionized water used throughout the experiment was obtained from a Millipore Milli-Q system (18.2 MΩ-cm). Glassware and plastics used were firstly pre-cleaned by soaking in diluted nitric acid solution overnight, and then rinsed with de-ionized water. Calibration solutions were prepared by serial dilution suitable aliquots of stock standard solutions (1000 mg L⁻¹), which were purchased from the Central Iron and Steel Research Institute, China.

Triplicate determinations were carried out for each sample. Procedural blanks covering the whole procedure were also run in parallel with the samples for control. The accuracy and precision of analysis procedure was checked using a certified sediment reference material, BCR-701 (European Community Bureau of Reference). For each extraction step, the concentration of each metal obtained was compared with the reference value, and recoveries (%) are shown in Table 3. Recoveries of the selected metals were 83-91% for digestion using the Electric Heating Board. The results indicate good agreement among experimental and certified values for the analytical procedure used in this study.

### TABLE 2 - Heavy metal sequential extraction procedure of the optimized BCR method [11].

<table>
<thead>
<tr>
<th>Step</th>
<th>Extractable fraction</th>
<th>Extraction procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>B1: Water soluble, exchangeable and carbonate bound fraction</td>
<td>Sediment sample 0.500 g, 20 ml 0.11 M acetic acid, shaking 16 h at 22 ± 2 °C. The extract was separated from the solid residue by centrifugation at 3,000 rpm for 20 min, decant supernatant and analyze. The residue was washed with 20 ml deionized water, shaken for 15 min, and centrifuged. The supernatant was decanted and discarded carefully to avoid loss of any solid residue.</td>
</tr>
<tr>
<td>2</td>
<td>B2: Iron-manganese oxides bound fraction</td>
<td>Shaking with 20 ml 0.5 M hydroxylamine hydrochloride (pH 1.5 with 2 M HNO₃) for 16 h at 22 ± 2 °C. Centrifuge extract, wash, shake and centrifuge as per Step 1. Digesting with 5 ml 30% hydrogen peroxide for 1 h at room temperature; 85 °C, 1 h in a water-bath; add a further 5 ml H₂O₂; and 85 °C for 1 h; 25 ml 1 M ammonium acetate (pH 2), shaking 16 h at 22 ± 2 °C. The later process as per Step 1.</td>
</tr>
<tr>
<td>3</td>
<td>B3: Organics/sulfides bound fraction</td>
<td></td>
</tr>
</tbody>
</table>

### TABLE 3 - Recoveries (%) of reference material BCR-701 (mean ± standard deviation, n = 4).

<table>
<thead>
<tr>
<th>Metal</th>
<th>Fraction 1 (%)</th>
<th>Fraction 2 (%)</th>
<th>Fraction 3 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cd</td>
<td>97 ± 6</td>
<td>87 ± 30</td>
<td>114 ± 0.2</td>
</tr>
<tr>
<td>Cr</td>
<td>117 ± 9</td>
<td>92 ± 25</td>
<td>95 ± 9</td>
</tr>
<tr>
<td>Cu</td>
<td>113 ± 1</td>
<td>100 ± 4</td>
<td>110 ± 11</td>
</tr>
<tr>
<td>Ni</td>
<td>100 ± 5</td>
<td>101 ± 18</td>
<td>103 ± 7</td>
</tr>
<tr>
<td>Pb</td>
<td>117 ± 0.2</td>
<td>101 ± 6</td>
<td>88 ± 18</td>
</tr>
<tr>
<td>Zn</td>
<td>94 ± 5</td>
<td>94 ± 5</td>
<td>104 ± 9</td>
</tr>
</tbody>
</table>
2.4 Data analysis

Data were corrected from background concentrations by subtracting three times the average of metal levels in the procedural blanks, and were expressed as means ± standard deviation. The results were analyzed with Origin 7.5. Descriptive analysis was completed with a criterion of p < 0.05 as significant difference using SPSS 12.0 for Windows.

3 RESULTS AND DISCUSSION

3.1 Sediment characteristics

The characterization of surface sediments from different sampling sites in NCIP is given in Table 1. The amounts of TOC, TIC, TC and TN in sediments were within the ranges of 1.35-3.90, 0.45-5.50, 1.75-10.05, and 0.09-0.48%, respectively. The high recovery (104%) between the average value of $TC_{\text{TOC+TIC}}$ estimated derived from LOI and the measured TC contents obtained from elemental analysis further supported the validity of the LOI values.

The pH measurements of surface water and sediment samples are also listed in Table 1. The results revealed the slightly alkaline nature of the samples (varying between 7.38 and 8.28 with a mean value of 7.86 for water, and 7.26 and 7.90 with a mean value of 7.67 for sediments).

3.2 Metal concentrations in surface water and sediments

The averages of dissolved metal concentrations in water from different sampling sites are shown in Fig. 2. The magnitude order of metal concentrations was Zn > Pb > Cr > Ni > Cu > Cd. From Fig. 2, we can clearly see that, among all sites, the highest values of metals all appeared at site 1; for instance, the concentrations of Cr, Ni, and Cd in site 1 were 0.0302, 0.0398 and 0.0105 mg L⁻¹, respectively, which were significantly higher than those of the other sites.

![FIGURE 2 - Distribution of heavy metals in water from different sites of NCIP.](image)

### TABLE 4 - Total heavy metal concentrations (mean ± standard deviation) in surface sediments (mg kg⁻¹, dry weight).

<table>
<thead>
<tr>
<th>Site</th>
<th>Cd</th>
<th>Pb</th>
<th>Ni</th>
<th>Cu</th>
<th>Cr</th>
<th>Zn</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>1.15 ± 0.31</td>
<td>71.24 ± 12.33</td>
<td>105.69 ± 1.70</td>
<td>28.67 ± 2.34</td>
<td>318.50 ± 8.62</td>
<td>265.47 ± 14.74</td>
</tr>
<tr>
<td>S2</td>
<td>1.03 ± 0.16</td>
<td>16.19 ± 4.08</td>
<td>37.87 ± 0.01</td>
<td>32.78 ± 0.58</td>
<td>54.50 ± 4.99</td>
<td>105.23 ± 8.06</td>
</tr>
<tr>
<td>S3</td>
<td>1.07 ± 0.06</td>
<td>22.57 ± 5.36</td>
<td>37.41 ± 2.63</td>
<td>37.38 ± 2.64</td>
<td>69.20 ± 0.64</td>
<td>90.22 ± 5.38</td>
</tr>
<tr>
<td>S4</td>
<td>0.93 ± 0.05</td>
<td>22.13 ± 1.03</td>
<td>26.26 ± 2.70</td>
<td>41.06 ± 1.21</td>
<td>34.26 ± 3.08</td>
<td>121.5 ± 2.69</td>
</tr>
<tr>
<td>S5</td>
<td>1.89 ± 0.03</td>
<td>72.23 ± 2.87</td>
<td>36.78 ± 0.28</td>
<td>173.75 ± 1.38</td>
<td>63.84 ± 1.35</td>
<td>160.63 ± 3.60</td>
</tr>
<tr>
<td>S6</td>
<td>0.92 ± 0.04</td>
<td>9.73 ± 0.95</td>
<td>16.23 ± 0.01</td>
<td>11.07 ± 0.69</td>
<td>30.33 ± 1.03</td>
<td>34.33 ± 18.40</td>
</tr>
<tr>
<td>S7</td>
<td>1.22 ± 0.04</td>
<td>38.42 ± 2.46</td>
<td>37.00 ± 1.58</td>
<td>63.77 ± 3.61</td>
<td>64.29 ± 2.32</td>
<td>293.41 ± 8.50</td>
</tr>
<tr>
<td>S8</td>
<td>1.11 ± 0.12</td>
<td>31.79 ± 5.74</td>
<td>32.19 ± 2.85</td>
<td>57.17 ± 4.02</td>
<td>56.83 ± 5.94</td>
<td>130.76 ± 17.61</td>
</tr>
<tr>
<td>PEC</td>
<td>4.98</td>
<td>128</td>
<td>48.6</td>
<td>149</td>
<td>111</td>
<td>459</td>
</tr>
<tr>
<td>TEC</td>
<td>0.99</td>
<td>35.8</td>
<td>22.7</td>
<td>31.6</td>
<td>43.4</td>
<td>121</td>
</tr>
</tbody>
</table>

PEC, Probable Effect Concentration. TEC, Threshold Effect Concentration.
Table 4 summarizes the total concentrations of metals in surface sediments. They followed the order Zn (34.33-280.15 mg kg⁻¹) > Cr (30.33-318.50 mg kg⁻¹) > Cu (11.07-173.75 mg kg⁻¹) > Ni (16.23-105.09 mg kg⁻¹) > Pb (9.73-71.24 mg kg⁻¹) > Cd (0.92-1.89 mg kg⁻¹). On the whole, the spatial distribution of metals in sediments was consistent with that in water. Among all the sites, site 1 showed the highest metal concentrations because of its proximity to the industrial area. Meanwhile, the lowest levels were found in site 6 due to the Yangtze River’s largest water discharge and site 6’s relatively longer distance from the industries. Metal content in river sediments is a good indicator of their usage in local industries. The ranges of metal concentrations reported herein were below those of the river originating from an electroplating plant in Jiangsu Province [15], but were much higher compared to the previous study on a small-scale vegetable farming system of Nanjing [6].

In order to assess the possible relationships between concentrations of different metals and pH, TOC as well as IC of the sediments, and also to find the possible inter-elemental associations, a correlation analysis was performed. Due to the non-normal distribution of the above parameters, the non-parametric Spearman rank-order correlation was subsequently used. Significant positive correlations were found among Cr and Cd, Pb, Ni (p < 0.05), Pb-Cd (p < 0.01) (Table 5), which suggested that these metals may share a common potential contamination source and/or kindred geochemistry characteristics. And the analogous sources might be related to excessive anthropogenic input of industrial effluents in the studied area (such as plants dealing with steel manufacture, plating, petrochemical, leather, dyestuff and so on).

### 3.3 Fractionation of metals in surface sediments

Different metal fractions have different behavior with respect to remobilization under varying environmental conditions, such as pH, organic matter, and hydrodynamic conditions [16]. In this paper, results obtained from the BCR are shown in Fig. 3. The distribution of metals in four fractions varied greatly among different elements and sediment samples.

It is evident from Fig. 3 that the metals (Ni, Cr, Cu, Zn and Pb) were mostly found in the B4 fraction (77.2, 69.9, 59.9, 47.8 and 46.4% of total amount, respectively). As we all know, metals in this fraction are mainly bound to silicates and chemically stable. Therefore, they are unavailable to aquatic organisms. As a result, the greater percentage the metal presents in the B4 fraction, the smaller risk it has, and the reason is that this portion of metal cannot be re-released to water under normal conditions [17]. However, the fraction distribution of Cd was different from the other metals, and the greatest amount of Cd was found in the exchangeable and carbonate bound fraction (B1) which exceeded 50% of the total amount, followed by the B4 fraction (32%), and the lowest proportion was associated with the organics/sulfides bound fraction (B3). Metals in the B1 fraction are the most active, mobile, and available ones for aquatic organisms [18]. Other studies also reported that Cd in sediments was associated with labile fraction [19, 20]. For example, Jain et al. [27] suggested that amongst the different metals, Cd concentration was the lowest but a major percentage of it (30-50%) was present in the most mobile fraction (B1) and, therefore, can easily enter the food-chain.

Except the dominating B4 fraction, considerable proportions of Pb, Cu, Cr and Zn were present in the B3 fraction (31.4, 28.8, 27.3 and 20.5%, respectively), which was similar to the results of Pertsemli et al. [15] and Hang et al. [20]. Compared with other sites, site 4 with the highest TOC level (Table 1) also had a higher percentage of metals in the B3 fraction. The results indicated that organic matter and sulfide-absorbed metals played a significant role in controlling the mobilization of trace metals in sediments because these metals could be released following degradation of the organic matter, or oxidation of sulfides to sulfates [4]. The distributions of these metals in the B1 and iron-manganese oxides-bound (B2) fractions were relatively low. In terms of Pb, the percentage of the B1 fraction (10.4%) was approximately equivalent to the B2 fraction (11.8%), while the percents of Zn were 18.8 and 12.8% for the B1 and B2 fractions, respectively. Besides Zn and Pb, the percentages of Cr, Cu and Ni were all less than 10%; especially, a very small proportion of Cr was present in the B1 and B2 fractions (1.5 and 1.2%). Considering that metals bound to Fe-Mn oxides would be released under reductive conditions, they are unstable under anaerobic environmental conditions [17]. Our results suggested that Cr was unlikely to pose a direct threat to the aquatic organisms.

---

**Correlation is significant at the 0.01 level (2-tailed); * Correlation is significant at the 0.05 level (2-tailed).**

**TABLE 5 - Correlation matrices of metal concentrations and some other parameters in surface sediments.**

<table>
<thead>
<tr>
<th></th>
<th>Cd</th>
<th>Pb</th>
<th>Ni</th>
<th>Cu</th>
<th>Cr</th>
<th>Zn</th>
<th>pH</th>
<th>TOC</th>
<th>IC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cd</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pb</td>
<td>0.952**</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ni</td>
<td>0.429</td>
<td>0.381</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cu</td>
<td>0.690</td>
<td>0.619</td>
<td>-0.119</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cr</td>
<td>0.714*</td>
<td>0.738*</td>
<td>0.762*</td>
<td>0.190</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zn</td>
<td>0.381</td>
<td>0.524</td>
<td>0.143</td>
<td>0.381</td>
<td>0.262</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>0.347</td>
<td>0.443</td>
<td>-0.299</td>
<td>0.790*</td>
<td>0.036</td>
<td>0.635</td>
<td>1.000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TOC</td>
<td>-0.071</td>
<td>-0.071</td>
<td>0.214</td>
<td>0.143</td>
<td>0.143</td>
<td>0.476</td>
<td>0.263</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td>IC</td>
<td>-0.024</td>
<td>0.095</td>
<td>-0.452</td>
<td>0.238</td>
<td>-0.238</td>
<td>0.667</td>
<td>0.575</td>
<td>0.095</td>
<td>1.000</td>
</tr>
</tbody>
</table>

**Correlation is significant at the 0.01 level (2-tailed), * Correlation is significant at the 0.05 level (2-tailed).**
3.4 Risk Assessment

3.4.1 Water quality assessment

Metal concentrations of surface water were compared with the Chinese Environment Quality Standard for Surface Water (GB 3838-2002) (Table 6) which classifies water quality into five levels, grades one to five [21]. In accordance with the above standard, the concentration of Cu was below the highest standard, levels of Cr and Zn were also under the cutoff value for grade one water quality, except in site 1 (Cr, 0.0302 mg L⁻¹; Zn, 0.0503 mg L⁻¹) and site 3 (Zn, 0.0594 mg L⁻¹). However, the levels of Cd in four sampling sites were relatively high, exceeding the standard value (0.001 mg L⁻¹) of grade one, and Pb levels were also above the value for grade one in sites 1-4.

To further assess metal contamination in surface water, the measured concentrations of metals were also compared with USEPA criteria of Maximum Concentration (CMC) and Continuous Concentration (CCC) (Table 6) in aquatic
TABLE 6 - National recommended freshwater quality criteria for dissolved metals (mg L⁻¹).

<table>
<thead>
<tr>
<th></th>
<th>Cd</th>
<th>Pb</th>
<th>Ni</th>
<th>Cu</th>
<th>Cr</th>
<th>Zn</th>
</tr>
</thead>
<tbody>
<tr>
<td>GW a</td>
<td>≤ 0.001</td>
<td>≤ 0.01</td>
<td>≤ 0.01</td>
<td>≤ 0.01</td>
<td>≤ 0.05</td>
<td></td>
</tr>
<tr>
<td>CMC b</td>
<td>0.002</td>
<td>0.065</td>
<td>0.47</td>
<td>0.013</td>
<td>0.016</td>
<td>0.12</td>
</tr>
<tr>
<td>CCC c</td>
<td>0.00025</td>
<td>0.0025</td>
<td>0.052</td>
<td>0.009</td>
<td>0.011</td>
<td>0.12</td>
</tr>
</tbody>
</table>

a GW, grade one of the Chinese Environmental Quality Standards for Water (GB3838-2002); b CMC (acute), criterion maximum concentration; c Cr (VII); d CCC (chronic), criterion continuous concentration.

ecosystems; both criteria have been established to prevent from acute and chronic toxicity to aquatic organisms, respectively [15, 20, 22]. From Table 6 and Fig. 2, it can be seen that concentrations of Cu, Ni, Zn and Cr in all sites were below the recommended criteria, except for Cr in site 1; this indicated that contaminations of these four metals in NCIP were not serious. However, Pb and Cd were always over CCC criteria for most sites, except for Pb at sites 7 and 8 as well as Cd at sites 5 and 7. This is probably due to the fact that site 7 is located on the downstream of the Yuezi River Gate which had interception effect of contaminants, and there were fewer plants around this site. Levels of Cd exceeded CMC in sites 1, 3 and 4, showing that Cd in these sites was likely to pose a threat to aquatic organisms, probably due to discharges of industrial effluents.

3.4.2 Sediment quality assessment

In order to estimate sediment quality conditions for aquatic organisms, metal concentrations were compared with criteria for freshwater sediments recommended by the National Oceanic and Atmospheric Administration (USA) (Table 4) [23]. Concentrations of metals in all sites were far below the proposed Probable Effect Concentrations (PECs), except for Ni and Cr in site 1 as well as Cu in site 5. Concentrations exceeded the Threshold Effect Concentrations (TECs) in sites 1 and 5, while metals from site 6 were all below the TECs. These results indicated that Ni and Cr at site 1 and Cu at site 5 were likely to result in harmful effects. Other metals in sites 1 and 5 were of potential risk. However, harmful effects were unlikely to be observed in site 6. Water quality was safe for aquatic organisms there. This conclusion was the same as that of the water samples.

Enrichment factor (EF) was calculated for each metal to evaluate the level of contamination and possible anthropogenic inputs in surface sediments. EF was calculated using the following expression [3, 24-25]:

\[ EF = \frac{(X / Fe)_{sediment}}{(X / Fe)_{background}} \]

where, X is the target metal, Fe is the selected normalizing element (reference metal), \((X / Fe)_{sediment}\) is the ratio of metal to Fe concentrations in sample, and \((X / Fe)_{background}\) is the ratio of background value of metal to Fe in the river system sediments of the Yangtze River. Table 7 shows the calculated EF values (EFs).

According to a five-category ranking system [26], except Cd, the EFs of the other five metals in site 6, the drinking water intake on the Yangtze River, were all < 1, suggesting that there is hardly any anthropogenic contamination in this site. This result is also supported by the above evaluation for water quality. In terms of other sites, the EFs of Pb, Ni, and Cr were also not very high (almost < 2). Noticeable enrichment of Pb was observed in sites 1 (> 3) and 5 (> 2), possibly be caused by nearby road traffic powered by gasoline containing Pb (site 1), and ships carrying coal on the Macha River (site 5). In addition, results of the present work showed that Cd was moderately enriched in sediments since the EFs of Cd were all in the range of 2-5. The relatively high Cd concentrations mainly result from anthropogenic inputs including pigment industries, thermal power plants, textile, solid waste

TABLE 7 - Enrichment factor (EF) of heavy metals in surface sediments, and judge standard of contamination degree by EF [26].

<table>
<thead>
<tr>
<th>Sampling sites</th>
<th>Cd</th>
<th>Pb</th>
<th>Ni</th>
<th>Cu</th>
<th>Cr</th>
<th>Zn</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>2.89</td>
<td>3.35</td>
<td>5.62</td>
<td>1.93</td>
<td>7.66</td>
<td>3.81</td>
</tr>
<tr>
<td>S2</td>
<td>2.53</td>
<td>0.75</td>
<td>1.98</td>
<td>2.17</td>
<td>1.28</td>
<td>1.48</td>
</tr>
<tr>
<td>S3</td>
<td>4.68</td>
<td>1.85</td>
<td>3.49</td>
<td>4.40</td>
<td>2.90</td>
<td>2.26</td>
</tr>
<tr>
<td>S4</td>
<td>4.32</td>
<td>1.92</td>
<td>2.59</td>
<td>5.11</td>
<td>1.52</td>
<td>7.42</td>
</tr>
<tr>
<td>S5</td>
<td>2.95</td>
<td>2.11</td>
<td>1.22</td>
<td>7.28</td>
<td>0.95</td>
<td>1.43</td>
</tr>
<tr>
<td>S6</td>
<td>2.11</td>
<td>0.42</td>
<td>0.80</td>
<td>0.68</td>
<td>0.67</td>
<td>0.45</td>
</tr>
<tr>
<td>S7</td>
<td>2.77</td>
<td>1.63</td>
<td>1.79</td>
<td>3.89</td>
<td>1.40</td>
<td>2.70</td>
</tr>
<tr>
<td>S8</td>
<td>2.67</td>
<td>1.43</td>
<td>1.65</td>
<td>3.69</td>
<td>1.31</td>
<td>1.80</td>
</tr>
</tbody>
</table>

Backgroud level (mg kg⁻¹): 0.44 - 23.5

<table>
<thead>
<tr>
<th>EF value</th>
<th>EF-class</th>
<th>Contamination degree</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 2</td>
<td>1</td>
<td>minimal</td>
</tr>
<tr>
<td>2 ~ &lt; 5</td>
<td>2</td>
<td>moderate</td>
</tr>
<tr>
<td>5 ~ &lt; 20</td>
<td>3</td>
<td>significant</td>
</tr>
<tr>
<td>20 ~ 40</td>
<td>4</td>
<td>very high</td>
</tr>
<tr>
<td>&gt; 40</td>
<td>5</td>
<td>extremely high</td>
</tr>
</tbody>
</table>
treatment center, and other industries which are mostly concentrated in this area. Furthermore, the distribution of different fractions may also imply that an appreciable percentage of Cd could be remobilized and become readily available following a slight lowering of pH in sediments [6]. The sediments might have been contaminated by the discharge of some wastewater from factories entering the river system. Higher EFs of metals were also observed at sampling sites 1 and 3-5, located on rivers flowing through NCIP and indicating that, with the rapid development of industry, intensive human activities have aggravated the water quality.

4 CONCLUSIONS

The quality of water and sediments from NCIP has been established by evaluation of metals contents and their fractionation. As a consequence, we have shown herein the influence of industrial activities to the aquatic ecosystems. Among all sampling sites, metal concentrations at site 1 were the highest, while the lowest were found in site 6. Using the sequential-extraction procedure, Cd seemed to have a high potential bioavailability in the aquatic system because of its preferential association with the B1 fraction, while others were dominantly confined in the B4 fraction.

The comparison of metal concentrations with National/International Quality Criteria, together with enrichment factor, might provide a relatively useful croscheck for the risk assessment. The results all indicated that Cd in some sites was likely to pose a threat to aquatic organisms. Cd content was the lowest among these metals, but considering its comparatively high availability and toxicity, it is, therefore, a necessity to pay attention to Cd contamination in the studied area.

ACKNOWLEDGEMENTS

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REFERENCES


A NEW INNOVATIVE DECISION SUPPORT SYSTEM USING FUZZY REASONING FOR THE ESTIMATION OF MOUNTAINOUS WATERSHEDS TORRENTIAL RISK (TOR-SYS). THE CASE OF RIVER KOSYNTHOS

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Department of Forestry & Management of the Environment & Natural Resources, Democritus University of Thrace, 68200 N. Orestiada, Greece

ABSTRACT

This research effort describes the design, development and testing of a Decision support system that performs torrential risk estimation of mountainous watersheds. The system applies an innovative model of Fuzzy logic. The application of the system was done in one of the most risky watersheds of Greece river “Kosynthos” which is located in the North-Eastern part of the country. The system was tested successfully in the 32 most important subwatersheds of “Kosynthos”. Its main advantage is that it offers the chance to evaluate the torrential risk of the mountainous watersheds based on a small number of parameters that are easy to measure. The final target is the classification of the watersheds based on their risk.

KEYWORDS: Basin, torrential risk, fuzzy logic, classification.

1 INTRODUCTION

One of the major problems related to the hydrological behavior of torrential streams is the estimation of their torrential risk, which is defined as “the degree of floods caused by streams” [1].

Torrential risk is defined as the degree of floods caused by a stream. The determination of torrential risk has been achieved up to now via the combination of a wide variety of complicated methods. Common methodology initially requires the calculation of the sediment based on either various hydrological models or on heuristic-empirical Formulae. Next, the bulk of sediment discharge that is produced in a watershed is calculated (this is usually estimated in Greece using either RUSLE or Gavrilović methods). If the combination of the above results exceeds specific limitations, in crudest form, watersheds are classified into low torrential and extremely high torrential risk watersheds [1]. As it becomes obvious, this method is rather time-consuming and particularly inconvenient in its use due to the fact that it requires the calculation of many different factors. From an empirical point of view, we could say that its reliability is important; nevertheless, the boundaries between the groups in the classification may prove to be not too easily discernible [2, 3].

To eliminate the above limitations in this study, an innovative DSS using fuzzy logic was developed to evaluate the mountainous watersheds torrential risk. The system will be applied to estimate risk due to floods in the framework of Act 2007/60.

The validation of the results reliability was done by comparing with the outputs of existing approaches. The application was done for the “Kosynthos” river area in the North-Eastern part of Greece, which according to Kotoulas is one of the most risky watersheds of Greece [4, 5]. This watershed has rough topographical characteristics and a very dense hydrographical network.

The mountainous nature of the area combined with the extreme waterfalls and snowing and with the non solid geological forms, favour the erosion phenomenon and the transfer of a high volume of load of sediments.

2 MATERIALS AND METHODS

2.1 Area of research

The mountainous watershed of “Kosynthos” river is located in the South-Eastern part of Xanthi prefecture, close to the mountains of Western Rodopi. It starts from the “[Gyloastro” mountain top (altitude of 1.827m) near the Greek-Bulgarian border and it reaches the limits of the town of “Xanthi” (altitude 80m). Its orientation is North-West to South-East. It ends at the lake “Vistonida” which is filled by the water and the sediments of “Kosynthos”.

* Corresponding author
The total area of its watershed is 236.7 km². River “Kosynthos” causes significant torrential phenomena and it is one of the most torrentially risky rivers in Greece.

According to the climate classification of Koppen the area belongs to the type Cfb. The average annual temperature in the mountainous zone is less than 22°C due to the altitude and the rain is equally distributed throughout the year. From this point of view the mountainous Rodopi has a climate particularity in the framework of the Greek territory [6].

In the study area, there are no observed values as regards the water supply as well as to the sediment discharge that are necessary for the determination of the torrential risk. For this reason, specific models were used to estimate the sediment and the sediment supply of the watersheds in the study area.

The required data are divided into physiographic and climate. The topographic data of the area under study were obtained by the digitization of maps 1:50.000 of the Geographical Army Service. Through the map digitization, the design of the hydrographical network and the specification of the boundaries of the watersheds were achieved. Also the hypsometric curves (20m) were drawn. The reproduction of these curves (in 5m) was performed by the use of Arcmap 9.3 and the digital elevation model of the ground was created with raster dimension of 10m. The “Kosynthos” watershed was initially divided in 52 substreams. This allowed a more efficient study of the whole area. Then the watersheds that had an area less than 1 km² were ignored. The final number of substreams was reduced to 32 (see Fig. 2). Then, for each one of the 32 streams the most important morphometric characteristics were estimated by using Arcmap 9.3. [7, 8, 12]. These characteristics are shown in Table 1. Geology is one of the most important parameters regarding the ground erosion. For the area under study, the edaphological map was created. This creation was done by using the file WRBFU.kml (from the European Soil Data Center) and by digitizing the above file (JRC European Commission, Land management and Natural Hazard Unit, European Soil Database (ESDB), Raster library 1 Km × 1 Km).

The database Corine LC was used for the determination of the land use and especially for the specification of the land cover percentage. The data were used as they were recorded in the basic scale of Corine. The data of the meteorological stations of Xanthi, Gerakas and Oraio were used for the climate conditions study. The station of Xanthi is located in 80 meters altitude the one of Gerakas in 340m and the one of Oraio in 610 meters and they cover the whole torrential streams. For all stations, data from 1980 till 1998 were used. The meteorological data came from the forest administration and from the Land Improvements Service of the Prefecture of Xanthi.

Missing rainfall and average annual temperature data, especially in the meteorological stations of Oraio and Gerakas were substituted by the use of the “Replace Missing Values” command by using the regression method of the SPSS statistical package [9, 10].

2.2 Methods

For the purposes of the present paper, we have developed an authentic, easy-to-use fuzzy logic system in order to classify watersheds depending on their torrential risk. The results were compared with results obtained from the application of classic and more complicated methods that require the calculation of the sediment and of the sediment supply of every watershed. More specifically:

2.2.1 Estimating Maximum Water supply

Empirical formulae were employed for the determination of the maximum water supply due to missing data values for the area under study.

The most well established empirical formulae that were selected were the “Alexopoulou”, Friedrich, Klement-Wunderlich, Wundt, Coutagne, Valentini, Kursteiner, Henry Boot, Melli Kresnik, Muller and Melli-Muller [7, 11, 12]. These empirical formulae are widely applied today especially in the watersheds, where as they can be applied probably also in our country. Most of the above mentioned formulae estimate the runoff $q$ (m³/sec·km). The estimation of the corresponding total water supply is done with the application of the following relation:

$$ Q = q \cdot F \text{ (m}^3\text{/sec•km)}$$

2.2.2 Erosion estimation with empirical models

The estimation of ground erosion was based on the Universal Soil Loss Equation (RUSLE), and on the Gavrilović approach. Empirical models were used due to the lack of data [13], (mainly due to lack of time series) and also because there are no water supply measurements for many years.
TABLE 1 - Morphometric characteristics of the Subwatersheds of river Kosynthos

<table>
<thead>
<tr>
<th>A/A</th>
<th>Subwatershed Area (km²)</th>
<th>Perimeter (km)</th>
<th>Mean Subwatershed Slope (Jm)</th>
<th>Degree of the Roundness</th>
<th>Length of the main stream (km)</th>
<th>Mean Subwatershed Altitude (m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.11</td>
<td>5.05</td>
<td>26.8</td>
<td>0.3207</td>
<td>2.36</td>
<td>459.07</td>
</tr>
<tr>
<td>2</td>
<td>7.47</td>
<td>12.80</td>
<td>23.7</td>
<td>0.3839</td>
<td>5.44</td>
<td>522.90</td>
</tr>
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<td>3</td>
<td>5.11</td>
<td>9.44</td>
<td>24.6</td>
<td>0.3289</td>
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<td>650.48</td>
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<tr>
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<td>20.9</td>
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<td>0.1968</td>
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<td>18.9</td>
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<td>0.2105</td>
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<td>16</td>
<td>0.3599</td>
<td>3.81</td>
<td>366.25</td>
</tr>
</tbody>
</table>
2.2.2.1 RUSLE

The RUSLE is based on several years of measurements of the erosion volume on certain surfaces, which were conducted by Wischmeier and Smith in various areas of the USA in the period 1930-1952 [14, 15]. This research aims to apply the RUSLE method in high slope areas with various types of land use and it was performed by the use of modified parameters. The general equation of soil loss RUSLE is showed in the following equation.

\[ A = R \cdot K \cdot L \cdot S \cdot C \cdot P \]

Where,
- \( A \): is the estimated annual general erosion.
- \( R \): is the coefficient of erosionability of the sediments
- \( K \): is the coefficient of erosionability of the geological background,
- \( L \): is the topographic coefficient
- \( C \): is the plant cover coefficient
- \( P \): is the coefficient of soil protection

In the total erosion of the watershed, the erosion of the water streams is added, which is estimated empirically as the 20% of the surface erosion.

The determination of the coefficient \( R \) was based on the relation \( R=0.83N – 17.7 \) which was developed in Germany and it was adjusted for the Greek conditions [16] :

\[ R = 0.83 \cdot N -17.7 \]

where, \( R \) (in Mj · mm/(ha · h)) is the coefficient of erosion due to rainfall, and \( N \) (counted in mm) the average annual rainfall. The estimation of \( N \) was done by using the annual average precipitation in the meteorological station of Xanthi based on \( y = 0.7429 \cdot x – 283.12 \). The rain scale value is \( b=0.7429 \), which means that by increasing the altitude by 100m the precipitation increases by 74.29 mm [17].

The estimation of \( K \) was done based on the WRBFU.kml file from the European Soil Data Center, because we didn't find any values for the coefficient related to the available mother rock.

The estimation of \( L \) was done based on the Jianguo Ma formula [18]:

\[ L = (\text{Flow Accumulation} \cdot \text{Cell Size} / 22.13)^{0.4} \cdot (\text{Sin Slope} / 0.0896)^{1.3} \]

A value of one was given to the coefficient \( P \) for all the subwatersheds, because no technical works were found in them. The watershed number 5 is the only exception because in its central part, five dams have been built, aiming to keep the load of sediments. For the specific watershed this coefficient had the value 0.6.

Finally for the estimation of \( C \), the European Environmental Agency Corine 2000 was used and also the data from the following Table 2 were employed.

Table 2 presents the land use codes [19].

2.2.2.2 The Gavrilovič Method

The Gavrilovič method [20, 21, 22, 23] is a distributed parametric model, which was used widely for the annual forecasting of the percentages of soil erosion and for the estimation of the load of sediments in wide scale, for watersheds in Slovenia and Croatia during the last 35 years [24]. This method was developed in Serbia aiming to protect the land from erosion, through proper forest management and stream control actions. This is a method that has been applied also to watersheds of the Italian and Swedish Alpes [25, 26, 27].

The method of Gavrilovič is based on the fact that the load of sediments that is carried by the torrential streams (\( G \)) is related to the volume of sediments that are produced by the soil erosion \( W \) and to the quantity that is kept in the river basin (\( R \) is the coefficient of the placement of the sediments).

It is given by the following equation

\[ G = W \cdot R \]

The estimation of the quantity of sediments \( W \) is done by the use of empirical coefficients (erosion coefficient, soil protection coefficient) and also by natural characteristics (annual rainfall, temperature, average slope and watershed area). The method determines the average annual erosion in the mountainous watersheds of the torrential streams and is given by the following equation:

\[ W = T \cdot h \cdot \pi \cdot z^{1/3} \cdot F \text{ (m}^3\text{/year)} \]

where, \( T \) is the temperature index which can be calculated by the following equation:

\[ T = \sqrt{\frac{t_0}{10} + 0.1} \]

where, \( t_0 \) is the average annual temperature in the mountainous watershed in °C.

<table>
<thead>
<tr>
<th>Land use</th>
<th>Coefficient C</th>
<th>Land use</th>
<th>Coefficient C</th>
<th>Land use</th>
<th>Coefficient C</th>
<th>Land use</th>
<th>Coefficient C</th>
</tr>
</thead>
<tbody>
<tr>
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<td>312</td>
<td>0.001</td>
<td>241</td>
<td>0.18</td>
<td>324</td>
<td>0.02</td>
</tr>
<tr>
<td>211</td>
<td>0.3</td>
<td>313</td>
<td>0.001</td>
<td>242</td>
<td>0.18</td>
<td>331</td>
<td>0.6</td>
</tr>
<tr>
<td>213</td>
<td>0.15</td>
<td>321</td>
<td>0.3</td>
<td>243</td>
<td>0.1</td>
<td>332</td>
<td>0.45</td>
</tr>
<tr>
<td>221</td>
<td>0.2</td>
<td>322</td>
<td>0.45</td>
<td>244</td>
<td>0.05</td>
<td>333</td>
<td>0.45</td>
</tr>
<tr>
<td>222</td>
<td>0.2</td>
<td>323</td>
<td>0.03</td>
<td>311</td>
<td>0.001</td>
<td>512</td>
<td>0.000</td>
</tr>
</tbody>
</table>
The following equation was used for the calculation of the \( t_s \) coefficient:

\[
T_s = T_o - \alpha (z_s - z_o)
\]

where, \( T_s \) and \( T_o \) are the temperature of the watershed and the base station respectively, \( z_s \) and \( z_o \) are the watershed altitude and the base station altitude respectively, and \( \alpha \) is the heat scale, \( h \) is the average annual precipitation of the watershed in (mm).

The estimation of the \( h \) parameter was done by the same methodology as the one that estimates \( N \) in the USLE method.

\( F \) is the area of the watershed (km\(^2\))

\( z \) is the erosion index which is estimated by the following equation:

\[
z = x \cdot y \cdot (\varphi + J^{1/2})
\]

where, \( x \) is the index that expresses the decrease of the resistance of the geological background during the erosion, depending on the situation and on the cultivation of its surface and on the percentage of forest cover [24]. The values of the coefficient \( x \) for various types of land use according to Corine 2000 are:

<table>
<thead>
<tr>
<th>Land Use</th>
<th>Value of ( x )</th>
<th>Land Use</th>
<th>Value of ( x )</th>
<th>Land Use</th>
<th>Value of ( x )</th>
<th>Land Use</th>
<th>Value of ( x )</th>
</tr>
</thead>
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<td>0.05</td>
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<td>0.05</td>
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<td>243</td>
<td>0.4</td>
<td>243</td>
<td>0.4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\( y \): is the erosion index of the geological background depending on the geological and soil composition of the methods.

\( \varphi \): is the index that expresses the type and the degree of watershed erosion. For every watershed a different value was assigned. These values were determined after a visit to the area of research and after a careful examination of all parameters [12].

\( I \): is the average slope of the surface of the watershed.

The coefficient of the load of sediments’ placement in the watershed \( R \) was adjusted by [28] and it is estimated based on morphological characteristics of the watershed. It is given by the following equation

\[
R = \left[ \frac{(O \times D)^{1/2} \{ L + L_i \}}{F(L + 10)} \right]
\]

Where,

\( O \): is the perimeter of the watershed
\( D \): is the average altitude of the watershed
\( L \): is the length of the central stream
\( L_i \): is the length of secondary streams

2.3 Fuzzy logic

2.3.1 Logic of fuzziness

The theory of Fuzzy logic was introduced by Prof. Lotfi Zadeh in 1965. He recognized that Boolean Logic that uses two situations (true or false) does not cover the grey side of true problems. It is a fact that sometimes truth is grey, fractional and fuzzy something can be partly true or false [29]. Boolean logic has only 2 values O (false) and 1 (true). More specifically in Boolean algebra a data point either belongs to a set or it does not.

In fuzzy logic every piece of the universe belongs to each fuzzy set in a matter of degree. The degree of belonging to a fuzzy set can be partially true or false simultaneously. Contrary to the binary crisp sets, a fuzzy set does not have specific boundaries. The structure of a fuzzy set allows a natural way of data processing in problems where inaccuracy is due to lack of clearly defined rules that would define the relation of a set of elements, in order to describe the respective variables used [30]. The fuzzy sets break, in a way, the law of dichotomy. Data belong partly to a fuzzy set or to more than one fuzzy set. The fuzzy membership function is a basic concept introduced for the understanding of fuzzy sets by Zadeh in 1965. The membership values range from 0 to 1 and they determine the degree of memberships of a point a to a fuzzy set A. During the last decades, fuzzy logic is used in a large number of problems and the application area is quite wide including: process control, decision making and management process research, finance, pattern recognition and classification. The most important disadvantage is its difficulty in the design of a fuzzy controller. For this reason the knowledge of an expert is required [31].

2.3.2 Fuzzy sets

A fuzzy set \( A \) is defined through the following membership function:

\[
\mu_A(X) : X \rightarrow [0,1] \text{ where: } \mu_A(X) = \begin{cases} 
1 & \text{if } X \text{ totally in } A \\
0 & \text{if } X \text{ no in } A \\
(0,1) & \text{if } X \text{ partially in } A 
\end{cases}
\]

In the case of continuous values \( A \) can be expressed as a triangular, trapezoidal, Gaussian etc. function [32].
2.4. The software
2.4.1 Development of the model

The Mamdani approach was employed for the development of the fuzzy logic model. The main characteristics of the Mamdani approach are the following:

- The fuzzification operation, where actual values are translated to fuzzy ones.
- The use of a fuzzy inference mechanism where the antecedent and the consequent use proper Linguistics
- The potential use of a fuzzy aggregation operations
- The deffuzzification capability where fuzzy values are translated to actual ones [33].

In fuzzy logic fuzzy relation operates are used in order to obtain the final value of the consequent. These operators process several basic characteristics of the operators used in a wider set theory and they are divided in two major classes:

- The conjunction fuzzy operators called T-norms and the disjunction ones called S-norms.
- The average operators, which model connections between T-norms and S-norms.

The T-norms differ depending on their generality and on their adaptability and they are based on the way of their justification. Their justification may be empirical or axiomatical or even heuristic [30].

For every design problem there is a set of feasible solutions in which every proposed solution should have an assessment score. This overall score can be represented by a set of fuzzy sets $D^i_k$, k=1,2,3,... These fuzzy sets are divisions of the initial set and they are expressed as relation functions with values in the closed interval [0,1].

FS is a family of fuzzy sets and score is the variable used for the corresponding fuzzy set.

In this case, due to the nature of the model, we have chosen to make the deffuzzification of the fuzzy powerset in order to rank the mountainous watersheds.

The input includes the topographic coefficient LS, as it was defined by the method of the global soil loss method, the average annual precipitation in mm, the area of the watershed measured in km², the coefficient of technical works P, the coefficient of geological background, and the coefficient of the forest cover, as they are defined by the RUSLE method.

These rules were created using the knowledge and the personal experience of the system’s designer. The ruleset consisted of a number of rules, which were equal to the number of fuzzy sets raised to the number of input sets. Specifically the fuzzy sets were 3 for each of the six input parameters and consequently there should be $3^6=729$ rules. This made the process of building the rule set very complicated. An additional problem is that when we wish to add more parameters for further analysis the number of rules becomes so high that it makes the construction of the ruleset practically impossible. Eg $3^7=2187$ or $3^8=6561$ [34]. To solve this problem the input parameters were divided in two types: those that favour the phenomenon and those that act in an inhibitory manner.

![ICON 2 - Structure of the TOR-SYS Model](image-url)
Consequently the area of the watershed, the average annual precipitation, the surface coefficient and coefficient of geological background belong to the first type whereas the technical works coefficient and the forest cover belong to the second category.

So, from the first factors we can obtain the unified torrential risk index. This index is the result of the unification of all the negative parameters.

From the intersection of the other parameters we obtained the unified torrential risk index due to positive factors. The rule set that was used for the unified negative risk index (UNRI) was $3^4=81$ whereas for the unified positive risk index (UPRI) there were $3^2=9$ rules. The unified torrential risk coefficient (UTRC) was produced by a new Mamdani model where the UNRI and the UPRI were used as input features and the UTRC was the outcome. The number of rules in this case was equal to $3^2=9$. Totally, for the whole effort 99 rules were used. This is a very small number compared to the 729 that should be used initially.

During the data input several tests were performed in order to find the conditions that would stabilize the system. Thus, the fuzzy operator AND was given as a product, whereas OR was given as an algebraic sum, and the implication method was given as a product. Finally the centroid approach was used for defuzzification [35].

These rules were on the form ‘If x is A then y is B’ where OR was used to define either maximum (max) or the algebraic sum (probor) of the fuzzy sets A and B.

Operator AND was used to define either minimum (min) or the product of the fuzzy sets A [36].

$$
\mu_{A\cup B}(x) = \mu_A(x) \lor \mu_B(x) = \max[\mu_A(x), \mu_B(x)] \quad \forall x \in X
$$

$$
\mu_{A\cap B}(x) = \mu_A(x) + \mu_B(x) - \mu_A(x) \cdot \mu_B(x) \quad \forall x \in X
$$

$$
\mu_{A\cap B}(x) = \mu_A(x) \land \mu_B(x) = \min[\mu_A(x), \mu_B(x)] \quad \forall x \in X
$$

$$
\mu_{A\cap B}(x) = \mu_A(x) \cdot \mu_B(x) \quad \forall x \in X
$$

### 3 RESULTS AND DISCUSSION

After the application of the formulae for every torrential stream separately, the results were compared to the actual water supply measurements for river Kosynthos. These measurements were conducted in the bridge of Sminthi village for the Ground Improvement Service of Xanthi prefecture. From this comparison we concluded the following: The Friedrich formulae overestimate the maximum water supply (MWS) by 11% whereas the ones of Melli-Muller underestimate it by 5% and the Valentini-Coutagne overestimates it by 33% (Table 3). The rest of the models have an error higher than 35%.

Consequently the use of the optimal Melli-Muller formula produces the following ranking of the watersheds (based on the water supply) (Table 4).

Then, the 32 watersheds were ranked based on the estimation of their load of sediments based on the RUSLE and the Gavrilovič methods (Table 4).

Looking at the results we conclude that the watersheds with the highest torrential risk based on the maximum water supply and according to Melli-Muller model are the streams 17, 25, 22, 26, 18, 5, 11, 19, 12 and 2. On the other hand, based to the ground loss estimation method of RUSLE, the most dangerous streams are 25, 22, 2, 26, 31, 17, 24, 5, 21 and 19. The Gavrilovič method that estimates the load of sediments due to erosion characterizes watersheds 25, 26, 2, 22, 31, 24, 5, 23 and 3 as the most risky ones.

At this point, it has to be stressed out that the area of watershed 17 is 31.01km$^2$, 24.72 km$^2$ of watershed 25, 14.07km$^2$ of watershed 22, 10.82km$^2$ of watershed 26, 10.44km$^2$ of watershed 18 and 9.44km$^2$ of watershed 5.

The methods that were used (RUSLE, Gavrilovič and Melli-Muller) are linear and the result depends on the size of the watershed.

As regards the RUSLE method: $\Sigma A = F \cdot A$, Gavrilovič $W = T \cdot h \cdot \pi \cdot (z)^{1/3} \cdot F$, Melli-Muller $Q = q \cdot F$, where $F$ is area (km$^2$).

From the above ranking it is concluded that the most dangerous watersheds are the biggest ones, something that proves that the existing methods cannot evaluate rationally the small streams.

### TABLE 3 - Maximum water supply in Sminthi Bridge

<table>
<thead>
<tr>
<th>$Q_{\text{max}}$ Smynthi bridge (YEB 1991-1998)</th>
<th>Friedrich ($Q_{\text{max}}$)</th>
<th>Melli-Müller ($Q_{\text{max}}$)</th>
<th>Countagne ($Q_{\text{max}}$)</th>
<th>Valentini ($Q_{\text{max}}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000</td>
<td>1108.56</td>
<td>947</td>
<td>1333.92</td>
<td>1333.92</td>
</tr>
</tbody>
</table>
TABLE 4 - Ranking of the watersheds based on the RUSLE and Gavrilović models. Estimation of the MWS according to the Melli-Muller formula and TOR-MIK system (Unified torrential risk index).

<table>
<thead>
<tr>
<th>A/A</th>
<th>RUSLE (t/y)</th>
<th>A/A</th>
<th>Gavrilović (tn/y)</th>
<th>A/A</th>
<th>Melli-Muller (Qmax)</th>
<th>A/A</th>
<th>TOR-MIK</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>1282.09</td>
<td>17</td>
<td>1258.95</td>
<td>17</td>
<td>129.940</td>
<td>17</td>
<td>0.87</td>
</tr>
<tr>
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<td>23</td>
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<tr>
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<tr>
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<td>27</td>
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<td>10</td>
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<td>9.737</td>
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<td>62.26</td>
<td>27</td>
<td>7.763</td>
<td>29</td>
<td>0.1302</td>
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</tbody>
</table>

The results from the developed system TOR-SYS show that the most risky watersheds are 17, 12, 5, 14, 15, 1, 16, 19, 25 and 4. It is concluded that the TOR-SYS model considers the most important morphometric and hydrographic characteristics of the watersheds. According to the classical approaches the area is the feature that determines to a great extend the final output. However, this innovative model considers the area as one of the parameters that affect the torrential risk estimation to some extent.

Especially the ranking by the use of TOR-SYS gives similar ranking results to the Melli-Muller model, which was checked and it has been proven that works well for the area under study.

The two rankings agree in the 50% of the cases. More specifically they give exactly the same outcome in 5 out of 10 cases.

Totally the RUSLE, Gavrilović and Melli-Muller models agree with the TOR-SYS approach in seven out of ten risky watersheds and the level of agreement reaches 70%.

The particularity of the TOR-SYS model is that it also indicates some risky watersheds that are characterized by high slope and altitude and high values of rainfall, which makes them potentially risky.

At this point, it has to be emphasized that the classic methodology most commonly-used for the classification of watersheds as regards their torrential risk yields a wide range of results depending on the way that the sediment and the sediment supply are calculated.

FIGURE 3 - Ranking according to torrential risk based on the TOR-SYS model. The case of the 32 subwatersheds of river Kosynthos.
The decision support system that we have developed far outweighs the classic methods in terms of reliability and speed of calculation. Another thing that has to be pointed out is that TOR-SYS performs the classification based on what causes the phenomenon in contrast to the classic method that relies on the ‘consequences’ (i.e. sediment and sediment supply) of the degree of torrential risk. As a result, TOR-SYS can be used for the classification of watersheds of every type irrespective of its morphological characteristics. Finally, TOR-SYS system restricts the degree of fuzziness compared to the classic method.

In the future, one will be able to develop new methods of classification based on the principles of fuzzy logic and technical neural networks.

4 CONCLUSIONS

In the classical approach for assessing torrential risk, we add algebraically the values of water and sediment flow, expressed in m³/year, and, on condition that the total value is between pre-determined limits, the basin is classified in a risk category [12]. In this methodology, both the water flow and the transfer of sediment deposits participate equally in the final result. From table 4 and by using the traditional approach we observe that basins no 17, 25, and 22 have the greatest torrential – flood risk while TOR–SYS model gives the basins 17, 12, and 5 as the most risky. Therefore, we conclude that the classic method in relation to the model of fuzzy algebra lacks precision, which is confirmed by both the history of flooding in the region and by bibliographic sources [2, 4, 37, 38].

An important issue in the algebraic approach is the determination of strict limits. In the case of torrential risk classification these limits are defined as follows:

<table>
<thead>
<tr>
<th>Total Load (m³/year)</th>
<th>Torrential Risk</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;300.000</td>
<td>Extreme High</td>
</tr>
<tr>
<td>300.000-100.000</td>
<td>Very High</td>
</tr>
<tr>
<td>100.000-50.000</td>
<td>High</td>
</tr>
<tr>
<td>50.000-20.000</td>
<td>Middle</td>
</tr>
<tr>
<td>20.000-10.000</td>
<td>Small</td>
</tr>
<tr>
<td>10.000-5.000</td>
<td>Very Small</td>
</tr>
<tr>
<td>&lt;5.000</td>
<td>Extreme Small</td>
</tr>
</tbody>
</table>

It can be observed that these limits are independent of the topographic and hydrological factors of each basin, which affects the accuracy and sensitivity of the method for application in various areas. In addition it should be noted that, when the methodology of Kotoulas is applied, some water basins, even though they have similar aggregated values, they are classified into different categories because of the strict limits, without having a substantial difference. For example, in the basins 8 and 7, the sediment flow is estimated using the USLE method at 1960,477 tn/year and 1935,702 tn/year, while the water flow was 24,550 m³/sec and 25,803 m³/sec, respectively. On can see that the basins have a matching hydraulic behavior but, as regards risk classification, the first was characterized as medium-risk while the second was characterized as low risk. On the contrary, the TOR-SYS model classifies basins into the same category, considering that fuzzy logic is a many-valued function [29] resulting in having basins into the category where they belong according to their degree of membership.

Comparing model results with both measurements in the study area as well as results from other relative studies [2, 4] one can come to the conclusion that the TOR-SYS is advantageous in accuracy and sensitivity. TOR-SYS is more user-friendly since it does not require any specific scientific knowledge, and, concurrently, it is less time consuming. This is due to the precise calibration of input and the architecture of the model where initially data are separated into those who interact positively and the rest who interact negatively. There is also a conversion of data into fuzzy sets, including interactions, and finally a defuzzification of the results. At this point we should note that there is a lack of scientific work based on modern classification techniques such as fuzzy sets, artificial neural networks and neurofuzzy logic, concerning this area of expertise.

In conclusion, the water supply and the transfer of sediments are the results of the floods, caused by a torrential stream. So far we have tried to evaluate the phenomenon by analysing the results and not the characteristics of the streams.

This research effort was motivated by the need to overcome limitations and problems of classical approach, and, for this reason, the TOR-SYS was developed. The use of fuzzy Algebra and the fuzzy ranking of the torrential streams are suggested. This approach overcomes the errors caused by the empirical approaches. Additionally it performs a more accurate evaluation, it is more adjustable and flexible and no special knowledge is required by the user of the system.

It has to be noted that it is the only method that considers morphometric and hydrographic characteristics of the watersheds that influence the torrential risk. This effort will be extended in the near feature for the improvement of the system and for its application in all of the mountainous watersheds of Greece based on the act 2007/60 of the European Union.

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DISTRIBUTION OF TOTAL MERCURY AND METHYLMERCURY IN THE SEDIMENT OF THE NANSI LAKE, CHINA

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ABSTRACT

The objectives of this study were to determine the contents of total mercury (THg) and methyl mercury (MMHg) in order to understand the geochemical relationships between mercury (Hg) and mineral phases of the sediment of the Nansi Lake. Twenty samples of surface sediment were collected and analyzed for the THg and MMHg contents. The results indicated that the average THg content was 0.089 mg/kg but that levels ranged from 0.025 to 0.168 mg/kg in the sediment. In addition, the content of THg in the sediment was significantly and positively correlated with the contents of clay and total organic carbon (TOC), respectively. Thus, the TOC content and the grain size of sediment dominated the distribution of THg in the sediment. The MMHg content in the sediment ranged from 0.033 to 0.764 ng/g and averaged 0.268 ng/g. Moreover, the content of MMHg was significantly and positively correlated with the contents of THg, clay, and TOC, respectively. Therefore, the MMHg content in the sediment of the Nansi Lake was primarily controlled by the THg content and the organic material content in fine-grained sediments. The methylation rate of Hg (%MMHg) in the sediment ranged from 0.096 to 0.476%, generally lower than 0.5%. The %MMHg showed a significant and positive relationship with the content of THg, but a weak relationship with the contents of clay and TOC. Thus, the %MMHg was primarily limited by the supply of Hg. In addition, regressions analysis indicated that the AVS content in the sediment limited the methylation rate.

KEYWORDS: Mercury, Methylation, Distribution, Sediment, Nansi Lake

1 INTRODUCTION

Mercury (Hg) pollution in natural environments has become a serious problem across the world due to its persistence, bioaccumulation, and toxicity (PBT) and increasing widespread presence [1-3]. Both natural and anthropogenic sources of Hg have led to elevated levels in the environment [2, 4, 5]. Among the Hg species, methylmercury (MMHg) is the most toxic and bioaccumulated species. Methylmercury is mainly produced by microbial Hg methylation in sediment, and sulfate-reducing bacteria (SRB) are generally thought to be the most important methylating agents [6]. Methylmercury poses a threat to human and ecological health through its accumulation in fish and other wildlife [7].

Mercury cycles between air, water, sediment, soil, and organisms [8,9]. Mercury that enters water bodies may either remain suspended in the water column, be taken up by aquatic biota, or settle on the bottom and become incorporated into the sediment [2, 10]. Sediment has been widely recognized as both a source and a sink for contaminants [11-13]. The high content of Hg in sediment can be leached into pore or surface waters through diffusion, resuspension, desorption or dissolution, and also enter the aquatic food web through Hg uptake by benthos that feed at the sediment-water interface [14]. Therefore, determining the level of Hg content in sediment is an important method of assessing Hg contamination and risk in aquatic environment. A large number of studies have been published on Hg distribution and speciation in soil, coastal sediment, freshwater, and organisms of contaminated areas [15-19]. However, few studies have focused on the distribution of Hg in the sediment of the Nansi Lake.

Therefore, the objectives of this study were to measure the content of the THg and MMHg in the sediment of the Nansi Lake and to investigate the geochemical relationships between Hg and sediment mineral phases.

2 MATERIALS AND METHODS

2.1. Study area

The Nansi Lake (34°27′-35°20′N, 116°34′-117°21′E) is a typical shallow lake with an area of 1,266 km² and average depth of 1.46 m (Fig. 1). It is the largest freshwater lake in the southwest of Shandong province and has become one of the largest buffering reservoirs in the East
Route of China’s South-North Water Transfer Project. After the building of a dam in 1960s, Nansi Lake was divided into two parts: the upper lake and lower lake. The urban streams are often contaminated by effluents and other wastes from industrial and domestic sources. There has been a rapid economic development in the region around the Nansi Lake since 1970s and this rapid industrialization and urbanization might lead to an excessive release of pollutants into it. Generally, the untreated effluents from industrial and municipal activities, the runoff from mining sites and agricultural land, and the deposition of air pollutants all contribute to the increase of heavy metal and Hg levels in the sediment. According to the “Water pollution Prevention Planning of the South-to-North Water Diversion Project (east route) of Shandong Section”, water quality of the lake should be better than the Grade III of the “China surface water quality standard”. However, previous researches indicated that the sediment of the Nansi Lake and its main inflow rivers were polluted by heavy metals [20]. But no literature reports the THg and MMHg levels in the sediment of the Nansi Lake.

FIGURE 1 - Location map of sampling sites in the Nansi Lake.

2.2. Sediment sample collection

In August 2010, 20 surface sediment samples (a depth between 0 and 15 cm from the surface) were collected from the Nansi Lake (Fig. 1) using cable operated sediment samplers (Van Veen grabs, Eijkelkamp) from a boat. The samples were collected from 3 to 4 locations at each site and then mixed to make composite samples to improve site representation. The sediment samples were transferred to acid-washed dark-colored polyethylene bags and transported to a laboratory where they were freeze-dried (FD-1A, China), slightly crushed, passed through a two millimeter sieve and stored at 4°C in glass bottles until analysis.

2.3. Analytical methods

Total organic carbon (TOC) of the sediment was determined on sub-samples of sediment after a pre-treatment with 3 M HCl by LiquiTOC (Elementar). Particle size analysis of the sediment was performed with an LS 230 laser diffraction particle analyzer (Beckman Coulter), and the percentages of clay (< 2 µm) and silt (2–63 µm) were calculated. A modified distillation method was employed to analyze AVS in the sediment samples based on EPA method 9215 [21,22]. Approximately 1 g of wet sediment sample, with 20 mL of 6 M deoxygenated HCl, was distilled for 1.5-2 h at room temperature under a flow of nitrogen gas. A sulfide antioxidant buffer solution (SAOB,
25 mL) was added to the plastic container to trap sulfide volatilized from the distillation vessel. The total sulfide trapped in the SAOB solution was measured using a sulfide ion specific electrode (Istek Co., Seoul, Korea).

For the measurement of the THg, sediment samples were prepared by digesting sediment (1 g) in a mixture of HNO₃ and H₂SO₄ acid, followed by 12 h oxidation with 0.2 N BrCl [23,24]. Analyses of THg were performed using tin chloride reduction, gold amalgamation trapping, and quantification by cold vapor atomic fluorescence spectrophotometry (CVAFS). The minimum detection limit (MDL) for THg analysis was 0.4 ng/g (n=20). The analytical protocol for MMHg was based on the method described by Horvat et al. [25]. Briefly, approximately 1 g of wet sediment was mixed with 0.5 mL of KCl and 1.0 mL of 8 M H₂SO₄, the volume was adjusted to 25 mL with deionized water, and then distilled. The distillate was buffered with acetate buffer, reacted with 1% sodium tetraethylborate, and purged with N₂ gas. Volatile Hg was absorbed onto a solid-phase trap (Tenax-TA), separated on a gas chromatographic column, converted to Hg⁰ via a pyrolytic column, and then detected by CVAFS. The MDL for MMHg analysis by this method was 0.006 ng/g.

Quality assurance and control of THg and MMHg analysis were determined by using duplicates, method blanks, matrix spikes, and certified reference materials (GBW07405; IAEA405). The average THg concentration of the geological standard of GBW07405 was 0.31 ± 0.01 mg/kg (n = 6), which is comparable with the certified value of 0.29 ± 0.04 mg/kg. A mean MMHg concentration of 5.77 ± 0.25 ng/g (n = 6) was obtained from IAEA 405 with a certified value of 5.29 ± 0.33 ng/g. Recoveries on matrix spikes of MMHg in sediment samples were in the range 85-115%. The relative standard deviation was < 8.5% and the relative percentage difference of sample duplicates was < 7.5%.

3 RESULTS AND DISCUSSION

3.1. General properties of the sediment of the Nansi Lake

A selection of general physical and chemical characteristics of the sediment is indicated in Table 1. The results indicated that the sediment consisted predominantly of clay and silt. The portion of clay (< 2 µm) ranged from 9.48 to 22.76% and silt (2-63 µm) from 60.84 to 75.48%, indicating the relative stability of the sedimentary environment. The AVS content ranged from 45.76 to 246 µmol/g and averaged 122.01 µmol/g. The high AVS concentration in the sediment of the Nansi Lake may be caused by deterioration of water quality. The TOC content in the sediment ranged from 0.75% to 5.62% and averaged 3.34%. High TOC content was observed at some sites located in the upper lake. A long-term monitor indicated that annually about 2,900 million tons of untreated domestic sewage and industrial wastewater from the cities around the Nansi Lake, such as Jining, Zaozhuang, and Heze, were discharged into the Nansi Lake through the thirteen main inflow rivers flowing into the upper lake, and the levels of chemical oxygen demand (COD) and biological oxygen demand (BOD) in these rivers were higher than the Grade V of the “China surface water quality standard” [26]. Inorganic and organic materials are carried and deposited by water from the upper lake to the lower lake under natural conditions. Therefore, the TOC content was higher in the sediment of the upper lake than that of the lower lake. In addition, faster development of aquaculture as well as richness in aquatic plants in the upper lake than that in the lower lake might also contribute to high TOC content in the upper lake sediment [27].

3.2. Contents of THg and MMHg in the sediment of the Nansi Lake

The THg content in the sediment of the Nansi Lake ranged from 0.025 mg/kg at site 19 to 0.168 mg/kg at site 5 and averaged 0.089 mg/kg (Table 1, Fig. 2). The Nansi Lake was formed by bedload from flooding of the Yellow River [28], so the average content of Hg in the main river sediment of Yellow River was used as the background content in the present study and it was 0.015 mg/kg [29]. Therefore, the THg content in the sediment of the Nansi Lake was higher than that of the main river sediment of Yellow River. In addition, the THg content of all sediment samples in this study was lower than the threshold values of the Chinese soil environmental quality criteria (0.15mg/kg) [30] and the control standards for pollutants in sludge from agricultural use (5 mg/kg) [31], respectively.

<table>
<thead>
<tr>
<th>Station</th>
<th>TOC (% dry)</th>
<th>Clay (% &lt;2µm)</th>
<th>Silt (%2–63µm)</th>
<th>AVS (µmol/g)</th>
<th>THg (mg/kg)</th>
<th>MMHg (%)</th>
<th>MMHg (ng/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Max</td>
<td>5.62</td>
<td>22.76</td>
<td>75.48</td>
<td>246.00</td>
<td>0.168</td>
<td>0.476</td>
<td>0.764</td>
</tr>
<tr>
<td>Min</td>
<td>0.75</td>
<td>9.48</td>
<td>60.84</td>
<td>45.76</td>
<td>0.025</td>
<td>0.096</td>
<td>0.033</td>
</tr>
<tr>
<td>Ave</td>
<td>3.34</td>
<td>14.35</td>
<td>71.05</td>
<td>122.01</td>
<td>0.089</td>
<td>0.262</td>
<td>0.268</td>
</tr>
<tr>
<td>Std</td>
<td>1.36</td>
<td>3.84</td>
<td>3.43</td>
<td>51.45</td>
<td>0.042</td>
<td>0.138</td>
<td>0.225</td>
</tr>
</tbody>
</table>

Max: the maximum value,
Min: the minimum value,
Ave: average content,
Std: standard deviation.
Yuan et al. [32] found that the THg content ranged from 0.024 to 1.6 mg/kg and averaged 0.198 mg/kg in the sediment of the Taihu Lake. Liu et al. [33] found that the THg content ranged from 0.42 to 1.18 mg/kg and averaged 0.66 mg/kg in the sediment of Hongfeng Lake in Guizhou, China. Sa et al. [34] found that the THg content ranged from 0.0637 to 0.113 mg/kg and averaged 0.089 mg/kg in the sediment of the Nanhai Lake. Therefore, the THg content in the sediment of the Nansi Lake was lower than that of the Taihu Lake and Hongfeng Lake. Some previous studies reported that the THg content in the sediment from Canada, Sweden, and USA were in the range of 0.01-0.159, 0.02-0.5%, which was similar to the results reported by some previous studies [14, 43, 44].

The coal resources are very rich in the region around the Nansi Lake. There are some big coal-fired power plants such as Jining, Zoucheng, Jiaxiang, and Liyan power plants nearby the Nansi Lake, and their total installed capacity is more than 10 million kilowatts. Previous research indicated that the average content of Hg was 0.17mg/kg in the coal of Shandong province [38], and more than 80 percent of them were discharged into the environment [39]. Therefore, the coal mining waste might be one important pollution source of Hg for the sediment of the Nansi Lake.

Main Hg emission industries included farming, mining and quarrying, power industry, smelting and pressing of ferrous metal, raw chemical material and chemical products. These industries emitted 63.2% of THg that was sent into atmosphere in 1995 [38]. In the cities around the Nansi Lake these industries mentioned above developed extremely quickly, which might result in the emission of large number of Hg pollutants. It was reported that $9.34 \times 10^5$, $7.41 \times 10^5$, and $1.98 \times 10^5$ tons of untreated industrial wastewater were discharged into the Nansi Lake from Jining, Zaozhuang, and Heze cities annually, respectively [26]. A long-term monitor indicated that thirteen inflow rivers were all polluted by heavy metals and Hg [20]. Therefore, industrial effluents resulted in the relatively high Hg content in the sediment of the Nansi Lake. In addition, water flows from the upper lake to the lower lake in the Nansi Lake, so the sediments of the upper lake are affected firstly by the effluents. After aggregation and decontamination in the upper lake, sediment in the lower lake is at a corresponding low-grade contamination level. Therefore, the THg content of the sediment in the upper lake is higher than that of the lower lake.

The MMHg content in the sediment of the Nansi Lake ranged from 0.033ng/g at site 20 to 0.764 ng/g at site 5 and averaged 0.268 ng/g (Table 1, Fig. 2). Previous studies showed that the MMHg content in the sediment from Canada, Sweden, and USA were in the range of 1.09-2.32, 0.6-2.0, and 1.94 ng/g, respectively [40-42]. Normalizing the solid phase MMHg concentrations to THg levels allows us to develop another indicator for methylation rate (%MMHg). The %MMHg in the sediment of the Nansi Lake ranged from 0.096 to 0.476% and averaged 0.268% (Fig. 2). The %MMHg was lower than 0.5%, which was similar to the results reported by some previous studies [14, 43, 44].

3.3. Relationships between Hg and mineral phases of the sediment of the Nansi Lake

Correlation analysis showed that the THg content in the sediment of the Nansi Lake was significantly and positively correlated with the clay and TOC content, respectively (Fig. 3). Generally, clay-rich sediment contain higher contents of Al and most trace metals, including Hg, due to the greater abundance of metal-rich aluminosilicates and greater surface area for the adsorption of metals. Moreover, it is noted that TOC correlated very well with clay in the sediment of the Nansi Lake, suggesting a common mode of THg delivery from the water column in depositional area. “Sticky” fine-grained sediments that are high in organic carbon effectively scavenge metals like Hg as they settle out of the water column [45]. Thus, the TOC content and the grain size of surficial sediment dominate the areal distribution of THg content across all stations sampled resulting in the distribution shown in Fig. 2. Some other studies also found that THg was significantly and positively correlated with TOC [43,44]. These results indicated that organic matter had an important effect on the distribution of THg in the sediment.

As it is shown in Fig. 4, the MMHg content was significantly and positively correlated with the THg, TOC, and clay contents in the sediment of the Nansi Lake, respectively. These results suggested that the MMHg content was primarily controlled by the THg content and the oxidation of organic material in fine-grained sediments. The Hg entering the lake is readily methylated in situ and...
Correlation analysis showed that the %MMHg showed a significant and positive relationship with THg, but a weak relationship to TOC and clay (Fig. 5). These results suggest that MMHg production in these sediments may be limited by the supply of THg. Other reasons may also have influenced methylation; thus, further investigation is necessary.

However, the AVS in the sediment of the Nansi Lake were not significantly and positively correlated with the preserved. Therefore, the THg concentration is a good predictor of the MMHg level in the sediment of the Nansi Lake. Sunderland et al. [46] observed that the MMHg content was significantly and positively correlated with both the THg ($R^2 = 0.45$, $p < 0.001$) and TOC contents ($R^2 = 0.56$, $p < 0.001$) in the sediment of the Bay of Fundy. Conaway et al. [47] found that the MMHg content showed a positive relationship with the THg ($R^2 = 0.39$), TOC ($R^2 = 0.68$), and clay ($R^2 = 0.58$) contents in the northern reach sediment of the San Francisco Bay. These results showed that reduction in inorganic mercury inputs would result in a proportional decrease in MMHg production in the sediment if all other factors remained constant.
THg, MMHg, and %MMHg, respectively (Fig. 6). In the presence of high sulfide level, Hg\(^{2+}\) methylation can be suppressed almost completely [6]. Hammerschmidt and Fitzgerald [48] also reported that Hg methylation decreased when AVS increased. Ouddane et al. [49] found that AVS could have two types of effect on sediment MMHg, i.e., AVS can promoted binding of MMHg to the sulfide or inhibit methylation by limiting available dissolved inorganic Hg. The results of this study indicated that AVS limited the methylation of Hg in the sediment of the Nansi Lake.

\[y = 0.0005x + 0.0311\]
\[R^2 = 0.3352\]

\[y = 0.0013x + 0.1094\]
\[R^2 = 0.0878\]

\[y = -3E-07x + 0.2619\]
\[R^2 = 1E-08\]

FIGURE 6 - Correlations between AVS and THg, MMHg, and %MMHg in the sediment of the Nansi Lake: (a) AVS vs. THg, (b) AVS vs. MMHg, (c) AVS vs. %MMHg.

4 CONCLUSIONS

The THg content in the sediment of the Nansi Lake were in the range of 0.025-0.168 mg/kg and averaged 0.089 mg/kg. The THg levels in the sediment of the upper lake were higher than that of the lower lake. The coal mining waste and industrial effluents might be the major sources of Hg found in the sediment of the Nansi Lake. The MMHg content in the sediment was in the range of 0.033-0.764ng/g and averaged 0.268ng/g. Regression analysis showed that the contents of the THg and MMHg were significantly and positively correlated with the content of the clay and TOC, respectively. Moreover, the MMHg content was significantly and positively correlated with the THg content. Thus, the TOC content and the grain size of sediment dominated the distribution of THg in the sediment of the Nansi Lake. The MMHg content in the sediment was primarily controlled by the THg content and the organic material content in fine-grained sediments. The %MMHg was in the ranged from 0.096-0.476% in the sediment. The %MMHg showed a significant and positive relationship with the THg content, but a weak relationship with the clay and TOC content, which indicated that the methylation rate of Hg was mainly limited by the supply of Hg. In addition, a weak correlation between THg and AVS was detected and there was no relationship between MMHg and AVS. Therefore, AVS in the sediment of the Nansi Lake limited the methylation of Hg.

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The authors are grateful for the financial Support of Encouraging Foundation for Outstanding Youth Scientists of Shandong Province (No.2008BS09011), the Natural Science Foundation of Shandong Province (No. 2009ZRZB01461), and the Science and Technology Project of Institutions of higher Education of Shandong Province (No.J10LC13). Financial support to T.G. Shi and S.L. Wang by the Environment Protection Foundation of Shandong Province, China is acknowledged.

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STIMULATING THE EFFECTS OF CO-EXIST BROMIDE AND IODIDE IONS ON MONOCHLORAMINE DECAY DURING DISINFECTION OF DRINKING WATER

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ABSTRACT

In this study, a comprehensive kinetic model has been developed to stimulate chloramines decay in co-exist of bromide and iodide ions in chloraminated the distribution system. The model incorporates simultaneous monochloramine auto-decomposition and inorganic species (e.g., bromide and iodide ions) demand pathways that occurs parallel to auto-decomposition. In addition, several key influencing factors, such as initial monochloramine concentrations, pH value, carbonate concentration, bromide concentration, and iodide concentrations on monochloramine decay were considered in this model. It was found that higher initial monochloramine concentrations, carbonate, bromide, and iodide concentrations lead to higher decay rate of monochloramine, while the rate of monochloramine decay decrease as the pH increased. Finally, this paper presented here validates and extended this model for use in distribution system water samples and under realistic chloramination conditions. The developed models provided satisfactory estimations of the concentration of the monochloramine, and the model regression coefficient ($R^2$) for two distribution system water samples are 0.968. The results indicated that the monochloramine decay in drinking water can be described by the model, which incorporates inorganic demand pathways (e.g. bromide and iodide ions) and is parallel to auto-decomposition.

KEY WORDS: Monochloramine decay, kinetics model, disinfection, bromide ion, iodide ion, stimulating

1. INTRODUCTION

The use of chlorine as a primary disinfectant has been widely practiced in drinking water to eliminate pathogens and protect of public health. However, chlorine reacts with naturally occurring organic material to form numerous disinfection by-products (DBPs), some of which are classified as probable or possible carcinogens. To meet disinfection requirements and minimize DBPs formation, many water utilities have altered their disinfection practice from the use of free chlorine to monochloramine as alternative disinfectant in drinking water distribution systems due to form much less DBPs [1-4]. Unfortunately, despite the long history of chloramines in drinking water treatment, chloramines are nonetheless inherently unstable at neutral pH values, even without the presence of reactive inorganic or organic substances, and auto-decompose by a complex set of reactions that ultimately result in the oxidation of ammonia and the reduction of active chlorine. As a result, the fate of chloramines in distribution system and the characteristics and processes that influence their stability are of increasing concern.

Bromide and iodide ions are naturally present in the raw or drinking water supplies. According to the previous surveys, the concentration of bromide ion in water resources are usually around 62 µg/L, but it can exceed 500 µg/L, especially in coastal areas or under special geological circumstance [5]. Total concentrations of iodide in water resources are then usually in the concentration range of 0.5-20 µg/L, but can exceed 50 µg/L up to 212 µg/L [6-8]. Some studies showed that the presence of bromide and iodide ions have significant impacts on monochloramine decomposition in drinking water. For instance, bromide can be immediately oxidized by chlorine to the active oxidant hypobromous acid (HOBr), which can react with natural organic matter (NOM) to form brominated DBPs [9, 10]. For iodide, the oxidation kinetics of I react with monochloramine and the product of formation have been investigated earlier [11]. Previous studies have demonstrated that the taste and odor problems in drinking water were frequently linked to the presence of iodoorganic compounds (i.e., CHI 3) [12, 13]. Further, Toxicological studies suggested that iodinated DBPs might be more toxic than the brominated and chlorinated analogues [14]. In recent years, some kinetic models to predict chloramine decay in aqueous environment have been proposed [15-
20]. However, most of these only considered the transient formation of chloramines in a bench-scale batch or effect of single factor. Very few studies are focused on the kinetic model to stimulate monochloramine decay in co-presence of bromide and iodide ions, especially in realistic water samples. Therefore, the co-effects of bromide and iodide ions on monochloramine decay are necessary to be taken into accounts under chloramines conditions.

The objective of this study was to develop a kinetic model stimulating monochloramine decay in co-presence of bromide and iodide ion under more realistic water quality and chlorination conditions. In addition, the effects of some influencing factors such as initial monochloramine concentrations, pH, carbonate concentrations, bromide, and iodide ions concentrations on monochloramine decay are also investigated in this study. Finally, the general utility and validity of the model were determined by excluding it to include reactions involving bromide and iodide ions, which can exert or catalyze monochloramine decomposition examined by using two water samples of distribution system. This work can be helpful for the further evaluating of the monochloramine decay and minimizing of DBPs formation during chloramination, especially in coastal areas or estuaries.

2 MATERIALS AND METHODS

2.1 Model development

2.1.1. Monochloramine auto-decomposition model development

Monochloramine auto-decomposition involved very complicated process and has been well characterized by Jafvert, Valentine, and Ozekin [15, 16]. The model includes four principle reaction schemes: (1) substitution/hydrolysis reactions involving free chlorine and ammonia or the chlorinated ammonia derivatives (reaction 1.1-1.4); (2) disproportionation reactions of chloramine species (reaction 1.5 and 1.6); (3) redox reactions that occur in the absence of measurable amounts of free chlorine (reactions 1.7-1.14); and (4) equilibrium reactions for pH dependent species (reactions E.1-E.4).

2.1.2. Incorporation of reaction of bromide and iodide

2.1.2.1. Bromide reactions

The reaction mechanism for the oxidation of bromide ion with monochloramine was presented in Table 1 (part II) [21-23]. Under chloramination conditions, reaction of bromide ion with monochloramine usually dominates. It accelerates monochloramine loss via complex reaction mechanism resulting in the formation, disproportionation, and decomposition of numerous haloamine species. Additional assumptions in the simplified reaction mechanism are that nitrogen gas would be the main oxidized nitrogen-containing end product of bromide catalyzed auto-decomposition. In this model, assuming bromide would be constant, monochloramine is low in concentration and are at pseudo-steady state, and that bromochloramine (NBrCl) reacts rapidly with monochloramine above neutral pH [24]. According to the net reaction (reaction 2.1-2.4), bromide may be primarily act to catalyze the overall decay rate of chloramine. Thus, equation (1) is given as follows:

\[ 3\text{NH}_2\text{Cl} + \text{Br}^- \rightarrow \text{N}_2 + 3\text{NH}_3 + 3\text{Cl}^- + 3\text{H}^+ + \text{Br}^- \] (1)

All reactions of bromamine species are assumed to be very fast, leading to rapid conversion of one form to the other in excess monochloramine [25]. The following differential equation was derived and included in the model for monochloramine decay (Eq.2). The second term of Equation 2 considers the oxidation of bromide by HOCl produced from monochloramine hydrolysis. The factor of two is derived from the consideration of the overall stoichiometry of this specific loss pathway.

\[ \frac{d([\text{NH}_2\text{Cl}])}{dt} = -3k_{1.3}[\text{NH}_2\text{Cl}][\text{Br}^-][\text{H}^+] + 2k_{1.4}([\text{HOCl}][\text{Br}^-]) \] (2)

2.1.2.2. Iodide reactions

In oxidative drinking water treatment, I\text{I} is first oxidized to HOI by chlorine or chloramines in a fast stage [11]. In a second step, some of these disinfectants could only oxidize HOI to IO\text{I}\. It has been shown that the presence of ammonia during chlorination [26] or chloramination favors the formation of iodinated compounds because monochloramine can oxidize iodide to HOI but does not further oxidize it to iodate [27,28]. Since HOCl is not stable in aqueous solution, it can also disproportionate to IO\text{I} and I\text{I} as above - mentioned. As a result, the disproportionation of HOI is described in reactions 3.5.

\[ \frac{d([\text{NH}_2\text{Cl}])}{dt} = 2k_{1.3}[\text{NH}_2\text{Cl}][\text{I}^-][\text{H}^+] + k_{1.4}([\text{HOCl}][\text{I}^-]) \] (3)

Where \(k_{1.1}\) is the rate coefficient for reaction between NH\text{II}Cl and H\text{II}; \(k_{1.2}\) is the rate coefficients for reaction between NH\text{II}Cl\text{I} and I\text{I}; \(k_{1.3}\) is the rate coefficients for reaction between HOCl and I\text{I}, respectively. Rate coefficients were determined by Kumar and Day et al. [11]. To consider monochloramine loss reacting with iodide ion, the expression given by Eq. (3) is included as a monochloramine loss pathway in the comprehensive model. The second term accounts for the reaction oxidation of iodide by HOCl produced from monochloramine hydrolysis. The factor of one is derived from the consideration of the overall stoichiometry of this specific loss pathway. Additionally, assuming that iodide is constant and iodide reaction with NHCl\text{I} and NCl\text{I} is not considered because of iodide oxidation by monochloramine ion usually dominates. Nitrogen Trichloride (NCl\text{I}) is not relatively stable in acidic solution. It appears that the yield of NCl\text{I} decreases with a lower excess of NH\text{III} at alkaline pH. The reaction of NCl\text{I} with iodide ion is not quantitative, in agreement with previous report by Dowell and Bray [29]. Therefore, the reaction of NCl\text{I} with iodide ion is not taken into account in this model.
TABLE 1- Stoichiometric equations for monochloramine decay in the presence of bromide and iodide ion

<table>
<thead>
<tr>
<th>Reaction stoichiometry</th>
<th>Rate coefficient/equilibrium constant</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Part I Auto-decomposition</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.1 HOCl+NH3→NH2Cl+H₂O</td>
<td>k₁=1.2×10⁻⁹ M⁻¹h⁻¹</td>
<td>Morris and Isaac (1981) [30]</td>
</tr>
<tr>
<td>1.2 NH₂Cl+H₂O→HOCl+NH₃</td>
<td>k₂=2.5×10⁻³ M⁻¹h⁻¹</td>
<td>Morris and Isaac (1981) [30]</td>
</tr>
<tr>
<td>1.3 HOCl+NH₂Cl→NHCl₂+H₂O</td>
<td>k₃=2.0×10⁻² M⁻¹h⁻¹</td>
<td>Margerum et al. (1978) [30]</td>
</tr>
<tr>
<td>1.4 NHCl₂+H₂O→HOCl+NHCl</td>
<td>k₄=5×10⁻³ M⁻¹h⁻¹</td>
<td>Margerum et al. (1978) [30]</td>
</tr>
<tr>
<td>1.5 NHCl₂+NH₂Cl→NHCl+NHCl₂</td>
<td>k₅=1.0×10⁻² M⁻¹h⁻¹</td>
<td>Vikesland and Ozekin (2000) [31]</td>
</tr>
<tr>
<td>1.6 NHCl₂+NH₃→NHCl₂+NH₃</td>
<td>k₆=1.0×10⁻² M⁻¹h⁻¹</td>
<td>Hand and Margerum (1983) [32]</td>
</tr>
<tr>
<td>1.7 NHCl₂+H₂O→H⁺</td>
<td>k₇=2.0×10⁻³ M⁻¹h⁻¹</td>
<td>Jafvert and Valentine (1987) [15]</td>
</tr>
<tr>
<td>1.8 I+NHCl₂→HOCl+products</td>
<td>k₈=1.0×10⁻³ M⁻¹h⁻¹</td>
<td>Leao (1981) [33]</td>
</tr>
<tr>
<td>1.9 I+NH₂Cl→products</td>
<td>k₉=3.0×10⁻³ M⁻¹h⁻¹</td>
<td>Leao (1981) [33]</td>
</tr>
<tr>
<td>1.10 NH₃Cl₂+ICl₂</td>
<td>k₁₀=55 M⁻¹h⁻¹</td>
<td>Leao (1981) [33]</td>
</tr>
<tr>
<td>1.11 HOCl+NHCl₂→NCl₃ + H₂O</td>
<td>k₁₁=1.0×10⁻³ M⁻¹h⁻¹</td>
<td>Hand and Margerum (1983) [32]</td>
</tr>
<tr>
<td>E.1 HOCl→OCI⁻ + H⁺</td>
<td>E.1pKₐ=7.5</td>
<td>Snoeyink and Jenkins (1980) [34]</td>
</tr>
<tr>
<td>E.2 NH₂⁺→NH₃ + H⁺</td>
<td>E.2pKₐ=9.3</td>
<td>Snoeyink and Jenkins (1980) [34]</td>
</tr>
<tr>
<td>E.3 H₂CO₃→HCO₃⁻ + H⁺</td>
<td>E.3pKₐ=6.3</td>
<td>Snoeyink and Jenkins (1980) [34]</td>
</tr>
<tr>
<td>E.4 HCO₃⁻→CO₃²⁻ + H⁺</td>
<td>E.4pKₐ=10.3</td>
<td>Snoeyink and Jenkins (1980) [34]</td>
</tr>
<tr>
<td>Part II Bromide reactions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.1 NH₂Cl₂ + H⁺→NH₂Cl</td>
<td>k₂=28 M⁻¹</td>
<td>Gray et al. (1978) [35]</td>
</tr>
<tr>
<td>2.2 NH₂Cl + Br⁻→NH₂Br + Cl⁻ + H⁺</td>
<td>k₃=1.8×10⁵ M⁻¹h⁻¹</td>
<td>Trofe et al. (1980) [21]</td>
</tr>
<tr>
<td>2.3 HOCl + Br⁻→BrO⁻ + Cl⁻</td>
<td>k₄=5.6×10⁵ M⁻¹h⁻¹</td>
<td>Kumar et al. (1987) [36]</td>
</tr>
<tr>
<td>2.4 BrO⁻+2NH₂Cl→N₂+3H⁺ + 2Cl⁻ + Br⁻</td>
<td>k₅=1.0×10⁸ M⁻¹h⁻¹</td>
<td>Gazda et al. (1994) [37]</td>
</tr>
<tr>
<td>Part III Iodide reactions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.1 NH₂Cl₂ + H⁺→NH₂Cl⁻</td>
<td>K₁=0.036 M⁻¹</td>
<td>Kumar and Day et al. (1986) [11]</td>
</tr>
<tr>
<td>3.2 NH₂Cl⁻→ICl⁻ + NH₃</td>
<td>K₂=6.7×10⁴ M⁻¹h⁻¹</td>
<td>Kumar and Day et al. (1986) [11]</td>
</tr>
<tr>
<td>3.3 HOCl⁻→HOCl+Cl⁻</td>
<td>k₃=5.0×10⁵ M⁻¹h⁻¹</td>
<td>Kumar and Day et al. (1986) [11]</td>
</tr>
<tr>
<td>3.4 ICl⁻+2OH⁻→IC⁻ + Cl⁻ + H₂O</td>
<td>pKₐ=10.6</td>
<td>Chia (1958) [38]</td>
</tr>
<tr>
<td>3.5 HO⁻+H⁺→OH⁻</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2kę equals pKa of HA = pK₁ = pKₐ = kₙC₀[HCO₃⁻] + kₙC₀[CO₃²⁻] + kₙC₀[H₂CO₃⁻] = 4×10⁴ M⁻¹h⁻¹, kₙC₀=800 M⁻¹h⁻¹, kₙ=2.5×10⁻⁸ M⁻¹h⁻¹; k₈C₀=1.2×10⁻⁸ M⁻¹h⁻¹; k₉C₀=1.0×10⁻⁸ M⁻¹h⁻¹; k₀H₂O=10⁻⁶ M⁻¹h⁻¹; k₀H⁺=10⁻⁶ M⁻¹h⁻¹; k₀Cl⁻=10⁻⁶ M⁻¹h⁻¹; k₀OH⁻=10⁻⁶ M⁻¹h⁻¹, t is the unidentified monochloramine auto-decomposition intermediate.

2.2 Experimental and analytical method

Monochloramine loss kinetic experiments were conducted using laboratory prepared “model” waters as well as two types of distribution system water samples collected from the drinking water treatment plant. The effects of initial monochloramine concentrations, pH, carbonate concentrations, bromide, and iodide concentrations on monochloramine loss were also investigated. All in the solution was prepared by using deionized (DI) water (18.2 MΩ cm) produced with a Millipore Super-Q water purification system. All chemicals used in experiments were of analytical grade. All bottles used in this study were soaked in concentrated free chlorine (5000 mg/L as Cl₂) for 24 h, rinsed with deionized water, and dried in an oven at 105°C prior to use.

Stock solutions of monochloramine were prepared by mixing an appropriate amount of NaOCl stock (5% sodium hypochlorite from Shanghai) to a solution of (NH₄)₂SO₄ buffered at pH 8.5 or greater by the addition of NaOH. It was mixed for 30 min before using it in an experiment. The chlorine to ammonia molar ratio was maintained at 0.7:1, according to previously published procedures [29]. The initial monochloramine concentration was determined by using N, N-diethyl-p-phenylenediamine (DPD)-ferrous ammonium sulfate (FAS) titrimetric method [39]. The solution was then transferred to 40 mL amber bottles and stored in a dark at 25±1°C. The ionic strength in each experiment was adjusted to 0.1 mol/L with the appropriate amount of sodium perchlorate. The pH measurements were carried out with a pH-S-25 (Raze Corp, China). Total organic carbon (TOC) was measured with a Shimadzu TOC-VPN (Shimadzu Scientific, Japan) according to standard methods 505A. Alkalinity was measured by titration with methyl purple indicator. Bromide and iodide ion were analyzed using a Dionex 1000 ion chromatography (IC) system (Dionex, Sunnyvale, CA) according to draft EPA method 300.1 [39]. All experiments were run in duplicate, the average error between replicate monochloramine measurement was <0.00141 mmol/L.

Water sources were collected from the Yangtze River (31°324′N, 121°516′E) and Huangpu River (31°321′N, 121°535′E) which is located in East China Sea. All water samples were collected at the end of each plant’s treatment scheme prior to disinfectant addition during two seasons in Shanghai. They were filtered with a 0.45-μm filter (cellulose acetate), and stored at 4°C in the dark until the experiments were conducted. Some of water qualities for four water samples are summarized in Table 2. The level of bromide ranged from 46.5 to 250 µg/L (Table 2), with an average concentration of 150 µg/L. The results are consistent with the reported data by Amy et al. and Fuge [4-6, 40]. Interestingly, the ratio of bromide to iodide here varied considerably between 4 and 13%, which is referred in this study.
TABLE 2—Characteristics of distribution system waters (2009)

<table>
<thead>
<tr>
<th>Water samples</th>
<th>pH</th>
<th>TOC (mg/L)</th>
<th>UV254 (cm⁻¹)</th>
<th>Alkalinity (mg CaCO₃/L)</th>
<th>Br (µg/L)</th>
<th>I (µg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>YRw sample collected in spring</td>
<td>7.9</td>
<td>2.2</td>
<td>0.053</td>
<td>109.4</td>
<td>250</td>
<td>10</td>
</tr>
<tr>
<td>YRw sample collected in autumn</td>
<td>7.7</td>
<td>1.5</td>
<td>0.024</td>
<td>110.2</td>
<td>46.5</td>
<td>6.5</td>
</tr>
<tr>
<td>HPw sample collected in spring</td>
<td>7.3</td>
<td>3.4</td>
<td>0.136</td>
<td>103.45</td>
<td>80</td>
<td>7</td>
</tr>
<tr>
<td>HPw sample collected in autumn</td>
<td>7.2</td>
<td>3.2</td>
<td>0.074</td>
<td>95.6</td>
<td>225</td>
<td>11.5</td>
</tr>
</tbody>
</table>

Note: YRw represents Yangtze River water; HPw represents Huangpu River water.

2.3. Modeling methodology

The comprehensive model for monochloramine decay was used here to stimulate the loss of monochloramine via its auto-decomposition, oxidation of bromide and iodide ion. There are 24 reactions, which occur when free chlorine and ammonia are mixed. In order to evaluate the kinetic model predicted values, a MATLAB program was designed to solve the set of stiff differential equations of \( \text{NH}_2\text{Cl}, \text{NHCl}_2, \text{Br}^-, \text{and I}^- \). The kinetic model for monochloramine decomposition with the appropriate rate coefficients obtained from the previous literatures (shown in Table 1) was then programmed into a mathematical code to predict the decay of several species. Furthermore, the model considered ionic strength effects for an equilibrium using the extended Debye-Hückel relationship to calculate activity coefficients. The pH may be fixed in the buffered solution.

3 RESULTS AND DISCUSSION

3.1 Effect of monochloramine concentration

The effect of initial concentration on monochloramine decay was studied. Fig. 1 shows that the monochloramine auto-decomposition model could predict monochloramine decay for an initial concentration range of 0.01-0.055 mmol/L (i.e., 0.71-3.58 mg/L as Cl₂), typically used in drinking water treatment. As shown, changes of the initial monochloramine concentration could affect the rate decay of monochloramine. The result shows that the predicted ability of the model to account for monochloramine decay at low concentrations. However, for the higher concentration levels, the model would not well able to predict monochloramine decay (data is not shown), this result is in agreement with a previous study [24].

3.2 Effect of pH

The influence of pH on monochloramine decay was investigated in the pH range of 6.6-8.3 with similar initial monochloramine concentration (0.055±0.005) mmol/L (Figure 2). The results show that an increase of pH from 6.6 to 8.3 resulted in a lower rate of monochloramine decay. It was found that monochloramine lost up to 40% at pH 6.6, and then up to 10% at pH 8.3. The reason may be explained that decreasing pH accelerated monochloramine loss due to enhancing formation rate of dichloramine at lower pH values. Once formed, dichloramine rapidly decomposes thereby leading to monochloramine loss. Besides, the previous results of study showed that at pH 7.0, the fraction of hydrolysis of monochloramine to free chlorine is about three times of those at pH 8.3 for a monochloramine concentration of 10 mg/L as Cl₂ [32]. Our experimental results were found that the half-life of monochloramine is 290 h at pH 8.3, which decreased to 70 h at pH 6.6, respectively, which is consistent with reported data by Trofe et al. [21]. The correspondence between the model calculations and the measured monochloramine concentra-
tions was quite good for all of the tested reaction conditions.

3.3 Effect of carbonate concentration

The effect of carbonate concentrations on monochloramine decay ranging from 1 to 10 mmol/L was investigated, while other experimental parameters were kept the same. As shown in Figure 3, it was found that increasing carbonate concentration can enhance monochloramine decomposition under given pH conditions, especially at 10 mmol/L carbonate concentration. For example, the Half-life of 0.05 mmol/L monochloramine at pH 6.6 is approximately 40 h in 4 mmol/L bicarbonate but only 25 h in 10 mmol/L bicarbonate concentrations. Besides, previous studies have showed that general acid, such as bicarbonate, phosphate, sulfate, and acetic acid have the potential to act as catalysts [33]. The possible reason is that with pH value reduced, the reaction species have stronger ability to donate H⁺ under acid conditions than that under basic conditions, so that general acid catalysts are more obvious. In this model, the rate coefficients for monochloramine decay in carbonated buffer could obtain from Valentine and Jafvert’s study [34].

3.4 Effect of bromide

Bromide is considered to be the most important parameters of water quality affecting the formation and relative speciation of DBPs in drinking waters. Figure 4 shows that the effect on monochloramine loss is evident at several tenths of a mg/L bromide. It can be observed that at a concentration of 0.1 mg/L Br⁻, the effect of bromide ions was minimal at pH 7.6, while with a further increase of bromide concentrations to 2 mg/L, the rate of monochloramine decay significantly increased. Because the low bromide concentrations (<0.1 mg/L) often are found in many distribution systems, reactions involving bromide should be considered a major monochloramine loss pathway. However, it should not have a significant effect at the lower concentrations often observed in many source waters. Based on the standpoint of fundamental chemistry, HOBr and bromamines are formed as disinfection by-products when chlorine or chloramine is applied to treat bromide-containing natural waters. Then, hypobromous acid acts as a key intermediate. In the presence of ammonia, HOBr is scavenged in a fast reaction forming bromamine (NH₂Br), dibromamine (NHBr₂) and chlorbromamine (NHBrCl) [41, 42]. All three bromamines which are taken as active bromine (Br(+)I) are low in concentration and unstable so as to be difficulty measured. Margerum and Huffman (1978) demonstrated monochloramine which belongs to alkalescence of compound can not rapidly obtain H⁺ form NH₃Cl⁺, which reacts with bromide ion form Br(+I) under alkaline conditions [43]. Hence, as the bromide concentration is increased and the effect of bromide becomes more prominent, the model does a good job at accounting for its effects.

3.5 Effect of iodide

The influence of iodide on monochloramine decay was also investigated by varying iodide concentrations (0-0.5 mg/L) at pH 7.6. Figure 5 shows that the rate of monochloramine decay increased with increasing iodide concentration. Notably, when I⁻ concentration is 0.5 mg/L, iodide exerted a significant monochloramine demand, espe-
cially in the initial fast stage. The oxidation reaction of iodide ion with monochloramine is very fast, being complete within a few seconds. The possible reason is that $\text{I}^-$ is rapidly oxidized to HOI by HOCl and monochloramine due to chemical reactivity of iodide ion. In a second step, HOI reacts with NOM to form the I-DBPs [26]. In addition, monochloramine decay consists of fast and slow stage [17]. Consequently, the model results obtained by including the reaction scheme from Table 1 (part III) in the comprehensive model correspond quite well to the experimental data (Fig. 5).

### 3.6 Cumulative effect of bromide and iodide

To investigate the effects of co-presence of bromide and iodide ions on monochloramine decay, experiments were conducted to examine monochloramine decay by varying in range of bromide (0-1.0 mg/L) and iodide ion concentrations (0-0.25 mg/L) under different pH conditions (Figure 6). The concentration range was chosen because the concentrations ranges of two kind ions were often found in many water sources. The results indicated that the combined model for monochloramine decay, including auto-decomposition as well as reactions of bromide and iodide ions, was able to predict monochloramine decomposition at accounting for its effects. However, it should be pointed out that the kinetic model was only validated by both bromide and iodide ions independently react via parallel reaction pathways so far and not considered synergistical effect if the reactions of one species influence the reactions of another species.

### 3.7. Monochloramine decay in collected distribution system

Comparison between measured and predicted monochloramine decay in waters collected from two different treatment plants in two seasons is conducted to examine the applicability of the model in realistic water samples (Fig. 7). The modeling results showed that by using the kinetic model, it is possible to estimate monochloramine level in two distribution systems and under realistic chloramination conditions. However, as shown in Figure 7, the good agreement for YZW is better than that of HPW in this model. This possible reason may be attributed to the relatively high NOM than YZW in this water. The result is in agreement with the studies of Vikesland and Ozekin et al. [24]. It should be noted that the relative complexity of monochloramine decomposition reactions makes it difficult to precisely evaluate how these competi-

---

**FIGURE 5** - Effect of iodide concentrations on monochloramine decay. Cl/N=0.7 mol/mol, pH=7.6, $C_{\text{TOT},\text{CO}_3}=1$ mmol/L, $I=0.1$ mol/L, $T=25^\circ\text{C}$. (Symbols are experimental data points and line represents model prediction).

**FIGURE 6** - Effect of different initial bromide and iodide ions on monochloramine decay at different pH values. Cl/N=0.7 mol/mol, $C_{\text{TOT},\text{CO}_3}=1$ mmol/L, $I=0.1$ mol/L, $T=25^\circ\text{C}$. (Symbols are experimental data points and line represents model prediction).

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3.8. Model validations

The model developed was used to predict monochloramine residual levels, and their predictions are compared with measured experimental data in this study. Fig. 8 shows the results of the validation analysis for the model. The model validation presented very satisfactory with \( R^2 \) value of 0.968. Specifically, 113 of the 120 observed values lay within \( \pm 10\% \) of the predicted values, with 53 of the 120 observed values lying between \( \pm 5\% \) of the predicted values. The result showed that the kinetics model was able to predict monochloramine levels in chloraminated two distribution system water samples. The modeling results also showed that, using our kinetic model, it is

![FIGURE 8 - Validation of the model for predictions and measured values of monochloramine decay levels (n=120).](image-url)
possible to stimulate monochloramine decay affected by bromide and iodide ions.

4. CONCLUSIONS

A comprehensive kinetic model predicted for monochloramine decomposition was established by considering fundamental reactions involving inorganic demand caused by bromide and iodide ions and applied to chloramine drinking water. In addition, it also included the several key influencing factors, such as initial monochloramine concentration, pH, carbonate concentration and ionic strength. It was found that monochloramine decay rate increased with increasing carbonate concentration, bromide and iodide ion, while the rate of monochloramine decay is decreased as pH increased. The good agreement between the predicted decay and the measured decay indicated that the monochloramine decay in the co-presence of bromide and iodide ion presented to demonstrate the ability of the model to incorporate inorganic demand pathways that occur parallel to auto-decomposition under realistic water quality and chloramination conditions. The results of the validation indicated very satisfactory predictions with significantly high regression coefficient ($R^2$) value of 0.968 were obtained. However, the kinetic model was only validated by both bromide and iodide ions react independently via parallel reaction pathways so far and not considered synergistically if the reactions of one species influence the reactions of another species. Additional research in this area is needed to better to understand the monochloramine decay among of NOM, bromide and iodide ions.

ACKNOWLEDGEMENTS

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EVALUATION OF FUMIGANT TOXICITY OF
*Mentha pulegium* ESSENTIAL OIL AGAINST
*Tetranychus cinnabarinus* UNDER GREENHOUSE CONDITIONS

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**ABSTRACT**

The carmine spider mite, *Tetranychus cinnabarinus* (Boisduval) (Acarina: Tetranychidae), is one of the most economically important pests of greenhouse-grown vegetables and ornamentals in southwestern part of Turkey (Antalya). In the present study, the fumigant toxicity of *Mentha pulegium* L. (Lamiaceae) essential oil was tested against *T. cinnabarinus* on cucumber under greenhouse conditions in the autumn of 2007. Two successive applications with the oil using a concentration of 4 µL/L air were made at a time when the majority of mites were at active stages (larva, nymph and adult) of development. The first application in mid-October was applied in mid-day at a temperature of ~ 45°C (inner temperature at the greenhouse). The second application in mid-November was made in the evening (at 12°C) in the same greenhouse. After an exposure period of 12 h, treatment efficacy was determined by counting live/dead individuals (larva, nymph and adult) from leaf samples, compared with that of a non-treated control. Sampling was done 1 day before and 1, 3, 7 and 14 days after treatments. Fourteen days after each application, the first one resulted in 89.25% and 72.93% reduction in the populations of larva + nymphs and adults of the pest, respectively, compared to the non-treated control, whereas the second one in the evening was less effective (54.13% and 50.85%, respectively). Pulegone, determined by gas chromatography-mass spectrometry, was the major constituent of the oil (94.99%). The results obtained suggest that the essential oil from *M. pulegium* has potential to be used as a fumigant for management of *T. cinnabarinus* under greenhouse conditions.

**KEYWORDS:** Carmin spider mite; *Tetranychus cinnabarinus*; *Mentha pulegium*; essential oil; fumigant activity; greenhouse

1. **INTRODUCTION**

Spider mites are known to feed on several hundred species of plants. Two species of spider mites, *Tetranychus cinnabarinus* (Boisduval) and *T. urticae* Koch, (Acarina: Tetranychidae), attack vegetables, fruits and ornamentals in southwestern part of Turkey (Antalya). The first species, in general, is more common in greenhouses in warm coastal areas (up to 20 km from the sea), causing considerable damage to vegetable and ornamental plants, whereas the latter is found more often in the inner parts of the region, maintaining high populations on outdoor vegetables, fruits and ornamentals [1].

The carmine spider mite, *T. cinnabarinus*, seriously damages both leaves and fruits of plants in greenhouses of Antalya region. Traditionally, the species is controlled by excessive use of acaricides in greenhouses. Its high reproductive potential and short life cycle, combined with frequent acaricide applications, result in development of strains of this mite that are highly resistant to pesticides [2]. In addition, excessive use of chemical acaricides can result in high residues on vegetables and fruits that are dangerous for consumers and the environment. Therefore, efforts have been focused on alternative control materials to these chemicals used in controlling spider mites.

In the search for alternatives to conventional chemical pesticides, essential oils extracted from aromatic plants and their components have been widely investigated. Their toxicities, arresting and repellent effects to stored-product insects and greenhouse pests have been of special interest during the last two decades [3-10]. There are many studies worldwide screening thousands of species of plants not only in search of pharmaceuticals, but also for pest control products. These studies have pointed to numerous plant species possessing potential pest-controlling properties under laboratory conditions, but the step from the laboratory to the field eliminates many contenders, even when judged only on their efficacy against pests under realistic field conditions. Hence in this study an attempt was made to explore the potential and utilization of a
common medicinal plant, mint, *Mentha pulegium* L. (Syn. *Pulegium vulgare* Mill.) (*Lamiaceae*), essential oil against *T. cinnabarinus* under greenhouse conditions, which had been previously found by Topuz [11] to have a strong fumigant activity against the pest under laboratory conditions.

*Mentha pulegium* is native to Africa, Temperate Asia and Europe [12]. This mint species is smaller than other mints and creeps along the ground and spreads rapidly through its underground root system. Stems are red-purple and highly branched while leaves are scales like. Flowers have verticillasters arrangement [13, 14]. In the last few decades many studies have been reported on the biological activity of *M. pulegium* essential oil against various pests. For instance, Pavela [15] investigated the insecticidal efficacy of oils of *Mentha spicata* L., *M. pulegium*, *M. citrata* Ehrh. and *M. arvensis* L. for larvae of *Spodoptera littoralis* (Boisdurval) (*Lepidoptera: Noctuidae*) in fumigation and topical application. In fumigation bioassay, *M. pulegium* was found to be most effective (LD₅₀ = 11.5 mg/l) while for topical assay *M. citrata* was best performer (LD₅₀ = 0.11 mg/l). In another study by Pavela [16], the essential oil of *M. pulegium* showed fumigant activity on the common housefly, *Musca domestica* L. (*Diptera: Muscidae*), and it was stronger than the 13 essential oils tested. Mahmoudvand et al. [17] studied the fumigant toxicity of essential oil extracted from *M. pulegium* on adults of stored-product insect pests, including *Tricholium castaneum* (Herbst) (*Coleoptera: Tenebrionidae*), *Sitophilus granarius* L. (*Col.: Curculionidae*), *Callosobruchus maculatus* F. (*Col.: Bruchidae*) and *Plodia interpunctella* (Hubner) (*Lepidoptera: Pyralidae*). They reported that the oil had good fumigant toxicity on all the stored-product insect pests and indicated that moths are more susceptible than coleopterans and low essential oil concentrations are more effective on moths than on beetles. Topuz [11] evaluated the fumigant toxicity of several plant essential oils on various biological stages of *T. cinnabarinus* under laboratory conditions and reported *M. pulegium* essential oil to be more effective for the pest than the other essential oils tested. The present work portrays a comprehensive picture of the fumigant activity of *M. pulegium* essential oil against *T. cinnabarinus* under greenhouse conditions. On the other hand, the goal of this study was to seek much safer and cheaper agents for controlling greenhouse pests.

### 2. MATERIALS AND METHODS

#### 2.1. Mite culture

Mites used in the present study were obtained from a laboratory culture of *T. cinnabarinus* maintained for 2 years without any pesticide exposure at the West Mediterranean Agricultural Research Institute, Antalya, Turkey. They were reared on 3 week old kidney bean (*Phaseolus vulgaris* L.) seedlings at 26 ± 1°C, 65 ± 3% RH, and a photoperiod of 16 : 8 h (L : D).

#### 2.2. Plant material and extraction of essential oil

Aerial parts (leaves and succulent stems) of *M. pulegium*, abundantly found in the Turkish flora, were collected in July 2007 from its natural habitats in Antalya. The choice of collection time was based on previous studies, which had indicated that the most appropriate time to collect them taking into consideration their essential oil level and its composition was from mid-Jun to the end of July [18-20]. Taxonomic identification was performed by botanists from the Biology Department, Faculty of Arts and Science, Akdeniz University in Antalya, where a voucher specimen of the species has been deposited.

The collected plant materials were dried at room temperature (26±1°C) for seven days. Essential oil was extracted from the shade-dried plant materials by hydrodistillation in a Cleveenger-type apparatus for 3 h [8]. The oil was obtained with a yield of 1.92% (v / w). The obtained essential oil was stored in a refrigerator at 4°C in glass vials until used for analysis and biological tests. The vials were covered with aluminum foil to avoid exposure to light.

#### 2.3. Fumigant toxicity assays under greenhouse conditions

Fumigant toxicity of *M. pulegium* essential oil was tested at two different application times in the autumn of 2007 against *T. cinnabarinus* on cucumber plants grown in low plastic tunnels in the Antalya-Serik district, where greenhouse cultivation is very common. For this purpose, two low plastic tunnels (Fig. 1a), one of which was for essential oil application and the other for control, were constructed in the study area (each 16 m² in floor area and 1.2 m high, corresponding to 14 m³). The tunnels were well ventilated by a 1-m high side opening with insect protective net and forced ventilation by an electrically operated fan.

After soil disinfection, 3 weeks old cucumber (*Cucumis sativus* L. cv. Poinsette) seedlings from a local company (Fitar Seedling Co., Ltd., Antalya, Turkey) were transplanted into the tunnels in early September, arranging in six rows with 10 seedlings. Thus, each tunnel consisted of 60 plants. Beith Alpha, which is one of the most common cucumber varieties grown in the district and susceptible to spider mites, was used throughout the study. The transplanted plants were watered using drip irrigation, applying 8 L of water per plant each week. All other cultural practices were applied uniformly in both tunnels.

Two weeks after transplanting, plants in both tunnels were inoculated with *T. cinnabarinus* (20 adult females per plant). The inoculated plants were isolated on the greenhouse bench using a ‘moat’ system [21] to prevent interplant dispersal of spider mites. For 3 weeks following release of spider mites, plants were sampled twice a week to track the spider mite populations on the plants. Three weeks after inoculating plants with spider mites (5 weeks after transplanting), the oil treatment was applied at a time, when the majority of mites were active stages (larva, nymphs and adult) of development. Two successive applications with the oil using a concentration of 4 µL/L air
FIGURE 1 - Low plastic tunnels used for fumigant toxicity of *Mentha pulegium* essential oil against *Tetranychus cinnabarinus* (a), blotting papers (each 29.7 x 42.0 cm) attached to the inner surface of the plastic cover at the ceiling for the application of the essential oil (b).

FIGURE 2 - Temperature (°C) and relative humidity (%) values inside the essential oil-treated tunnel recorded at 30-minute intervals throughout an exposure period of 12 h at the first and second application times.
were made. The first application in mid-October was applied in mid-day at a temperature of ~ 45°C (inner temperature at the tunnel), and the second application in mid-November was made in the evening (at 12°C) in the same tunnel. Temperature and relative humidity values inside the essential oil-treated tunnel were recorded at 30-minute intervals by using a portable temperature-humidity monitoring device (HOBO ‘H’ Series Loggers) throughout the exposure period (Fig. 2).

In the essential oil-treated tunnel, the oil was applied using an automatic pipette on 6 blotting papers (each 29.7 x 42.0 cm), attached separately from each other to the inner surface of the plastic cover at the ceiling (Fig. 1b). The oil was equally divided on each of the papers, and total amount of the essential oil applied to the whole tunnel (14 m³) was 56 mL, corresponding to 4 μL/L air. This concentration level, resulting in approx. 100% mortality against spider mites at an exposure period of 12 h, was based on our previous dose-finding studies in the laboratory. We made some dose-finding studies using glass desiccators with a capacity of 11 L as test chambers under laboratory conditions to determine the most appropriate dose for our field study. However, the findings of these studies have not been published yet. No material was applied to the control tunnel. Both essential oil-treated and untreated control tunnels were kept tightly closed during the exposure period (12 h). For the second application in mid-November, all the procedures were applied as those in the first application.

2.4. Phytotoxicity test

Although no phytotoxicity was observed during the field trials, the phytotoxic effect of the oil was evaluated ones more by exposing 3 weeks old potted cucumber (Cucumis sativus L., cv. Poinsette) seedlings in desiccators of 10 L capacity. The dose of essential oil used in the field trials was applied using a micropipette on to a blotting paper stripe of 3 x 8 cm attached to the underside of the desiccators’ lid. After an exposure period of 12 h, the plants were observed over a 7-day period for any signs of phytotoxicity. There were five replicates for the essential oil and the control. No material was applied in control desiccators.

2.5. GC-MS analysis

The essential oil was analyzed by Agilent 6890N (USA) gas chromatography (GC) with a jeol MS route JMS.600H (Japan), equipped with a split-splitless injector and a PTE-5 capillary column (30 x 0.25 mm x 0.25 μm). The carrier gas was Helium (Vlinear =30.0 cm/s), ionization voltage was 70 eV, and GC conditions were heating from 60 to 240°C at 3°C/min. Resolved compounds were identified by using NIST98 mass spectra library [22].

2.6. Data collection and statistical analysis

The oil applications were evaluated by counting all active stages (larva, nymphs and adult) of the mite, live or dead, on leaf samples, taken periodically. Sampling times were the 1st, 5th, 10th and 14th day after each application. At each sampling, 36 young/fully-expanded leaves were collected from each tunnel (6 per row) and examined under a stereo-microscope in the laboratory. Mites were considered dead if they did not move when prodded with a fine pin.

During sampling, incidental predators such as spiders and thrips were destroyed as they were encountered. In the first test we found approximately one incidental predator for every 10 leaves sampled; in the second test we found approximately one for every 30 leaves.

The efficiency of the essential oil applications was calculated according to the following formula described by Henderson and Tilton [23].

\[
\text{Efficiency (\%)} = \frac{1- (A1 \times B1 / A2 \times B2) \times 100}{A1}
\]

where A1 = number of living mites on the treated leaf after treatment,

A2 = number of living mites on the treated leaf before treatment,

B1 = number of living mites on the control leaf after treatment,

B2 = number of living mites on the control leaf before treatment.

All percentage data from both applications were transformed to arcsin values prior to analysis and then subjected to analysis of variance (ANOVA). However, untransformed means are presented here. Significant differences among the treatment means were separated using the Duncan’s multiple range test (DMRT), and a probability (P) of 0.05 was accepted as statistically significant [24].

3. RESULTS

3.1. Fumigant toxicity of essential oil

The fumigant toxicity of M. pulegium essential oil against T. cinnabarinus was evaluated by the percentage reduction in the population of active stages (larva + nymphs and adult) of the mite over the untreated control (Table 1).

The essential oil treatment resulted in significant reduction of population density of both larva + nymphs and adult of the mite in both applications (DMRT, \( P < 0.05 \)). Responses varied depending on application time, biological active stage of the mite and sampling time (only for the second application) (Table 1). The internal temperature of the tunnel throughout the exposure period (12 h) was the most effective factor on fumigant activity of the oil (Fig. 2, Table 1). Mortality in all active stages of the mite increased as the temperature inside the tunnel increased. There were significant differences between the population reductions of both larva + nymphs and adult stage of the mite in terms of the application time (i.e., temperature) and sampling time (valid only for the second application) at \( P < 0.05 \) (Table 1). Especially, higher mortality was observed as the internal tunnel temperature increased. Fourteen days after the first and second applications, the population reductions of T. cinnabarinus
TABLE 1 - Percentage of population reduction (%) of the spider mite, *Tetranychus cinnabarinus* exposed to the first and second applications of *Mentha pulegium* essential oil as a fumigant for 12 h under greenhouse conditions

<table>
<thead>
<tr>
<th>Sampling times</th>
<th>1st application</th>
<th>2nd application</th>
<th>F, df and P values</th>
<th>Larva+nymphs</th>
<th>Adult</th>
<th>Larva+nymphs</th>
<th>Adult</th>
<th>Larva+nymphs</th>
<th>Adult</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 day after appl.</td>
<td>71.03 ± 7.28 Aa</td>
<td>67.55 ± 5.18 Aa</td>
<td>22.64 ± 6.67 Bb</td>
<td>18.64 ± 3.38 Bb</td>
<td>17.77, 1, 0.0018</td>
<td>62.60, 1, 0.0001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 days after appl.</td>
<td>79.65 ± 5.60 Aa</td>
<td>71.58 ± 5.22 Aa</td>
<td>35.85± 7.63 Abb</td>
<td>16.98 ± 6.41 Bb</td>
<td>14.66, 1, 0.0033</td>
<td>45.52, 1, 0.0001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 days after appl.</td>
<td>84.36 ± 3.11 Aa</td>
<td>74.50 ± 3.79 Aa</td>
<td>57.60 ± 5.95 Aa</td>
<td>45.97 ± 9.71 Ab</td>
<td>2.71, 1, 0.1306</td>
<td>6.92, 1, 0.0251</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14 days after appl.</td>
<td>89.25 ± 2.10 Aa</td>
<td>72.93 ± 5.58 Aa</td>
<td>60.55 ± 4.83 Ab</td>
<td>50.85 ± 7.39 Ab</td>
<td>27.26, 1, 0.0004</td>
<td>4.87, 1, 0.0517</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values within a column with different capital letters and values within a row for each stage (adult or larva+nymph) with different lower-case letters are significantly different (DMRT, P < 0.05).

* The first application of *M. pulegium* essential oil was made in mid-day (at about 45°C; inner temp. of the tunnel) in mid-October 2007, the second application was made in the evening (at 12°C; inner temp. of the tunnel) in mid-November in the same greenhouse.

TABLE 2 - Chemical composition of *Mentha pulegium* essential oil, identified by GC/MS and quantified by gas chromatography

<table>
<thead>
<tr>
<th>Constituent</th>
<th>RI</th>
<th>Quantity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-pinene,</td>
<td>C_{10}H_{16}, 136</td>
<td>937</td>
</tr>
<tr>
<td>β-pinene,</td>
<td>C_{10}H_{16}, 136</td>
<td>978</td>
</tr>
<tr>
<td>Limonene</td>
<td>C_{10}H_{16}, 136</td>
<td>1029</td>
</tr>
<tr>
<td>1,8-cineole</td>
<td>C_{10}H_{15}O, 154</td>
<td>1032</td>
</tr>
<tr>
<td>Terpinen-4-ol</td>
<td>C_{10}H_{15}O, 154</td>
<td>1172</td>
</tr>
<tr>
<td>(+)-Pulegone</td>
<td>C_{10}H_{15}O, 152</td>
<td>1233</td>
</tr>
</tbody>
</table>

RI: retention indices from literature.

3.2. Phytotoxicity

After an exposure period of 12 h, no phytotoxicity was observed over a 7-day period, and there was no difference in appearance of essential oil-treated plants (even in young cucumber foliage) when compared with the untreated controls.

3.3. Chemical composition of essential oil

The essential oil of *M. pulegium*, with the yield of 1.92% (v / w) on dry weight basis, was analyzed by GC–MS and the chemical constituents with their percentage and retention indices from literature are summarized in Table 2. A total of 6 components were identified and accounted for 97.85% of the essential oil. (+)-Pulegone was the major constituent of the essential oil, with a quantity of 94.99%. Another important constituent in the oil was terpinen-4-ol (at 1.59%).

4. DISCUSSION AND CONCLUSION

In recent years, essential oils have received much attention as resources of potentially useful bioactive compounds. Particular emphasis has been placed on their antimicrobial, antifungal, antioxidant and pesticidal action [25]. In Turkey, there are a very rich and diversified flora and aromatic plants widely separated; however, they are commonly used as spices, flavorings in foods and medicines. Recent studies in the world showed that essential oils and their constituents could be used in the plant protection field [5, 6, 8, 26-29].

*Mentha pulegium*, essential oil of which was used in this study, is a well known species in the Lamiaceae family [30,31], and many species of this family are generally used in the food and beverage industry as flavourings, in the cosmetic and perfume industry for fragrances and in the pharmaceutical industry for medicinal purposes [32, 33]. They are therefore considered to have limited or less harmful effects on human health than most conventional pesticides, and they are perceived as ‘natural’ pesticides.

The results of the present study showed that essential oil obtained from *M. pulegium* plant significantly reduced the population of larva, nymph and adult stages of *T. cinnabarinus* in each of the two application times. The fumigant toxicity of the oil was relatively enhanced with increasing sampling time (only in the second application). However, a substantial increase in efficiency of the oil occurred with increasing temperature and relative humidity inside the tunnel (Table 1 and Fig. 2). Similar findings were also reported in other studies showing the toxic effects of essential oils obtained from some other aromatic plants against various mite species [10, 34].

A survey of literature revealed that the present study is the first study demonstrating that the essential oil of *M. pulegium* has fumigant activity against spider mites under greenhouse conditions. Although there are some studies on fumigant toxicity of *M. pulegium* essential oil against mites, all of them have pointed to the oil possessing potential mite-controlling properties under laboratory conditions. For example, Choi et al. [35] tested fumigant toxicity of 53 plant essential oils against the two-spotted spider mite, *Tetranychus urticae* (Acarina: Tetranychidae) under laboratory conditions and obtained more than 90% mortalities with vapours of essential oils from *M. pulegium* and a related species, *Mentha piperita* L., against adults of...
the pest in a concentration of $14 \times 10^{-3}$. In another laboratory study by Topuz [11], essential oil of *M. pulegium* was found to be fumigant toxic against adults (LD$_{50} = 0.49$ μL/L air), eggs (LD$_{50} = 0.60$ μL/L air) and nymphs (LD$_{50} = 0.75$ μL/L air) of *T. cinnabarinus* 96 h after an exposure period of 12 h. Unlike the findings of Topuz [11], in this study we found that young active stages (larva and nymphs) of *T. cinnabarinus* were more sensitive to the essential oil vapours of *M. pulegium* than its adults.

The chemical composition of the essential oil tested in this study was also studied, and the results showed that the major constituent of the oil was pulegone, with 94.99% (Table 2). Similarly, Muller et al. [20] reported that the essential oil levels of *M. pulegium* plant samples collected from different localities of Turkey varied from 1.6% to 5.2% and the major constituent of the essential oil was pulegone.

In conclusion, the results from the present study suggest that essential oil of *M. pulegium* may have great potential to be used for effective management of *T. cinna- barinus* under greenhouse conditions; however, the important point is that oil treatments must be applied in mid-day, in a higher value of daily temperature in greenhouses. Relative humidity inside greenhouses is also another important point is that oil treatments must be applied in mid-day, in a higher value of daily temperature in greenhouses. Relative humidity inside greenhouses is also another important point. The oil treatments in this study had different efficacy rates against different biological stages of the pest. Therefore, all oil treatments must be timed for maximum effectiveness. The results of this study indicate that the young (larva and nymphs) active stages are the most susceptible stages of the mite to essential oil treatments. That is why it is necessary that such applications be made at a time when the majority of mites are at a susceptible stage of development. A further study is also necessary to determine the toxicity of this essential oil on other economically important pests in greenhouse conditions where pest management depends on chemical applications, which is causing environmental pollution and resistance in pest populations.

**ACKNOWLEDGEMENTS**

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**REFERENCES**


GENOTYPE VARIATION IN Cd ACCUMULATION AND, CHEMICAL FORMS AND HISTOCHEMICAL DISTRIBUTION OF Cd IN LOW- AND HIGH-Cd CULTIVARS OF CHINESE LEAF MUSTARD

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State Key Laboratory for Biocontrol, School of Life Sciences, Sun Yat-sen University, Guangzhou 510275, China

ABSTRACT

In the present study, the first pot experiment was conducted to screen for Cd pollution-safe cultivars (Cd-PSCs) from 27 cultivars of Chinese leaf mustard (Brassica juncea L. Czern. et cross. var. juncea) under three Cd treatments (0.31, 0.71 and 1.71 mg kg$^{-1}$). The results indicated that eight cultivars could be considered as typical Cd-PSCs and only one cultivar was typical non-Cd-PSC (high Cd accumulative cultivar). Shoot Cd concentrations showed significant correlations between any two treatments ($p < 0.05$), which suggested that Cd accumulation in shoot of Chinese leaf mustard was genotype-dependent. In the second pot experiment, the accumulation of Cd in the nine typical cultivars was investigated under three levels of co-exposures to Cd (0.55 mg kg$^{-1}$) and Pb (48.84, 78.06 and 116.79 mg kg$^{-1}$). The results showed that shoot Cd concentrations were increased according to the levels of Pb in soil, which may indicate that the presence of Pb in soil facilitated the Cd accumulation in shoot of Chinese leaf mustard. Two cultivars (low-Cd cultivar (cv. YXZ) and high-Cd cultivar (cv. JTN)) were employed to determine the chemical speciation and trace the path of Cd. Results showed that the greatest amount of Cd was found in the extraction of 1M NaCl and 2% HAC, and the least in residues in all test tissues. Histochemical detection of cadmium (with dithizone) in tissues showed that it was uptaked by the root hairs and then transported through vascular bundles to the leaves. The Cd in root hairs of JTN was much more than those of YXZ, which may be due to the higher ability to uptake cadmium in root hairs of JTN.

KEYWORDS: Chinese leaf mustard (Brassica juncea L. Czern. et cross. var. juncea); cadmium (Cd); pollution-safe cultivar (PSC); chemical forms; dithizone.

1 INTRODUCTION

There is a growing public concern over the potential accumulation of heavy metals in agricultural soils owing to mining and smelting activities, wastewater irrigation, electroplating, and fertilization [1]. In China, about one fifth of farmland is contaminated by cadmium (Cd), arsenic (As), and lead (Pb) [2].

Excessive accumulation of heavy metals in agricultural soils results in elevation of heavy metal uptake by crops and further affects food quality and safety [3]. Some researchers tried to find an avenue way to reduce the potential health risk of pollutants entering the human food chain from soils via agricultural products [4, 5]. The concept of PSCs (pollution-safe cultivars), that is, the cultivars in which edible parts accumulate certain pollutants at a level low enough for safe consumption even when grown in contaminated soil, has been proposed [5-7]. The PSCs strategy is based on the fact that the uptake and accumulation of pollutants in plants vary not only among species but also among cultivars. Wide variations in the accumulation of Cd have been documented among cultivars of rice (Oryza sativa L.) [6, 8-11], wheat (Triticum aestivum L.) [12-14], maize (Zea mays L.) [15, 16], barley (Hordeum Vulgare L.) [17], peanut (Arachis hypogaea L.) [18], asparagus bean (Vigna unguiculata subsp. sesquipedalis L.) [19], leaf lettuce (Lactuca sativa L. var. crispa) [20], sunflower (Helianthus annuus L.) [21], and so on.

In order to explain the genotype difference in Cd accumulation, investigation of Cd uptake, transportation and detoxification is helpful. The translocation of Cd from roots to shoots has been studied in several species, including maize [22], bean [23], tomato [24], and wheat [25]. The movement of Cd from roots to shoots is likely to occur via the xylem, driven by transpiration from the leaves [26]. Preliminary study in Thlaspi caerulescens [27] showed that Cd is mainly stored in the root apoplast and, to a lesser extent, in vacuoles. There are different methods for determining elements distribution in plant tissues such as histochemical [28, 29], cell fractionation [30], particle-induced X-ray emission (micro-PIXE) [31] and nuclear micro-probe technique (NMP) [32]. Histochemical detec-
tion e.g. by using the silver sulfide staining method, is highly sensitive and allows detection of very small Cd concentration at both the light and electron microscope [33].

Mustard belonging to Brassica genus is widely cultivated in Asia and Europe [34], which is easily polluted by Cd and Pb [35]. Cultivars of Indian mustard (Brassica juncea L.) have been demonstrated the ability to concentrate Pb to a level as high as 1.5% in their shoots when grown in nutrient solution containing high concentrations of Pb [36]. However, there are few studies on heavy metal accumulation in Chinese leaf mustard (Brassica juncea L. Czern. et cross. var. juncea), and lack of evidences on whether variations in Cd accumulations among cultivars of Chinese leaf mustard is sufficient to establish the PSC strategy.

In the present study, 27 cultivars of Chinese leaf mustard were grown in three levels of Cd contaminated soils to screen for Cd-PSCs. In addition, two selected cultivars with different Cd accumulating ability were used to compare their histochemical traits by using dithizone dyeing method. Determination of chemical speciation of Cd in Chinese leaf mustard is also carried out. It is hypothesized that variations in Cd among the current using cultivars of Chinese leaf mustard is large enough to identifying Cd-PSCs and is unrelated to Cd level in soil. It is expected that the proofs from experiments of histochemistry and chemical speciation of Cd in Chinese leaf mustard can explain the mechanisms in difference of Cd uptake from soil to plant and Cd translocation from root to shoot between the low- and high-Cd accumulating genotypes.

2 MATERIALS AND METHODS

2.1 Cultivars of Chinese leaf mustard

There were 27 cultivars (Table 1) of Chinese leaf mustard used in the first pot experiment (exp. 1) for screening of Cd-PSCs. Nine cultivars selected from the exp. 1 were used in the second pot experiment (exp. 2) for validating the stability of the low Cd accumulation in shoot of Cd-PSCs and to investigating effect of soil Pb on the Cd accumulations. Seeds of the cultivars were obtained from seed companies in Guangdong, Jiangxi and Hubei provinces of China.

2.2 Experimental design and treatments of the exp. 1

The experiment was conducted in an experimental garden in the suburb of Qingyuan city (111°55'E, 23°30'N), Guangdong province, China.

Experimental soil was collected from farmland adjacent to the garden, air-dried, and ground to pass through a 5 mm sieve. The soil was treated as the low-Cd soil. The middle-Cd and high-Cd were prepared by mixing the low-Cd soil and Cd-contaminated soil (33.28 mg kg\(^{-1}\) dw) with different ratios [5]. Each soil was thoroughly mixed in a large basin and then watered and left to balance outdoors under a waterproof tarpaulin for two weeks before analyzing the soil properties (Table 2). Cd concentrations of the low-Cd, middle-Cd and high-Cd soils were 0.31, 0.71 and 1.07 mg kg\(^{-1}\), respectively. According to the maximum level (ML) of Cd (0.3 mg kg\(^{-1}\)) in the state standard of Farmland environmental quality evaluation standards for edible agricultural products (HJ332-2006), the low-Cd soil was lightly Cd contaminated soil, indicating that the agricultural soil of the farmland where the experimental soil was collected has high risk of Cd contamination. The middle-Cd and high-Cd soil were both Cd contaminated soils which Cd level exceeded the Cd maximum level of HJ332-2006 standard for 1.4 folds and 2.6 folds, respectively.

The experiment was conducted in a greenhouse with air temperature of 15-25 °C under a randomized complete block design with three replicates per treatment. Each plastic pot, (top diameter: 24cm; bottom diameter: 19cm; height: 20cm), was filled up with 4 kg prepared soil (DW). For each pot, seeds of the tested cultivar of Chinese leaf

<table>
<thead>
<tr>
<th>Cultivar Code</th>
<th>Provider</th>
<th>Cultivar Code</th>
<th>Provider</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shuidongxijinjancui A</td>
<td>Techunzhuhecai</td>
<td>TCZ</td>
<td>H</td>
</tr>
<tr>
<td>Siqichuncui A</td>
<td>Tiaiczuijuncui</td>
<td>TCZ</td>
<td>J</td>
</tr>
<tr>
<td>Fengweijiecai A</td>
<td>Gejiajancui</td>
<td>KJ</td>
<td>J'</td>
</tr>
<tr>
<td>Shuidongdenglong B</td>
<td>Daroubianjancui</td>
<td>DRB</td>
<td>K</td>
</tr>
<tr>
<td>Shuidongtiancuijui C</td>
<td>dagenpuzaoxunjancui</td>
<td>DPP</td>
<td>K</td>
</tr>
<tr>
<td>Houroudabanjie C</td>
<td>Gaojiaojancui</td>
<td>JD</td>
<td>L'</td>
</tr>
<tr>
<td>Nanhaijidaroujie C</td>
<td>Chaqijancui</td>
<td>CJ</td>
<td>M</td>
</tr>
<tr>
<td>Youxuanzhjuihe C</td>
<td>Youzhoubaxunjancui</td>
<td>AZB</td>
<td>N</td>
</tr>
<tr>
<td>Shuidongjancui D</td>
<td>Xiuyuezhujancui</td>
<td>XYX</td>
<td>O</td>
</tr>
<tr>
<td>Erhaocuihe E</td>
<td>Jinxuexuezhuijancui</td>
<td>JXP</td>
<td>P</td>
</tr>
<tr>
<td>Daroubaxunjancui E</td>
<td>Jinxuexuezhuijancui</td>
<td>JXP</td>
<td>Q</td>
</tr>
<tr>
<td>Youxuanzhjuihe F</td>
<td>Jiutouxiaoxuezhuijancui</td>
<td>JTN</td>
<td>R</td>
</tr>
<tr>
<td>Tianshuangzhuihe G</td>
<td>Dayuexuezhuijancui</td>
<td>DXY</td>
<td>S</td>
</tr>
<tr>
<td>Tiansuexuezhuijancui G</td>
<td>TCK</td>
<td>C</td>
<td></td>
</tr>
</tbody>
</table>

mustard were sown at the rate of 15 seeds per pot on Nov. 22, 2008. After germination, seedlings were thinned to 5 plants pot⁻¹ left in a week and watered with tap water daily. The solid compound fertilizer (N:P:K = 15:15:15) was applied into the soils for 2.5 g pot⁻¹ in the 20th day after germination.

Soil pH was determined by a pH meter (PHS-3C, Shanghai, China) in a soil to water ratio of 1:2.5 [37]. Organic matter content was determined by wet digestion following the method of Nelson and Sommers [38]. Total N was determined by titration of distillates after Kjeldahl sample preparation and analysis [39]. Available P was determined by molybdenum blue colorimetry [40]. Available K [37] was measured by using a atomic absorption spectrophotometer (AAS, Hitachi Z-5300, Japan). Total and extractable concentrations of Cd and Pb were also determined by the AAS system following mixed acid digestion (HNO₃–HClO₄–HF) [41] and DTPA extraction, respectively.

2.3 Experimental design and treatments of the exp. 2

The experiment was conducted in the experimental garden mentioned in the exp. 1. Native soil collected from farmland adjacent to the garden was used and three soil treatments varying in Pb concentration were conducted. Cd concentrations of the soils ranged 0.52-0.57 mg kg⁻¹ (Table 2), and thus the tested soils were all Cd contaminated according to the HJ332-2006 standard. By mixing the native soil and a Pb contaminated soil at ratios of 100:1, 1 and 15:1 (w/w), low-Pb (48.84 mg kg⁻¹), middle-Pb (78.06 mg kg⁻¹) and high-Pb soils (116.79 mg kg⁻¹) were obtained (Table 2). According to the ML of Pb (≤50mg kg⁻¹) in the HJ332-2006 standard, the middle- and high-Pb soils were Pb contaminated. Pot size, soil volume per pot and fertilization in this experiment were the same as the exp. 1.

2.4 Tissue sampling and chemical analyses

TABLE 2 - Properties of soils in exp. 1 and exp. 2 (dry weight basis).

<table>
<thead>
<tr>
<th></th>
<th>The first pot experiment</th>
<th>the second pot experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>low-Cd</td>
<td>middle-Cd</td>
</tr>
<tr>
<td>pH</td>
<td>6.90</td>
<td>6.16</td>
</tr>
<tr>
<td>Organic matter (%)</td>
<td>1.29</td>
<td>1.27</td>
</tr>
<tr>
<td>Total N (g kg⁻¹)</td>
<td>0.134</td>
<td>0.131</td>
</tr>
<tr>
<td>Available P (mg kg⁻¹)</td>
<td>49.42</td>
<td>45.39</td>
</tr>
<tr>
<td>Available K (mg kg⁻¹)</td>
<td>27.73</td>
<td>26.32</td>
</tr>
<tr>
<td>Total Cd (mg kg⁻¹)</td>
<td>0.31</td>
<td>0.71</td>
</tr>
<tr>
<td>DTPA Cd (mg kg⁻¹)</td>
<td>0.21</td>
<td>0.39</td>
</tr>
<tr>
<td>Total Pb (mg kg⁻¹)</td>
<td>37.96</td>
<td>37.04</td>
</tr>
<tr>
<td>DTPA Pb (mg kg⁻¹)</td>
<td>4.90</td>
<td>4.91</td>
</tr>
</tbody>
</table>

Sampling for plant materials of each cultivar for determinations of chemical forms of Cd was carried out on the 20th day after the translocation, and that for observations of intracellular localization of Cd was carried out on the 25th day after the translocation.

2.5 Preparation of plant samples for extraction of Cd in different chemical forms and intracellular localization of Cd

Seeds of the low-Cd cultivar (cv. YXZ) and the high-Cd cultivar (cv. JTN) were germinated on filter paper dampened with distilled water in sealed Petri dishes at 25 °C in the light. They were then sown into vermiculite in small pots with top diameter of 5.0 cm placed in a glass house at 25 °C, and were watered with Hoagland’s nutrient solutions [42]. After 10 days, the seedlings were sampled and adhering vermiculite was washed carefully from their roots. The seedlings with uniform size and growth status were selected and then grown in Hoagland’s solutions containing 1 and 10 mg L⁻¹ of Cd²⁺, respectively. Nutrient solution was topped up at regular intervals to maintain the level of the culture solution in all reservoirs. There were 6 replicates for each cultivar.

Sampling for plant materials of each cultivar for detections of chemical forms of Cd was carried out on the 20th day after the translocation, and that for observations of intracellular localization of Cd was carried out on the 25th day after the translocation.

2.6 Extractions and analyses of chemical forms of Cd in plant tissues

Cd associated with various chemical forms of the prepared plant samples was successively extracted by designated solutions in the following order [43]:
(1) 80% ethanol, extracting inorganic Cd giving priority to nitrate, chloride, and aminophenol cadmium, \( F_{\text{EtOH}} \); 
(2) distilled water (d-H\(_2\)O), extracting water-soluble Cd with organic acids and Cd(H\(_2\)PO\(_4\))\(_2\), \( F_{\text{d-H2O}} \); 
(3) 1 M NaCl, extracting pectate- and protein-integrated Cd, \( F_{\text{NaCl}} \);
(4) 2% HAC, extracting insoluble CdHPO\(_4\), Cd\(_3\)(PO\(_4\))\(_2\), and other Cd-phosphate complexes, \( F_{\text{HAC}} \); 
(5) 0.6M HCl, extracting cadmium oxalate, \( F_{\text{HCl}} \);
(6) Cd in residues, \( F_R \).

Plant material (about 2 g) was cut into small pieces of 1-2 mm\(^3\), mixed with 37.5 ml of the appropriate extraction solution and incubated at 30 °C (18 h), and then the extraction solution was pooled. The residues were extracted again with the same extraction solution (37.5 ml) for another 2 h and this was repeated twice. The combined extracts (150 ml) were evaporated to constant mass and digested with an oxidizing mixture of acids (HNO\(_3\)-HClO\(_4\), 4:1, v/v). The residual plant material was extracted with the next extraction solution in the sequence, using the procedure described above. The Cd concentration of the plant material remaining after all of the extractions had been conducted was determined by digestion with HNO\(_3\)-HClO\(_4\) (4:1, v/v). The concentrations of Cd associated with different chemical forms were determined by AAS (Hitachi Z-5300, Japan).

2.7 Observation of intracellular localization of Cd

Cross- and tangential sections of the prepared plant materials were made for their root. Histochemical detection of Cd using dithizone dyeing method (diphenylthiocarbazone, 30 mg dissolved in 60 ml acetone and 20 ml distilled water; staining, 1.5 h) was carried out for microscopic examination of intracellular localization of Cd. The procedure was performed according to the method described by Seregin and Ivanov with slight modification [29]. After staining, the sections were rinsed in water and observed by light and stereoscopic microscopy. The presence of Cd in tissues was detected in dark red to black complexes of cadmium with dithizone [29, 44].

2.8 Standard for food safety and statistical methods

According to the General Standard for Contaminants and Toxins in Foods (Codex Standard 193-1995, Revision 4, 2008, http://www.codexalimentarius.net/web/more info.jsp?id sta=17), the Codex maximum levels (MLs) in leafy vegetables for Cd were 0.2 kg\(^{-1}\) (FW), respectively. The standard was used to evaluate the safety of consuming the tested Chinese leaf mustard cultivars.

Two-way ANOVA on shoot biomass, shoot Cd concentration, and Pearson correlation were conducted using the software package SPSS 17.0. To compare the relative response of cultivars to the different levels of Cd exposures, an index of biomass response to stress (BRS) [5] was calculated as follows:

\[
\text{BRS} \, (\%) = \frac{(B_H - B_L)}{B_L} \times 100
\]

where \( B_H \) and \( B_L \) are the shoot biomasses (dry weight, DW) under high and low Cd treatments, respectively.

To estimate Cd translocation to the shoot, translocation rate (TR) [45] was calculated as follows:

\[
\text{TR} \, (\%) = \frac{\text{Cd accumulation in shoot}}{\text{total Cd in the whole plant}} \times 100
\]

3 RESULTS

3.1 Biomass response to Cd stress

The averages shoot and root biomasses under high-Cd treatment were 7.06 g plant\(^{-1}\) and 0.69 g plant\(^{-1}\), respectively, which were higher than those under low-Cd treatment (5.48 g plant\(^{-1}\) and 0.63 g plant\(^{-1}\), respectively). According to the calculation of the BRS (Figure 1), there were 24 cultivars under high-Cd treatment having higher shoot biomasses than under low-Cd treatment. Among them, six cultivars showed significant difference (\( p < 0.05 \)) between the two treatments. There were only 3 cultivars (cv. DRB, FWD, and CQJ) under high-Cd treatment having insignificantly lower shoot biomasses (\( p > 0.05 \)) than under low-Cd treatment. The results show that the species has high tolerance to Cd toxicity.

![FIGURE 1 - Biomass response to stress (BRS) of the tested cultivars in the first pot experiment. Note: ns not significant between low and high Cd; ** significant between low and high Cd at \( p < 0.01 \) level.](image-url)
3.2 Cadmium accumulation and selection of Cd-PSCs

Cd concentrations in tissues of the tested cultivars under the low-, middle- and high-Cd exposures are shown in Table 3. According to 2-way ANOVA, variations from cultivar and Cd treatment and interaction between cultivar and Cd treatment were significant ($P < 0.01$), indicating that the tested cultivars may respond to Cd stress with different degrees or in varying ways. The average Cd concentrations in shoot and root under the high-Cd treatment were 3.28 and 3.27 fold higher than those under the low-Cd treatment, respectively, close to the difference of Cd concentrations in soil between high-Cd and low-Cd treatments (3.45 folds).

### TABLE 3 - Cd concentrations in different tissues of Chinese leaf mustard and under different soil Cd levels

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Range (Average) of Cd in the tissues (mg kg$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shoot</td>
<td>low-Cd 0.061-0.227 (0.103) middle-Cd 0.095-0.493 (0.197) high-Cd 0.172-0.779 (0.338)</td>
</tr>
<tr>
<td>Root</td>
<td>low-Cd 0.085-0.156 (0.117) middle-Cd 0.137-0.695 (0.374) high-Cd 0.281-0.848 (0.381)</td>
</tr>
</tbody>
</table>

FIGURE 2 - Cd concentrations in shoot of the tested cultivars under low-Cd treatment in the first pot experiment. Note: According to the LSD test, different small letters indicate significant difference at $p < 0.05$ level between different cultivars in the same soil; Error bars represent SD ($n = 3$). The Codex ML for Cd, the Codex maximum level for Cd according to the Codex General Standard for Contaminants and Toxins in Foods.

FIGURE 3 - Cd concentrations in shoot of the tested cultivars under middle-Cd treatment in the first pot experiment. Note: According to the LSD test, different small letters indicate significant difference at $p < 0.05$ level between different cultivars in the same soil; Error bars represent SD ($n = 3$). The Codex ML for Cd, the Codex maximum level for Cd according to the Codex General Standard for Contaminants and Toxins in Foods.
Cd concentrations in shoots of the tested cultivars under the low-, middle- and high-Cd exposure are shown in Figures 2, 3 and 4, respectively. Under the low-Cd exposure, shoot Cd concentrations (FW basis) of all the cultivars were lower than the Codex ML for Cd (0.2 mg kg\(^{-1}\)), except cv. YXC. Under the middle-Cd exposure (0.71 mg kg\(^{-1}\)), 16 of the 27 tested cultivars (59.3\%) still had shoot Cd concentrations lower than the ML. However, under the high-Cd exposure (1.07 mg kg\(^{-1}\)), shoot Cd concentrations of all the tested cultivars exceeded the ML. The present results indicated that there were at least 16 cultivars that could be considered as Cd-PSCs under the middle-Cd exposure. Among them, cv. TSZ, TCZJ, YXZ, TCZ, KJJ and GJD consistently showed lowest Cd concentration in shoot under all of the three soils. Contrarily, cv. YXC, JPX and JTN consistently showed the highest shoot Cd concentration.

Correlations of Cd concentrations of shoots between different Cd treatments are shown in Figure 5. Significant correlation coefficient \((p < 0.01)\) was observed between any two treatments. The results well-demonstrate that the Cd accumulation in Chinese leaf mustard can be charac-

FIGURE 4 - Cd concentrations in shoot of the tested cultivars under high-Cd treatment in the first pot experiment. Note: According to the LSD test, different small letters indicate significant difference at \(p < 0.05\) level between different cultivars in the same soil; Error bars represent SD \((n = 3)\). The Codex ML for Cd, the Codex maximum level for Cd according to the Codex General Standard for Contaminants and Toxins in Foods.

FIGURE 5 - Correlations of Cd concentrations of shoots in the first pot experiment between different Cd treatments.
characterized as genotype-dependent, which is less affected by the level of Cd contamination in soil.

However, although most tested cultivars showed stable ability in Cd accumulation, there were still some cultivars which changed their Cd concentrations accompanying with the change of soil Cd level, such as cv. HRD and DRB. This should be the reason of the significant interaction between cultivar and soil Cd treatment. Under the high Cd treatment, there was no experimental line that accumulated shoot Cd lower than the CAC ML, suggesting that PSC strategy is not adoptable when Cd concentration in soil exceeded 1 mg kg\(^{-1}\).

3.3 Validation of the low Cd accumulation in shoot of Cd-PSCs and effect of soil Pb on the Cd accumulations in the exp. 2

Shoot Cd concentrations in of the tested cultivars in the exp. 2 are shown in Figures 6. The shoot Cd concentrations ranged from 0.089 to 0.271 mg kg\(^{-1}\) with an average of 0.138 mg kg\(^{-1}\), similar to the average shoot Cd concentration (with an average of 0.198 mg kg\(^{-1}\)) under the middle Cd exposure in the exp. 1. Whatever the Pb concentration in soil, the shoot Cd concentrations in the typical Cd-PSCs were significantly lower than the high Cd cultivar (cv. JTN) and lower than the ML of Cd in the CAC standard. The high recurrence of the low or high Cd accumulation in the typical cultivars brought forth the genetic stability of the Cd accumulation as a cultivar character of Chinese leaf mustard.

The average shoot Cd concentrations in the low-Pb, middle-Pb and high-Pb soils were 0.123, 0.138, and 0.153 mg kg\(^{-1}\), respectively. Shoot Cd concentrations were increased accompanying with the increases of Pb levels in the soil (Figure 6), and the differences within the same cultivars were significant (\(p<0.05\)). These results indicate that the presence of Pb in the soil facilitates the absorption of Cd for Chinese leaf mustard.

3.4 Chemical forms of Cd

Cd concentrations bound to different chemical forms in root and shoot of typical low- and high-Cd cultivars of Chinese leaf mustard are shown in Table 4. In the T1 (Cd=1 mg L\(^{-1}\)) and T2 (Cd=10 mg L\(^{-1}\)) treatments, Cd concentrations in FE, FD, FNaCl and FHAC of shoots in cv. YXZ were lower than the CAC ML for Cd. The Codex ML for Cd, the Codex maximum level for Cd according to the Codex General Standard for Contaminants and Toxins in Foods.
JTN (high-Cd cultivar) were significantly higher than those in cv. YXZ (low-Cd cultivar) (p<0.05). Cd concentrations of FNaCl, FHAc and FD were predominant in both cultivars, and their average proportions to ratio total Cd amount among all the Cd treatments presented 42.4%, 37.9% and 14.2% in roots, and 41.2%, 16.9% and 38.9% in shoots, respectively. The Cd amounts in Fe and FHCl were very low, and their proportions in roots and shoots were only 2.7% and 2.8% and 2.2% and 0.8%, respectively.

When compared with the high-Cd cultivar (cv. JNT), the typical Cd-PSC (cv. YXZ) had lower proportions of FFe-Cd and higher proportions of FHAc-Cd in roots under the 10 mg L⁻¹ Cd treatment. This indicated that the behaviors of FFe-Cd and FHAc-Cd in root influenced the genotype difference in Cd accumulation. In the shoots, the proportions of the FNaCl-Cd and FFe-Cd were higher than the other forms in the two cultivars, demonstrating that the Cd binding to pectates/protein (extracted by NaCl), might play an important role in Cd accumulation and detoxification of Chinese leaf mustard.

3.5 Cadmium localization in tissues

The results of the histochemical study using dithizone dyeing method (Figure 7) showed that the greatest amounts of Cd were located in the root. The presence of Cd in the root hairs points to the route by which it is taken up by plants (as with other trace and macroelements). However, the amount of Cd combined with root hairs of cv. YXZ, a typical Cd-PSC (Figure 7 a, c), was much lesser than that with cv. JTN, a typical high-Cd cultivar (Figure 7 b, d), indicating that there was critical difference in affinity of Cd and root surface between the two cultivars.

It was visible that many Cd entered internal root tissues of Chinese leaf mustard. The metal was initially immobilized in the apoplast (Figure 7 e, f) and then accumulated in in root epidermis cells (Fig7 g, h). The largest amounts of cadmium in roots were found in the vascular bundles and epidermis. Cadmium was transported to the aboveground parts of the plant mainly through vascular bundles. This is indicated by its presence in the vascular bundles (Figure 7 i, j).

No qualitative but only quantitative differences were found in the distribution of cadmium in the roots of two typical cultivars (low-Cd cultivar (cv. YXZ) and high-Cd cultivar (cv. JTN)) of Chinese leaf mustard.

4 DISCUSSION

Under the high-Cd treatment, 88.9% of the tested Chinese leaf mustard cultivars had positive BRS, which is, yielding more than those under low-Cd treatment. Because Cd is still deemed to be one of the toxic elements to plant, the positive biomass responses to Cd in most cultivars of Chinese leaf mustard could be attributed to a kind of stress reaction which was also observed in Thlaspi species [46]. These results also showed that Chinese leaf mustard had higher tolerance to Cd toxicity in soil (about 1 mg kg⁻¹) when compared to some other leafy vegetables [47]. Accordingly, farmers may not distinguish the Cd contaminated plants, which will increase the health risk from the consumption of Chinese leaf mustard contaminated by Cd.

Chinese leaf mustard could be easily polluted by Cd, if the Cd concentration in soil has reached a level > 1 mg kg⁻¹, which is commonly found in sewage-irrigated areas and industrial areas in China [48]. Even under the low-Cd treatment (0.31 mg kg⁻¹ Cd in soil), there were some cultivars that accumulated high levels of Cd (> 0.2 mg kg⁻¹), which is above the ML of the CAC. Therefore, Chinese leaf mustard should not be cultivated in Cd-contaminated or potentially contaminated agricultural soils, such as sewage or wastewater-irrigated area and industrial area, especially mining areas. Significant correlations were observed between the shoot Cd concentrations under any two Cd treatments in soil (p < 0.01). These findings proved that Cd accumulation in many cultivars of Chinese leaf mustard was genetically stable and less affected by soil properties, which was also reported in some other leafy vegetables such as water spinach (Ipomoea aquatica Forsk.) [47, 49] and Lettuce (Lactuca sativa) [50].

Interaction between Cd and Pb in soils influenced obviously accumulations of the metals in Chinese leaf mustard. The presence of Pb greatly increased Cd accumulation. A similar finding was reported by Lin et al. [51] that the presence of Pb enhanced the activity of Cd in soil and increased Cd accumulation in rice plant as a result. It was reported that the decline of Cd uptake in lettuce was due to Pb antagonism and the increase of soil Cd concentration reduced Pb availability for binding and uptake at the root surface [52]. Interestingly, the great changes of Cd accumulations in Chinese leaf mustard depend on soil Pb level, which is worth further investigating.

It has been proved that metal-tolerant plants always accumulate higher concentration of toxic metal in roots and lower in shoots as compared with the non-tolerant ones [53, 54]. Therefore, higher Cd concentration in root than in other tissues could be considered as an important tolerance mechanism of Chinese leaf mustard. The toxicity degree and migration of Cd are also dependent on its chemical forms in plants. For instance, water-soluble Cd in inorganic form (extracted by 80% ethanol) and organic form (extracted by d-H2O) exhibits higher capacity to migrate and hence more deleterious effect on plant cells in comparison with the undissolved Cd phosphate (extracted by 2% HAC) and cadmium oxalate (extracted by 0.6M HCl). In different Chinese leaf mustard tissues, the majority of Cd was integrated with pectates and protein (extracted by 1M NaCl). By contrast, a Cd-sensitive barley genotype was found to have higher Cd concentration in inorganic and water-soluble forms, while lower in pectates and protein integrated Cd as compared with the Cd-resistant genotypes [43]. Therefore, it may be assumed that larger percentages of NaCl-extractable Cd are responsible for the adaptation of Chinese leaf mustard to Cd stress.
In Chinese leaf mustard, much of the Cd accumulated within the cell walls and intercellular spaces, with some Cd moving across the plasma membrane into the symplast. This observation is consistent with that reported for several other species including maize [55] and *Biscutella laevigata* [56]. Much of the Cd was quite finely divided and not obvious in crystalline form. However within the apoplast of cv. YXZ, clumps of needle-like crystals, possibly of Cd oxalate dihydrate were found in some cells and root hairs of JTN. Similar crystalline deposits have been observed in the roots of corn [57] and sweet vernal grass [58] which had been exposed to Pb. The Cd in root hairs of JTN was much more than those of YXZ, which may be due to the higher ability to uptake cadmium in root hairs of JTN.
5 CONCLUSIONS

This study investigated the accumulation of heavy metals (Cd and Pb) in shoot of Chinese leaf mustard. Eight cultivars, cv. SJC, TSZ, TCZJ, YXZ, TCZ, KJJ, TCK, and GJD, were treated as typical Cd-PSCs. Two cultivars, cv. YXZ and JTN, contained Cd in edible parts exceeding the Codex ML for Cd even under low-Cd treatment, and it was defined as typical non-Cd-PSC. Along with the increase of soil lead concentrations, the cadmium absorption of the Chinese leaf mustard also gradually increase, indicating the presence of Pb in the soil might have facilitated the accumulation of Cd in shoot. The greatest amount of Cd was found in the extraction of 1M NaCl and 2% HAC, and the least in residues in all test tissues. The Cd was found in the extraction of 1M NaCl and 2% HAC, and it was defined as typical non-Cd-PSC. Along with the increase of soil lead concentrations, the cadmium absorption of the Chinese leaf mustard also gradually increase, indicating the presence of Pb in the soil might have facilitated the accumulation of Cd in shoot. The greatest amount of Cd was found in the extraction of 1M NaCl and 2% HAC, and the least in residues in all test tissues. The Cd was found to accumulate primarily in the cell walls and intercellular spaces of the root epidermis cells of YXZ, although some Cd moved across the plasma membrane into the symplast. The Cd in root hairs of JTN was much more than those of YXZ, which may be due to the higher ability to uptake cadmium in root hairs of JTN.

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EFFECTS OF ASPIRIN AND ACETALDEHYDE ON LONGEVITY AND METAMORPHOSIS DURATION OF Drosophila melanogaster

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ABSTRACT

In this study, the effects of acetylsalicylic acid (ASA), the active agent in aspirin, and acetaldehyde (AcH), an intermediate metabolite in alcohol metabolism, on life span and some developmental characteristics of Drosophila melanogaster are investigated. Substance administration was done on adult Drosophila through feeding. The effects were investigated using both ASA and AcH individually (ASA; AcH), and together (ASA + AcH). The life span of female D. melanogaster was longer in the experimental groups in which ASA and AcH were administered together. There was no change in the life span of male D. melanogaster. The duration of metamorphosis was prolonged in the ASA and ASA + AcH administered F₁ generation, and AcH administered F₂ generation. Additionally, the survival rate was lower with both individual and combined administration of ASA and AcH in Drosophila melanogaster.

KEYWORDS: Acetylsalicylic acid, acetaldehyde, toxic effect, Drosophila melanogaster, life span, metamorphosis duration

1 INTRODUCTION

The phenomenon of ageing is highly complex, multifactorial, and heterogeneous due to various interactions between the genes and the environment. It is most likely to be induced through a build-up of damage in DNA, mitochondria, and other related structures. It also depends on the interplay of genetic inheritance and environmental factors, such as lifestyle [1]. It is still not possible to measure aging directly; however, demographic measurements comparing mean, median, and maximum life spans of different populations in various environments has formed most of our knowledge about this phenomenon. Parameters, such as age at death and survivorship curves in demographic studies, provide crucial information needed to analyze the aging process. It is presumed that changes that shorten or prolong life span may be caused by an effect on the aging process itself [2].

Aspirin is a drug with positive side effects alongside its therapeutic use. It has been pointed out that aspirin can reduce the rate of malignant tumors [3], and prevent secondary cancer formation through inhibiting the damage to the DNA caused by mutation and H₂O₂ [3, 4]. Moreover, aspirin may suppress aging symptoms by interfering with oxidant production and cytokine response processes, and by blocking glycoloxidation reactions [5, 6].

On the other hand, acetaldehyde is used in the production of certain chemicals [7], alcohol denaturation, cosmetics products, and as a preservative and synthetic sweetener agent in certain foods [8]. Additionally, it is found in blood as a metabolite in trace amounts and a by-product of ethanol and sugar metabolism [9]. It has been shown that acetaldehyde is metabolically highly reactive and toxic, and that it binds to proteins and macromolecules [10]. It has also been shown to cause sister chromatid exchange, chromosomal deviations, and cross-links in the DNA in mammalian cell cultures [11]. Moreover, in individuals who consume large amounts of alcohol, acetaldehyde has been observed to bind to the DNA in granulocytes and lymphocytes [12].

ASA as a painkiller and AcH as an intermediate metabolite of alcohol metabolism are two common chemicals that human metabolism encounter in daily life. With that in mind, the effects of both chemicals on longevity were investigated in Drosophila melanogaster. Discovering life-prolonging drugs through feeding alone is obviously a major goal of mankind. D. melanogaster were chosen since they have most of the metabolic activation enzymes that mammals have, and their genetic characteristics and reproduction processes are very well-known. Due to this similarity in enzymes, any substance that affects the DNA of Drosophila can have similar effects on the human DNA. Therefore, it can be suggested that, if longevity and maintenance of strength can be prolonged through feeding, high throughput screening is possible [13]. Drosophila
is a perfect model organism for observing the effects of synthetic materials on developmental processes through several generations. Experiments using an organic compound in this model should provide useful data on the effects on humans. In this study, we attempt to evaluate the effects of aspirin on longevity and observe whether ASA has positive effects on the toxicity of AcH of which the genotoxicity has been determined, even though it is an intermediate metabolite for *Drosophila* [14]. Therefore, ASA and AcH were used together in some of the experimental groups. Aging process is defined by the deprivation of homeostatic maintenance functions and physiological fitness. The use of *Drosophila*, or fruit flies, as a model system is perfect for studying aging, because the genetic background, developmental processes, and some more aspects are thoroughly known. They are also highly similar to mammalians in terms of genetic makeup [1].

### 2 MATERIALS AND METHODS

Aspirin (CAS No: 50-78-2) and acetaldehyde (CAS No: 75-07-0) were obtained from Sigma and Merck, respectively. In the experiments, acetylsalicylic acid was used in crystal form, and acetaldehyde was dissolved in distilled water.

A highly homogeneous, wild-type Oregon-R strain of *D. melanogaster* was used in the experiments. This strain has round, red eyes, and no mutant genes. Cultures used in the experiments were stored in cooled incubators at 25±1 °C without any light. A 50 ml Standard Drosophila Medium (SDM) consisting of corn flour, sugar, yeast, agar, water, and propionic acid was used as the medium. SDM was used alone in the control group and F2 generation. Virgin female and male flies at the same age were used in the experimental and control groups [15]. Longevity was analyzed separately in male and female populations. Lethal concentration levels of the chemicals were determined initially. 300 mg acetylsalicylic acid (ASA), 10 mM, 20 mM, and 30 mM of acetaldehyde (AcH) were added to the experimental group medium. Each medium had 50 female and 50 male flies, adding up to populations of at least 100 flies in each group. Media of both control and experimental groups were checked daily. Flies were transferred to new media in 2-3 day periods without the use of anesthesia. Transfers were continued until every fly died and the longevity of each group was determined [16, 17].

In order to observe the duration of metamorphosis, 20 female and 20 male flies from the control and experimental groups were added to the media containing ASA and AcH. Adult flies were removed from the media following copulation and oviposition. Media were checked daily to determine the emergence of eggs, larvae, pupae, and adults. Adults from the F1 generation were transferred to chemical-free media giving rise to the F2 generation on which the same observations were recorded [18].

Evaluation of longevity was performed using one-way ANOVA and Duncan’s multiple comparison test.

### 3 RESULTS

Various amounts of crystal ASA were added to the media in order to determine the lethal concentration levels. Fifty female and 50 male flies were introduced to each medium. Mortality and survival after 24 h were determined in order to calculate the survival rates. LD50 of ASA was determined to be between 500 - 600 mg. The same method was used to determine the lethal concentration levels of acetaldehyde. However, despite several trials, no mortality was observed, even at high concentrations. Therefore, LC50 values for acetaldehyde in literature were considered [14], and AcH concentrations of 10, 20, and 30 mM were used in this study.

<table>
<thead>
<tr>
<th>Sex</th>
<th>Concentration</th>
<th>Average Life Span</th>
<th>Standard Error (±)</th>
<th>Standard Deviation</th>
<th>Max. Value</th>
<th>Min. Value</th>
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<tbody>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>65.5</td>
<td>1.5</td>
<td>2.1</td>
<td>67</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>10 mM AcH</td>
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<td>0.0</td>
<td>59</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>20 mM AcH</td>
<td>58.5</td>
<td>7.5</td>
<td>10.6</td>
<td>66</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>30 mM AcH</td>
<td>71.5</td>
<td>3.5</td>
<td>4.9</td>
<td>75</td>
<td>68</td>
</tr>
<tr>
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<td>2.8</td>
<td>68</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>ASA + 10 mM AcH</td>
<td>65.0</td>
<td>1.0</td>
<td>1.4</td>
<td>66</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>ASA + 20 mM AcH</td>
<td>57.0</td>
<td>7.0</td>
<td>9.9</td>
<td>64</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>ASA + 30 mM AcH</td>
<td>63.0</td>
<td>0.0</td>
<td>0.0</td>
<td>63</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>59.0</td>
<td>8.0</td>
<td>11.3</td>
<td>67</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>10 mM AcH</td>
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<td>0.0</td>
<td>0.0</td>
<td>65</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>20 mM AcH</td>
<td>70.5</td>
<td>4.5</td>
<td>6.4</td>
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<td>66</td>
</tr>
<tr>
<td></td>
<td>30 mM AcH</td>
<td>63.0</td>
<td>7.0</td>
<td>9.9</td>
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<tr>
<td></td>
<td>ASA</td>
<td>72.5</td>
<td>3.5</td>
<td>4.9</td>
<td>76</td>
<td>69</td>
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<tr>
<td></td>
<td>ASA + 10 mM AcH</td>
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<td>0.0</td>
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<td>70</td>
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<tr>
<td></td>
<td>ASA + 20 mM AcH</td>
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<td>4.0</td>
<td>5.7</td>
<td>83</td>
<td>75</td>
</tr>
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<td></td>
<td>ASA + 30 mM AcH</td>
<td>85.5</td>
<td>0.5</td>
<td>0.7</td>
<td>86</td>
<td>85</td>
</tr>
</tbody>
</table>

### TABLE 1 - The effects of ASA and AcH on longevity of fruit flies.
TABLE 2 - ANOVA table for longevity of female and male flies.

<table>
<thead>
<tr>
<th>Sex</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>Between groups</td>
<td>326.938</td>
<td>7</td>
<td>46.705</td>
<td>1.498</td>
</tr>
<tr>
<td></td>
<td>Within groups</td>
<td>249.500</td>
<td>8</td>
<td>31.188</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>576.438</td>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>Between groups</td>
<td>1040.438</td>
<td>7</td>
<td>148.634</td>
<td>3.676</td>
</tr>
<tr>
<td></td>
<td>Within groups</td>
<td>323.500</td>
<td>8</td>
<td>40.438</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>1363.938</td>
<td>15</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The difference is significant at the probability level of p < 0.05; SS: sum of squares; df: degrees of freedom; MS: mean square; F: F statistics.

TABLE 3 - Duncan’s MCT statistics table for longevity of female flies.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>n</th>
<th>Average Longevity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2</td>
<td>59.00</td>
</tr>
<tr>
<td>10 mM AcH</td>
<td>2</td>
<td>65.00</td>
</tr>
<tr>
<td>20 mM AcH</td>
<td>2</td>
<td>70.50</td>
</tr>
<tr>
<td>30 mM AcH</td>
<td>2</td>
<td>65.00</td>
</tr>
<tr>
<td>ASA</td>
<td>2</td>
<td>72.50</td>
</tr>
<tr>
<td>ASA+10 mM AcH</td>
<td>2</td>
<td>70.00</td>
</tr>
<tr>
<td>ASA+20 mM AcH</td>
<td>2</td>
<td>79.00</td>
</tr>
<tr>
<td>ASA+30 mM AcH</td>
<td>2</td>
<td>85.00</td>
</tr>
</tbody>
</table>

There is no statistical significance between the values with the same letters (alpha = 0.05); n: number of tests.

FIGURE 1 - Effect of ASA and AcH on longevity in D. melanogaster

Longevity data for this study are summarized in Tables 1, 2, and 3. According to the data, the longevity values for male flies were similar between the control and experimental groups, and there was no significant difference between the means (p > 0.05; Tables 1 and 2). For that matter, it can be stated that use of ASA and AcH does not affect longevity in male flies (Fig. 1).

There was a significant difference in longevity between certain groups in which female flies were treated with combined chemicals (ASA + 20 mM AcH and ASA + 30 mM AcH; Table 2). Longevity was higher in these groups. In order to obtain a more distinct value, this difference was also evaluated using Duncan’s multiple comparison test, and as a result of this test, a significant difference was again observed. On the other hand, there was no significant difference between the groups treated with AcH and ASA alone. In conclusion, the longevity of female flies remained the same when ASA and AcH were used alone; however, combined use of these chemicals in high concentrations increased their longevity (Fig. 2). Use
of these chemicals, both individually and combined, have shown no effect on male flies.

When the effects of these chemicals on the survival rates of male flies are observed, there is a difference after the sixth day compared to the control group (Fig. 3). While it was the 37th day when survival rate was 50% in the control group, it was the 28th day for the 10 mM AcH group, 26th day for the 20 mM AcH group, 30th day for the 30 mM AcH group, 32nd day for the ASA + 10 mM AcH group, 20th day for the ASA + 20 mM AcH group, 31st day for the ASA + 30 mM AcH group, and 14th day for the ASA group (Fig. 3). These values show that ASA has a negative effect on the number of flies. The survival rates have dropped faster with the use of ASA and the combination of ASA + 20 mM AcH when compared to the other experiments.

The data on survival rates of female flies is shown in Fig. 4. According to the data, the survival rates are determined as 50% on the 36th day in the control group, 29th day in the 10 mM AcH group, 27th day in the 20 mM AcH group, 34th day in the 30 mM AcH group, 21st day in the ASA group, 30th day in the ASA + 10 mM AcH group, 38th day in the ASA + 20 mM AcH group, and 27th day in the ASA + 30 mM AcH group. In this case, the experimental group treated with aspirin alone is the most negatively affected group (Fig. 4).
FIGURE 4 - Graph showing life span of female flies.

FIGURE 5 - The duration of metamorphosis in F1 generation.

FIGURE 6 - The duration of metamorphosis in F2 generation.
The effects of the chemicals used in this study on the duration of metamorphosis were also analyzed. It was observed that the F1 generation flies in the control and experimental groups continued their normal life cycles until the transition to the second phase of the third instar. However, a delay in the transition from second phase of the third instar to the pupa stage was observed in the ASA and ASA + AcH treated experimental groups. The transition to adult phase was delayed three days on average. Accordingly, ASA and the combined use of ASA + AcH have delayed the duration of metamorphosis (Fig. 5). In the F2 generation, development of flies in the AcH group was delayed, but normal life cycle of flies continued in the ASA and ASA + AcH groups (Fig. 6). While AcH had no effect in the F1 generation, its effects appeared in the F2 generation. This was an unexpected outcome since the F2 generation had no direct contact with the chemical.

4 DISCUSSION AND CONCLUSIONS

In a study on the delay of aging with geroprotectors in Drosophila melanogaster, it has been stated that average longevity values were between the 32nd and 69th days, and also that the average longevity is higher in females than males [19]. There are studies showing that aspirin prolongs longevity in various species. In a study on humans, elderly individuals were monitored for 5 years, and an increase in longevity was observed in individuals who take aspirin daily [20]. In another study, it was observed that aspirin prolonged longevity in male mice [6]. It was stated that when aspirin is administered to Sandhoff disease mice, an increase in life span and decrease in progression of the disease is observed [21]. Contrary to these studies, ASA alone had neither a positive nor a negative effect on longevity in male and female Drosophila melanogaster; however, it caused a decrease in the survival rates. While it was the 37th day in the control group, the survival rate dropped down to the 14th day, especially in male flies. In this case, it can be stated that only the resistant flies survived.

Similarly, the use of AcH had no effect on longevity in male and female flies. One of the crucial elements for Drosophila species is ethanol. While high levels of ethanol are lethal for this species, they can utilize lower levels as resources. Ethanol is converted to acetaldehyde by alcohol dehydrogenase (ADH), a catalyzing cytosolic enzyme, and acetaldehyde is then converted to acetate through oxidation by aldehyde dehydrogenase (ALDH), another catalyzing enzyme which has mitochondrial forms in both adults and larvae, but has a more important role in adults. Therefore, ALDH is the key component in preventing Drosophila mitochondria from acetaldehyde poisoning [22]. Even though the genotoxicity of AcH in Drosophila has been determined, its toxic effects on longevity have not been observed. However, in a study on rats, AcH has been observed to increase mortality [23]. These varying outcomes may be due to differences between the enzyme activities in mammals and Drosophila. In a recent study, the rate of oxidation of acetaldehyde to acetate by mammalian ADH was observed to be much higher than previously thought [22]. Moreover, despite the fact that AcH is a metabolite for Drosophila, it has caused a decrease in survival rates. Half of the flies in the control group were still alive on the 37th day, while in the experimental groups, half of them were already dead on the 26th day. Researchers testing Usnea longissima extracts on Drosophila have observed a decrease in longevity, caused by increased metabolic rate due to the toxic effects, leading to increased rates of respiration and oxygen-derived free radical formation, and eventually, cell damage through oxidative damages [16]. The decrease in longevity observed in this study might be due to the reasons mentioned above.

The longevity of male flies in groups treated with both chemicals (ASA + AcH) remained the same, while it was prolonged in female flies at high concentrations. It was stated that the reason for prolonged longevity in D. melanogaster with longer life spans than normal was due to an increase in the expression of genes belonging to the antioxidant system, the production of Cu/Zn-SOD (Cu/Zn-superoxide dismutase) proteins, and ADS (antioxidant defense system) enzyme activities. As a consequence of this, it was stated that longevity is prolonged in flies that are resistant to oxidative stress. Thus, in D. melanogaster, it was noted that antioxidative activities of enzymes, such as catalase and glutathione reductase, and total glutathione levels decrease with aging [16]. It is possible that a product of ASA and AcH interaction may have prolonged longevity in flies that show resistance. The effects of these products emerging from interactions of substances may indeed be antagonistic or synergistic.

Data on the durations of metamorphosis in F1 and F2 generations following the use of ASA, AcH, and ASA + AcH on D. melanogaster is shown in Figs. 3 and 4. The duration of metamorphosis was delayed when ASA was used alone, and in combination with AcH in the F1 generation. There are several studies supporting this outcome. In rat embryos, it has been observed that salicylates cause fetal death, delayed development, and congenital anomalies. It has been determined that ASA negatively affects the growth and development of mice [24]. It has also been observed that aspirin has teratogenic effects in mice fetuses [25]. In our study, ASA delayed metamorphosis in the F1 generation; however, toxic effects were not observed in the F2 generation. In fact, AcH delayed the duration of metamorphosis in the F2 generation while it had no effect in the F1 generation. It was stated that ALDH enzyme that converts acetaldehyde to acetate is much more important than ADH during the larval stage [22]. Therefore, it may be that the levels of this enzyme were enough for the natural metabolism of the organism in larval stage, but the increasing level of AcH was not enough for catabolism, and its accumulation had genotoxic effects. Consequently, the effects might have surfaced in the F2 generation. In fact, AcH has been found to have teratogenic
effects and cause embryolethality in rats [26], gene mutations in human lymphocytes [27], and sister chromatid exchange and chromosome deviations in mammalian cell cultures [11]. In addition, it was stated that 2-chloro-acetaldehyde caused cross-links in DNA molecules in *D. melanogaster* [28]. Considering the results of the present study, findings in the F2 generation can be associated with the genotoxic effects of AcH. The delay in metamorphosis observed in the F2 generation may be explained with the hereditary mutations caused by AcH in the developmental genes of *D. melanogaster* since flies in this generation had no direct contact with AcH.

In conclusion, ASA and ASA + AcH applications lead to a deterioration of development, and a decrease in the survival rate. However, higher concentrations of ASA + AcH prolonged longevity of female flies, which is quite intriguing. Positive effects of the combined use of these chemicals were observed in resistant flies that successfully avoided toxicity. Another interesting result is that AcH caused a delay in development in the F2 generation, even though AcH is a metabolite for *Drosophila*, and flies in the F2 generation had no direct contact with it.

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BIOSORPTION OF CONGO RED BY PELLETS OF LIVE Streptomyces sp. LH1

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ABSTRACT

In the present research, the potential of pellets of live Streptomyces sp. LH1 was investigated as a biosorbents for removal of an anionic disazo direct dye Congo red, from an aqueous solution. Live biomass of strain LH1 was found to be more effective with a removal rate of 74% compared with 65% of dead biomass for Congo red. The visual observation and ultraviolet-visible (UV-VIS) spectral analysis showed that dye removal using live biomass mainly involved adsorption by the cell surface including the intracellular bioaccumulation. Batch pH, initial dye concentration and isotherm studies were conducted to evaluate the biosorption capacity of live biomass. A maximum removal of 97.5% for 50 mg/L of Congo red by Streptomyces sp. LH1 pellets was observed at pH 2.0. The pellets seemed to exhibit high biosorption activity in the low pH range. Equilibrium was reached in 1 h. Increasing the initial dye concentration resulted in decreasing the color removal efficiency while higher adsorption yields were observed at lower concentrations of dye. Isotherm studies indicated that biosorption of Congo red on live biomass fitted both the Langmuir and Freundlich models well. Biomass of strain LH1 was found to be effective in removing the other five dyes from an aqueous solution. This study showed that it is possible to develop systems for dye removal using Streptomyces biomass which occurs as a byproduct in waste streams of fermentation industries.

KEYWORDS:
Streptomyces sp. LH1; biosorption; Congo red; dye

1 INTRODUCTION

The release of dyes into the environment is of concern due to coloration of natural waters, and the toxicity, mutagenicity and carcinogenicity of the dyes and their biotransformation products [1-4]. Thus, the decolorization of wastewaters containing these dyes prior to discharge is mandatory by environmental regulations in most countries [5]. The removal of dyes from wastewaters is based mainly on physical or chemical methods [6]. However, application of such techniques is always expensive and ineffective in terms of energy and chemical products consumption, especially at low dye concentration [6]. Therefore, there is a great need for an alternative technique, which is both economical and efficient. Biosorption, based on live or dead biomass, has been regarded as an efficient method for the removal of dyes from effluent due to its low initial cost, simplicity of design, ease of operation and insensitivity to toxic substances [7].

A wide variety of microorganisms including bacteria, fungi and algae are capable of removing a wide range of metal ions and dyes with high efficiency via adsorption [8-11]. In particular, of bacterial biosorbents, Streptomyces are noteworthy. Since these microorganisms are used widely in different food/pharmaceutical industries, they are generated as waste and can be acquired free or at low cost from these industries [12]. In recent years, some Streptomyces strains have been employed as inexpensive biosorbents in the removal of metal ions and dyes. The simultaneous biosorption of Cu²⁺, Zn²⁺ and Cr⁶⁺ from wastewater by Streptomyces rimosus biomass was reported by Chergui et al. [13]. In another study, Yuan et al. [14] investigated the comparative biosorption of cadmium by two different Streptomyces strains K33 and HL12. On the other hand, Naceria and Aicha investigated the biosorption of methylene blue by pretreated dead streptomyces rimosus [15]. Biosorption of acid fast red by Streptomyces globosus was reported by El-Sersy et al. [11]. However, there is still not much information on the biosorption of dyes by Streptomyces strain. Also, there is limited study on dye biosorption ability of live cells of given Streptomyces strain.

The objective of the present work is to investigate the biosorption potential of using pellets of live Streptomyces strain LH1, as a low cost adsorbent to uptake Congo red from aqueous solution. The comparative biosorption of Congo red by live and dead cells of strain LH1 was carried out. The effects of important factors, such as pH and initial concentration on Congo red adsorption were studied. Langmuir and Freundlich adsorption isotherms were employed to quantify the adsorption equilibrium. The bio-
sorption capacity of other classes of dyes by pellets of live *Streptomyces* strain LH1 was also evaluated.

## 2 MATERIALS AND METHODS

### 2.1 Chemicals

Congo red used as a model reactive dye in the present work was procured from Sigma-Aldrich, (Beijing, China). It has the molecular formula C$_{32}$H$_{22}$N$_6$O$_6$S$_2$Na$_2$ (MW 696.66g/mol) with Color Index Number 22,120 and CAS (Chemical Abstracts Service) Number 573-58-0. All other reagents and chemicals were of the highest purity available and of an analytical grade.

### 2.2 Preparation of pellets of strain LH1 as biosorbent

The *Streptomyces* strain LH1, isolated from the soil collected from a textile mill in Wuhan, China, was tested in this study. Stationary-phase cells of this strain were typically inoculated into modified Gause’s liquid medium (solute starch 16.0 g, glucose 4.0 g, NaCl 0.5 g, KNO$_3$ 1.0 g, KH$_2$PO$_4$·H$_2$O 0.5 g, MgSO$_4$·7H$_2$O 0.5 g, FeSO$_4$·7H$_2$O 0.01 g, water 1000 ml, pH 7.5±0.1) and 120rpm agitation was prepared by recording the absorbance values for a standard concentration 1000 mg/L and was subsequently diluted to the required value with diluted or concentrated H$_2$SO$_4$ and NaOH solutions before mixing the biomass suspension.

### 2.3 Identification of strain LH1

Genomic DNA was extracted in accordance with the methods described by Edwards et al. [16]. The PCR amplification of the 16S rRNA gene was conducted using two primers: 5’-AGAGTTTGATCCTGGCTCAG-3’ and 5’-AAGGAGGTGATCCAGCCGCA-3’, as described by Weisburg et al. [17]. The sequence of the 16S rRNA gene (1,409 bp) was obtained using an Applied Biosystems DNA sequencer (model 3730) and deposited in the GenBank database under accession number GU270857. The sequence was submitted to a BLAST search of the NCBI GenBank database to identify the organism.

### 2.4 Analytical Techniques

The dye was made up in a stock solution of concentration 1000 mg/L and was subsequently diluted to the required concentrations. The calibration curve for this dye was prepared by recording the absorbance values for a range of known concentrations of dye solution at the wavelength of maximum absorbance of the dye. The value of $\lambda$ max (490 nm) was used in all subsequent investigations. The absorbance spectra of the dye, before and after adsorption, were scanned by a Varian CARY50 UV-VIS spectrophotometer (Varian, Salt Lake, UT, USA), and the changes in its absorbance spectrum (250-800 nm) were recorded.

### 2.5 Effects of pH and initial concentration on biosorption

All the samples were incubated for 24 h in 50 mg/L of dye. Unless otherwise stated, standard conditions for biosorption experiments included an initial pH 5, being agitated at 150 rpm and having a dosage of 2.0 g. The range of concentrations of prepared dye solutions was 20-60 mg/l. The pH of each solution was adjusted to the required value with diluted or concentrated H$_2$SO$_4$ and NaOH solutions before mixing the biomass suspension.

### 2.6 Batch biosorption studies

The experiments were conducted in 250 ml Erlenmeyer flasks containing 100 ml of dye solutions. The flasks were agitated on a shaker at 150 rpm for 48 h to ensure that equilibrium was reached. Samples (5 ml) were taken before mixing the biosorbent solution and dye bearing solution and at predetermined time intervals for the residual dye concentration in the solution. Before analysis the samples were centrifuged at 8000 rpm for 5 min and the supernatant liquid was analyzed for the remaining dye. Control experiments were carried out without adsorbent to estimate the dye removal due to adsorption onto the walls of the flasks. It was observed that adsorption on the container walls was negligible. All the experiments were carried out in triplicates and it was noted that the deviation in results were below 5%.

### 2.7 Calculations

The amount of dye adsorbed at time $t$, $q_t$, was calculated from the mass balance equation. When time $t$ (min) is equal to the equilibrium contact time, $C_i = C_e$, $q_t = q_e$, then the amount of dye ions adsorbed at equilibrium, $q_e$, was calculated using Eq. (1):

$$q_e = \frac{(C_0 - C_e)V}{m}$$

where $q_e$ and $q_t$ are the amount of solute adsorbed onto the unit mass of the adsorbent at time $t$ (min) and at equilibrium (mg/g), respectively; $C_0$, $C_t$, and $C_e$ are the concentrations of the solute in the initial solution and in the aqueous phase at time $t$, and at equilibrium (mg/L), respectively; $V$ is the solution volume of the aqueous phase (L); and $m$ the amount of adsorbent used (g). The removal efficiency (%) was calculated according to Eq. (2):

$$removal(\%) = \frac{C_0 - C_e}{m} \times 100$$

## 3 RESULTS AND DISCUSSION

### 3.1 Isolation and identification of strain LH1

A strain of bacterium, LH1, with strong adsorption ability on Congo red was isolated. The bacterium was Gram-positive. The colony of strain LH1 was circular and gray-colored. The aerial mycelium of strain LH1 is short-napped, powdery, and mouse-gray in color. The sporopho-
res are spiraled. Sequence analysis of 16S rDNA showed that strain LH1 had highest similarity with the genus *Streptomyces* (99%). Based on the phenotypic characteristics and phylogenetic analysis, strain LH1 was identified as *Streptomyces*. The 16S rDNA sequence of *Streptomyces* sp. LH1 has been deposited in GenBank with the accession number GU270857 (http://www.ncbi.nlm.nih.gov/nuccore/GU270857).

3.2 UV-visible spectrophotometric analysis

The ultraviolet and visible absorbance (250-800 nm) of dye samples during biosorption process was monitored by a UV-VIS spectrophotometer to examine the change of the structure of Congo red. The dye has the maximum absorption peak at 332 nm and the second peak at 490 nm (Fig. 1). As soon as the dye solution was contacted with the live biomass, the peaks rapidly declined, and after 70 min, the peaks did not change significantly. Therefore, the contact time to allow the sorption system to reach an equilibrium state was less than 70 min. As can be seen in Fig. 1, the shape of absorption spectra did not alter during the removal process, implying that Congo red was adsorbed without any change in its original chemical structure. In Fig. 2, the color of pellets turned from white to deeply red after 60 min contact with the dye solution, indicating a significant adsorption effect on the cells of LH1. These results suggest that biosorption plays a major role in the removal of Congo red by live biomass of *Streptomyces* sp. LH1.

3.3 A comparative adsorption between live and dead biomass of *Streptomyces* sp. LH1

A comparative study on removal of Congo red by pellets of live and dead LH1 was carried out. The effect of contact time on biosorption equilibrium and the removal efficiency by pellets of live and dead LH1 at initial concentrations of 50 mg/L were represented in Fig. 3. In the case of the pellets of dead LH1, the time required for equilibrium was about 80 min and the removal efficiency was 65%, after that the removal value was nearly constant. Volesky et al. indicates that the first phase of biosorption is always rapid, and it is considered to be a spontaneous process with no energy consumed [18]. For the biosorption by the pellets of live LH1, the time required for equilibrium was about 60 min and removal efficiency was 74%, after that the removal value was increase slowly. The result showed that the pellets of live LH1 appeared to have higher efficiency for removal of the dye than those of dead LH1. It suggested that the intracellular bioaccumulation might also contribute to the uptake of the
dye, in addition to the rapid adsorption by the cell surface. This result was consistent with the former research focusing on the adsorption of zinc ions onto live cells of *Streptomyces cicaucasicus* [19]. The pellets of live LH1 were used in all subsequent investigations.

### 3.4 Effect of initial pH on dye biosorption

The solution pH significantly influenced the dye biosorption properties of *Streptomyces* sp. LH1 pellets. Removal of Congo red by the adsorbent was high at lower pH, particularly when the solution pH was decreased below 5.0 (Fig. 4). A maximum removal of 97.5% for 50 mg/L of Congo red by the adsorbent was observed at pH 2.0. Above pH 5.0, the removal of Congo red remained almost constant till pH 10.0. Similar results were reported by several researchers for adsorption of reactive dyes [12].

![FIGURE 4 - Effect of pH on adsorption of Congo red onto Streptomyces sp. LH1 pellets.](image)

Solution pH influences both the cell surface dye binding sites and the dye chemistry in water. Congo red has two sulphonate groups, which have negative charges in aqueous solution. Higher uptakes obtained at lower pH may be due to the electrostatic attractions between negatively charged dye anions and positively charged cell surface. At higher pH levels, excess OH- ions compete with dye anions for adsorption sites on the adsorbent surface resulting in less adsorption.

### 3.5 Effects of concentration of dye on adsorption

Initial concentration provides an important driving force to overcome all mass transfer resistances of the dye between the aqueous and solid phases. The effect of initial dye concentration on the dye sorption capacity was investigated between 20 and 60 mg/L at the initial pH 5.0, the results are presented in Fig. 5. The equilibrium time of Congo red adsorption for the pellets was about 60 min and removal was in the range of 54-78% for 20-50 mg/L Congo red solution. Increasing the initial dye concentration resulted in decreasing the color removal efficiency while higher adsorption yields were observed at lower concentrations of dye. This is due to the fact that at lower initial solute concentrations, the ratio of the initial moles of solute to the available surface area is low. However, at higher concentrations, the sites available for sorption on the biosorbent become fewer compared to the moles of solute present.

![FIGURE 5 - Removal efficiency of Congo red by streptomyces sp. LH1 pellets over initial concentration ranging from 20 to 60 mg/L.](image)

### 3.6 Adsorption Isotherms

The Langmuir and Freundlich isotherm models were examined in this study to describe the biosorption equilibrium. The Langmuir isotherm [20] can be stated as follows:

\[
q_e = \frac{q_m b c_e}{1 + b c_e}
\]

Where \(q_m\) (mg/g) and \(b\) (L/mg) are Langmuir constants which are indicators of the maximum adsorption capacity and the affinity of the binding sites, respectively.

The Freundlich isotherm [21] is shown in Eq. (4) below:

\[
q_e = k_f c_e^{1/n}
\]

Where \(k_f\) ((mg/g) (mg/L)\(^{-1/n}\)) and \(n\) (dimensionless) are the Freundlich constants, indicating adsorption capacity and adsorption intensity, respectively.

The Langmuir and Freundlich adsorption constants with the correlation coefficients are presented in Table 1. As listed in Table 1, high regression correlation coefficients (>0.950) were found under studied conditions. The Langmuir model considers sorption by monolayer type and supposes that all the active sites on the sorbent surface have the same affinity by the sorbate. On the other hand, the Freundlich isotherm is an empirical equation which assumes a heterogeneous biosorption system with different active sites [14]. The applicability of both Langmuir and Freundlich isotherms to the dye-Streptomyces sp. LH1 pellets systems implies that both monolayer adsorption and heterogeneous surface conditions exist under the experimental conditions used. Similarly, earlier study show that the dye adsorption equilibrium data of *Candida* yeast fitted very well to both Freundlich and Langmuir adsorption models in the studied concentration range [22].
TABLE 1 - Isotherm parameters for removal of Congo red by streptomyces sp. LH1 pellets

<table>
<thead>
<tr>
<th>Adsorbents</th>
<th>Langmuir</th>
<th>Freundlich</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Q0(mg/g)</td>
<td>b (L/mg)</td>
</tr>
<tr>
<td>Bacterial pellets</td>
<td>38.12</td>
<td>0.05</td>
</tr>
</tbody>
</table>

TABLE 2 - Removal of different dyes (50mg/L) by the pellets (2g) after 1 h of incubation under the conditions of 25°C and 150 rpm

<table>
<thead>
<tr>
<th>Adsorbents</th>
<th>Removal (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Toluidine blue</td>
</tr>
<tr>
<td>Bacterial pellets</td>
<td>78.4</td>
</tr>
</tbody>
</table>

The sorption of dye on the pellets is thus likely to be complex, involving more than one mechanism such as intracellular bioaccumulation.

3.7 Biosorption of other dyes

Adsorption ability of the pellets to other dyes was tested. According to Table 2, the pellets showed high dye adsorption activity for different classes of dyes. More than 60% removal was achieved by the pellets and removal efficiency of Malachite green (87.6%) was highest. Hu also demonstrated the ability of bacterial cells isolated from activated sludge process of a textile industry and soil to adsorb 11 reactive dyes [23]. Zhou and Zimmermann [24] used the actinomycete Streptomyces BW130 as an adsorbent for the decolorization of effluents containing anthraquinone, phthiocyanine, and azo dyes. Since the mode of dye uptake by the cells is extracellular, the chemical functional groups of the cell wall play vital roles in biosorption. Due to the nature of the cellular components, several functional groups are present on the bacterial cell wall, including carboxyl, phosphonate, amine and hydroxyl groups. These functional groups differ in their affinity and specificity for dye binding [12]. The dye molecules, which exist as dye cations/anions in solutions, are attracted towards negatively/positively charged groups via electrostatic interaction.

4 CONCLUSIONS

The strain LH1 was isolated from the soil and identified as Streptomyces genera based on the phenotypic characteristics and phylogenetic analysis. Biomass of strain LH1 was found to be effective in removing Congo red from an aqueous solution. Compared with live and dead cells of Streptomyces sp. LH1 used in the study, live cells proved to be more efficient than dead ones. The visual observation and UV-VIS spectral analysis showed that dye removal using live biomass mainly involved adsorption by the cell surface including the intracellular bioaccumulation. Initial pH of the dye solution strongly influenced the chemistry of dye molecules and live biomass in the aqueous solution. Removal of Congo red by the pellets of Streptomyces sp. LH1 was high at lower pH. Increasing the initial dye concentration resulted in decreasing the color removal efficiency, whereas higher adsorption yields were observed at lower concentrations of dye. The batch biosorption data of Streptomyces sp. LH1 pellets conformed to both Langmuir and Freundlich equations well. Streptomyces sp. LH1 pellets also showed a good biosorption capacity of different classes of dyes. Taking into consideration of present findings, pellets of Streptomyces is a promising biosorbent for dye removal from a dye wastewater.

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POTENTIAL INTERACTIVE EFFECTS OF
TEN HEAVY METALS (Pb, Cd, Cu, Zn, Ni, Cr, Co, Sb, Fe AND Mn) ON SOIL BIOTOXICITY IN AN OILFIELD FROM CHINA

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ABSTRACT

The potential interactions of ten heavy metals (Pb, Cd, Cu, Zn, Ni, Cr, Co, Sb, Fe and Mn) on soil biotoxicity were investigated using a modified principal component regression (MPCR) model in an oilfield in China. Principal component analysis (PCA) was used to mitigate the multicollinearity, and an Expectation-Maximisation (EM) algorithm was applied as a missing value treatment to make the principal components readily interpretable. It was found that the MPCR can be used to model the interaction between heavy metals in the biotoxicity analysis of the oilfield soil. According to the modified PCR model, the Cu acts as a micronutrient in the soil for the activities of luminescent marine bacteria (Photobacterium phosphoreum), and the other nine metals have a toxic effect on the bacteria. Additionally, the interactions of metals between Co-Zn, Co-Cu, Co-Cd, Cu-Pb, Cd-Sb, Pb-Zn and Pb-Cd can be described as antagonistic, while the interactions between Co-Ni, Co-Cr, Co-Sb, Co-Pb, Cu-Sb, Cu-Cd, Cd-Ni, Cd-Fe, Pb-Cr and Pb-Sb can be described as synergistic.

KEYWORDS: Heavy metal; Soil biotoxicity; Oilfield; Interaction; Modified principal component regression (MPCR)

1 INTRODUCTION

The largest marine oil spill in the history of the petroleum industry, which occurred in the Gulf of Mexico in 2010, has attracted greater attention to the issue of petroleum contamination. Numerous studies on petroleum contamination have been reported [1-4], and some research has focused on the inorganic contamination in soils generated by oil exploration and exploitation [5]. Biotoxicity in the soil can be associated with various factors, such as heavy metals [6], PAHs [7, 8], organic matter and clays [2, 3]. According to the early study, the contents of these organic pollutants did not relate to the biotoxicity, but the heavy metals did. So the heavy metals may be one of the most important factors influencing biotoxicity in soils [9], and there are interactions between some heavy metals. Other studies show that heavy metals can affect biotoxicity by interactions described as antagonism, synergism and additivity [10-12].

Different mathematical algorithms have been proposed to predict the toxicity of a mixture of several contaminants with the Microtox® test [13, 14]. Multiple regression analysis is one of the most widely used methods for expressing the dependence of a response variable on several independent variables [15]. However, the regression approach can face serious difficulties, such as multicollinearity [16, 17]. Principal component analysis (PCA) has been used to analyse voluminous environmental data to separate interrelationships into statistically independent basic components [18-20]. PCA is equally useful in regression analysis for mitigating the problem of multicollinearity and exploring the relationships among independent variables for model prediction [15]. In the earlier study, PCA was used to mitigate the problem of multicollinearity, and a least-squares regression model was established to reveal the relationship between biotoxicity and ten heavy metals (Pb, Cd, Cu, Zn, Ni, Cr, Co, Sb, Fe and Mn) without considering any interaction. However, the result showed that four heavy metals (Cd, Co, Pb and Cu) had a negative influence on the biotoxicity, which indicated the potential interactions among them [9]. So the potential interactions of the heavy metals will be investigated in this paper, after a missing value treatment.

Missing values are problems in statistical analysis; these occur when no data value is stored for the variable in the current observation. Missing values are a common occurrence, and they can reduce the representativeness of the sample and severely disturb the conclusions drawn from the data [21]. The treatment of missing values is an important task because it can improve the quality of knowledge discovery in the dataset (KDD) dramatically, especially if the dataset contains a large amount of missing data [22].

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The primary goal of this article is to establish a model to reveal the relationship between biotoxicity and ten heavy metals (Pb, Cd, Cu, Zn, Ni, Cr, Co, Sb, Fe and Mn), including the interactions between the heavy metals. A missing value treatment is applied to make the principal components readily interpretable. PCA was used to mitigate the problem of multicollinearity among the metal elements, as we did in the earlier article, and a modified principal component regression (MPCR) was used to obtain a model of the biotoxicity and the ten heavy metals for the purposes of qualitatively assessing the interactions of the ten heavy metals on the biotoxicity in the oilfield soil.

2 MATERIALS AND METHODS

2.1. Data source

Soil samples were collected from six oil wells in an oilfield in China following the time in serves (1-15 years), and no oil leakage accidents are recorded and no visible leaked crude oil around the oil wells are observed. A total of 292 soil samples were obtained.

The soil samples were pre-treated for the subsequent heavy metal analysis and biotoxicity test. Pseudo-total amounts of the selected ten heavy metals were determined by following the recommended standard method of USEPA Method 3050B using an AAAnalyst 400 absorption spectrometer (Perkin-Elmer, USA). The soil biotoxicity was determined against a biological test system: luminescent marine bacteria, Photobacterium phosphoreum. The Microtox® bacterial assay of soil was run according to the ASTM standard method D5660-96. A Microtox® toxicity analyser (Model DXY-2, the Institute of Soil Science, Academic Sciences, Nanjing PRC) was used to evaluate the inhibition of luminescence in the marine bacteria Photobacterium phosphoreum. The decrease in light emission by Photobacterium phosphoreum exposed to the soil extracts was measured to indicate the soil biotoxicity (BT). More details about the datasets are available in our earlier study [9].

2.2. Missing value treatment

Missing value treatment methods include removal and imputation methods [21]. The removal method removes all instances that have missing values. The imputation method is one of the most important missing value treatment methods. This method replaces the missing values with estimated values based on information available in the dataset. Imputation aims to determine the relationships among the values in the dataset and to estimate missing data under the help of these relationships [21].

In this paper, the Expectation-Maximisation (EM) algorithm was used as the missing value treatment method to develop maximum-likelihood estimates of the regression parameters and the variance-covariance matrix and to replace the missing values with the predicted values obtained from the regression.

2.3. Data analysis methods

PCA was applied to the treated data to assess the associations between variables. Then, digitalisation of the correlation matrix transformed the original correlated variables into uncorrelated (orthogonal) variables called principal components, which are weighed linear combinations of the original variables by the component score coefficient [23]. The characteristic roots (eigenvalues) of the principal components are a measure of their associated variances, and the sum of the eigenvalues coincides with the total number of variables [20]. The correlation of the principal components and their original variables is given by loadings, and the individual transformed observations are called scores [20]. Loadings of the principal components are the vector of coefficients for the principal components and can be used to describe the means of the principal components [23].

The transformed principal components were obtained as weighted linear combinations of the original variables as the following:

\[ PC_n = a_{1n}Z_1 + a_{2n}Z_2 + \ldots + a_{mn}Z_m \]  

where \( PC_n \) is the principal component; \( Z_{mn} \) is the z-transformed original variable; and \( a_{mn} \) is the component score coefficient.

According to equation (1) and the component score, the model with \( PC_n \) can be transformed into a new model of the original variables; this is the original principal component regression (PCR). In this study, the principal components were replaced by new variables according to the interpretations using a stepwise regression analysis. The new variables can embody the interactions of the ten heavy metals.

3 RESULTS AND DISCUSSION

3.1. Missing value treatment results of the variables

Descriptive statistics of the soil biotoxicity and heavy metal contents before and after the missing value treatment are shown in Table 1, and a summary of the estimated mean and standard deviations is shown at the right in the table. As seen from the table below, there are missing data in the univariate of the six heavy metals and the biotoxicity. Half of the Cd and approximately 10%–20% of the Sb, Co, Mn and Cr contents are missing. Conversely,
TABLE 1 - Descriptive statistics of biotoxicity and heavy metals in the soils

<table>
<thead>
<tr>
<th>Metals</th>
<th>N</th>
<th>Mean (mg/kg)</th>
<th>Std. Deviation</th>
<th>Count</th>
<th>Percent</th>
<th>Mean</th>
<th>Std. Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pb</td>
<td>292</td>
<td>18.15</td>
<td>9.14</td>
<td>0</td>
<td>0</td>
<td>18.15</td>
<td>9.14</td>
</tr>
<tr>
<td>Cd</td>
<td>146</td>
<td>1.58</td>
<td>0.91</td>
<td>146</td>
<td>50.0</td>
<td>1.39</td>
<td>0.95</td>
</tr>
<tr>
<td>Cu</td>
<td>291</td>
<td>29.14</td>
<td>26.97</td>
<td>1</td>
<td>0.3</td>
<td>29.11</td>
<td>26.95</td>
</tr>
<tr>
<td>Zn</td>
<td>292</td>
<td>31.91</td>
<td>9.01</td>
<td>0</td>
<td>0</td>
<td>31.91</td>
<td>9.01</td>
</tr>
<tr>
<td>Ni</td>
<td>292</td>
<td>20.39</td>
<td>8.00</td>
<td>0</td>
<td>0</td>
<td>20.39</td>
<td>8.00</td>
</tr>
<tr>
<td>Cr</td>
<td>236</td>
<td>16.76</td>
<td>7.63</td>
<td>56</td>
<td>19.2</td>
<td>15.15</td>
<td>8.30</td>
</tr>
<tr>
<td>Co</td>
<td>246</td>
<td>15.01</td>
<td>5.44</td>
<td>46</td>
<td>15.8</td>
<td>13.42</td>
<td>6.30</td>
</tr>
<tr>
<td>Pb</td>
<td>261</td>
<td>43.37</td>
<td>17.90</td>
<td>31</td>
<td>10.6</td>
<td>40.21</td>
<td>19.71</td>
</tr>
<tr>
<td>Fe</td>
<td>292</td>
<td>16348.17</td>
<td>17.69</td>
<td>3</td>
<td>1.0</td>
<td>76.91</td>
<td>17.69</td>
</tr>
<tr>
<td>Mn</td>
<td>292</td>
<td>308.84</td>
<td>17.69</td>
<td>1</td>
<td>0.3</td>
<td>16348.17</td>
<td>9370.82</td>
</tr>
</tbody>
</table>

only 3 and 1 values are unavailable in the case of the BT and Cu, respectively. After the EM treatment and the replacement of the missing values with the predicted values obtained from the regression, the univariate statistics of the mean and standard deviations show no significant alterations.

3.2. PCA of the heavy metal contents in the oilfield soils

The Kaiser-Meyer-Olkin (KMO) measure and Bartlett’s test of sphericity are two indicators of the strength of the relationships among variables. The KMO measure of sampling adequacy is 0.81 (> 0.70), which shows that a factor analysis of the variables is a good idea [25]. Bartlett’s test shows a chi-squared of 2648.37 with 45 degrees of freedom and a significance level of <0.005, indicating that the relationships among variables are strong and that it is a good idea to conduct a factor analysis for the data after replacing the missing value [25]. After the missing value treatment, the KMO was increased from 0.69 to 0.81, making the principal components readily interpretable.

The treated variables were first transformed into an equal number of principal components. The primary objective was to obtain a small number that would explain most (typically 80% or 90%) of the total variation in the predictor variables [15]. A principal component with an eigenvalue greater than or equal to 1 is usually considered to have statistical significance (the Kaiser criterion) [23]. Then, three principal components were retained, which had eigenvalues greater than unity and explained approximately 80% of the variance or information contained in the original dataset in our earlier study without the missing values treatment [9]. The loadings of the PCn are presented in Table 2. PC1 explains 60.5% of the variance to which almost all variables highly contribute with a loading of greater than 0.5, except Cu, which has a loading of -0.594. PC2 and PC3 explain 11.2% and 10.1% of the variance, including a higher loading of Cd (-0.753) and Pb (0.718), respectively. The eigenvalues of PC4 and PC5 are smaller than 1 to which Cu and Co contribute with a higher loading of 0.539 and 0.534, respectively. According to the multiple regression model in the earlier study [9], Cd, Co, Pb and Cu had a negative influence on biotoxicity, perhaps contributing to their interactions. Thus, in this study, PC2, PC3, PC4 and PC5 were selected to represent the interactions of Cd, Pb, Cu and Co, respectively, and PC1 was considered to be the independent action of the ten heavy metals without interaction. Then, the five principal components were selected for subsequent regression analysis, which explained approximately 90% of the information contained in the original dataset.

3.3. Regression analysis of the biotoxicity and the principal components of the heavy metals in the oilfield soils

The main objective of the last section was to provide the best prediction equation for modelling the biotoxicity with the multiple regression method. Factor scores can be

TABLE 2 - Principal component loadings of the heavy metals

<table>
<thead>
<tr>
<th>Metals</th>
<th>PC1</th>
<th>PC2</th>
<th>PC3</th>
<th>PC4</th>
<th>PC5</th>
<th>PC6</th>
<th>PC7</th>
<th>PC8</th>
<th>PC9</th>
<th>PC10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ni</td>
<td>0.930</td>
<td>0.226</td>
<td>-0.041</td>
<td>0.189</td>
<td>0.005</td>
<td>-0.025</td>
<td>-0.078</td>
<td>-0.118</td>
<td>-0.089</td>
<td>-0.134</td>
</tr>
<tr>
<td>Cr</td>
<td>0.910</td>
<td>0.153</td>
<td>-0.127</td>
<td>0.169</td>
<td>-0.197</td>
<td>0.131</td>
<td>0.057</td>
<td>0.140</td>
<td>-0.140</td>
<td>0.074</td>
</tr>
<tr>
<td>Fe</td>
<td>0.903</td>
<td>0.122</td>
<td>-0.195</td>
<td>-0.057</td>
<td>-0.018</td>
<td>-0.01</td>
<td>0.345</td>
<td>0.086</td>
<td>-0.028</td>
<td>0.028</td>
</tr>
<tr>
<td>Mn</td>
<td>0.841</td>
<td>0.475</td>
<td>0.033</td>
<td>0.005</td>
<td>0.052</td>
<td>-0.086</td>
<td>-0.096</td>
<td>-0.182</td>
<td>0.045</td>
<td>0.106</td>
</tr>
<tr>
<td>Sb</td>
<td>0.817</td>
<td>-0.298</td>
<td>0.156</td>
<td>0.364</td>
<td>-0.136</td>
<td>0.153</td>
<td>0.147</td>
<td>0.06</td>
<td>0.139</td>
<td>0.009</td>
</tr>
<tr>
<td>Zn</td>
<td>0.798</td>
<td>0.159</td>
<td>0.408</td>
<td>-0.107</td>
<td>0.021</td>
<td>-0.343</td>
<td>-0.056</td>
<td>0.191</td>
<td>0.033</td>
<td>-0.02</td>
</tr>
<tr>
<td>Co</td>
<td>0.772</td>
<td>0.126</td>
<td>-0.178</td>
<td>-0.188</td>
<td>0.534</td>
<td>0.171</td>
<td>-0.046</td>
<td>0.066</td>
<td>-0.015</td>
<td>0.006</td>
</tr>
<tr>
<td>Cu</td>
<td>-0.504</td>
<td>0.31</td>
<td>0.425</td>
<td>0.539</td>
<td>0.25</td>
<td>0.083</td>
<td>0.097</td>
<td>0.032</td>
<td>0.014</td>
<td>0.004</td>
</tr>
<tr>
<td>Cd</td>
<td>0.529</td>
<td>-0.753</td>
<td>0.194</td>
<td>0.211</td>
<td>0.082</td>
<td>-0.21</td>
<td>0.075</td>
<td>-0.099</td>
<td>-0.053</td>
<td>0.039</td>
</tr>
<tr>
<td>Pb</td>
<td>0.545</td>
<td>-0.115</td>
<td>0.718</td>
<td>-0.259</td>
<td>-0.121</td>
<td>0.301</td>
<td>0.015</td>
<td>-0.051</td>
<td>0.005</td>
<td>-0.009</td>
</tr>
</tbody>
</table>

Eigenvalues 6.046 1.122 1.013 0.648 0.430 0.338 0.180 0.125 0.061 0.038

% of Variance 60.5 11.2 10.1 6.5 4.3 3.4 1.8 1.2 0.6 0.4

Cumulative % 60.5 71.7 81.8 88.3 92.6 96.0 97.8 99.0 99.6 100

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computed for each respondent, which can then be used in regression analyses [24]. In a subsequent regression analysis, the scores of the five selected principal components were used as the independent variables, and biotoxicity was the dependent variable abbreviated as BT. The following model was obtained.

\[
BT = 25.017 + 6.147PC1 + 3.779PC2 - 1.747PC3 + 2.469PC4 - 2.591PC5
\]  

(2)

The model using the five principal components yielded an R of 0.577 (n=292), and the coefficients of the regressions were all statistically highly significant (P<0.01). For the prediction model, the distributions of the residuals were approximately normal, with zero means and no detectable serial correlation, an indication of adequate model fit. The coefficient of determination increased from 0.513 to 0.577 (n=292), and the principal component regression model was more robust after the missing value treatment.

According to equation (1) and the component score, the model with PCn can be transformed into a new model of the original variables as follows:

\[
BT = 11.623 - 0.140 \times PCn - 2.192 \times CTd + 0.011 \times CTd^2 - 0.012 \times CSb + 0.302 \times CNi + 0.433 \times CNi - 0.577 \times CCd + 0.114 \times CFe - 1.5 \times CTd - 0.021 \times CFe
\]

(3)

Using the same method, model (3) is an equiform formula of that obtained in the earlier study [9], with some slight alterations in the coefficients.

After applying the missing value treatment, the principal components can be interpreted readily. According to the principal component analysis, PC1 was considered to be the independent action of the ten heavy metals, and PC2, PC3, PC4 and PC5 were interpreted as the interactions of Cd, Pb, Cu and Co, respectively. Equation (2) can now be changed as follows:

\[
BT = 25.017 + IT + TCd + TPb + TCu + TCo
\]

(4)

Where IT represents the independent biotoxicity of the heavy metals; and TCd, TPb, TCu and TCo represent the interactions of the biotoxicity of Cd, Pb, Cu and Co, respectively.

In this study, then, the PCn were replaced by new variables according to the interpretations using a stepwise regression analysis. The new variables can embody the interaction of the ten heavy metals. IT can be still be replaced by ten heavy metals using the stepwise regression analysis or the transformed method demonstrated in equation (1). TCd, TPb, TCu and TCo were regressed stepwise as dependent variables and the products of Cn, CPb, CCd and CCo and the ten heavy metals as independent variables, respectively. Then, the new model of the original variables, including the interactions, was obtained as follows:

\[
BT = 25.017 + (0.059 \times CPb - 0.583 \times CPb - 0.026 \times CCo + 0.090 \times CPb + 0.116 \times CPb + 0.012 \times CCo + 0.139 \times CCo + 0.041 \times CCo + 9.706E-5 \times CFe + 0.008 \times CFe + 15.485 + (1.720 - 0.828 \times CCo^2 - 0.183 \times CCo - 4.135E-5 \times CCo - 0.172 \times CCo - 0.031 \times CCo - 0.001 \times CCo - 0.004 \times CCo - 0.004 \times CCo - 0.004 \times CCo - 0.002 \times CCo - 0.002 \times CCo - 0.002 \times CCo - 0.000 \times CCo - 0.001 \times CCo - 0.001 \times CCo - 0.002 \times CCo - 0.002 \times CCo - 0.004 \times CCo - 0.004 \times CCo - 0.006 \times CCo - 0.006 \times CCo - 0.003 \times CCo - 0.005 \times CCo - 0.050 \times CCo)
\]

(5)

All of the coefficients of the five regressions were statistically highly significant (P<0.01). The model summary and ANOVA are shown in Table 3.

The biotoxicity modelled by the equation (5) was plotted against the corresponding observed values, and the results are presented in Fig. 1. The biotoxicity modelled by the equation (5) was plotted against the corresponding observed values shown in the insets, and the solid line indicates the linear fit of 292 points with a R of 0.577 (n=292, P<0.01). The dashed lines indicate the 95% upper prediction limit (UPL) and 95% lower prediction limit (LPL). The points tended to cluster along the 45° tangent line, means the biotoxicity modelled by the equation was the observed one. This means the model can be used to describe the soil biotoxicity of heavy metals, including their interactions.

In the independent biotoxicity of the heavy metals (IT section), all coefficients are positive except Cu, which indicates that most of the metals can have a negative effect on the soil in contrast with the positive effect of Cu. As metalloenzymes and as cofactors of enzymes at catalytic

### TABLE 3 - Regression model summary and ANOVA

<table>
<thead>
<tr>
<th>Model</th>
<th>R</th>
<th>R Square</th>
<th>Adjusted R Square</th>
<th>Std. Error of the Estimate</th>
<th>F</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>IT</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
<td>0.00002</td>
<td>3.087E12</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>TCd</td>
<td>0.766</td>
<td>0.587</td>
<td>0.579</td>
<td>2.45195</td>
<td>72.824</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>TPb</td>
<td>0.932</td>
<td>0.869</td>
<td>0.866</td>
<td>0.63954</td>
<td>281.971</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>TCu</td>
<td>0.891</td>
<td>0.794</td>
<td>0.790</td>
<td>1.13027</td>
<td>197.864</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>TCo</td>
<td>0.948</td>
<td>0.899</td>
<td>0.895</td>
<td>0.84014</td>
<td>247.887</td>
<td>&lt;0.005</td>
</tr>
</tbody>
</table>

a. Dependent Variable: IT (Independent Biotoxicity); Predictors: (Constant), Mn, Cd, Cu, Pb, Co, Sb, Zn, Fe, Cr, Ni
b. Dependent Variable: TCd (Interactions Biototoxicity of Cd); Predictors: (Constant), Cd+Cd, Co+Cd, Fe+Ce, Ni+Cd, Sb+Cd
c. Dependent Variable: TPb (Interactions Biototoxicity of Pb); Predictors: (Constant), Cu+Pb, Zn+Pb, Cr+Pb, Pb+Pb, Cd+Pb, Sb+Pb
d. Dependent Variable: TCu (Interactions Biototoxicity of Cu); Predictors: (Constant), Sb+Cu, Cu+Cu, Pb+Cu, Cd+Cu, Co+Cu
e. Dependent Variable: TCo (Interactions Biototoxicity of Co); Predictors: (Constant), Co+Co, Cr+Co, Cu+Co, Pb+Co, Zn+Co, Ni+Co, Sb+Co, Cd+Co, Mn+Co
amounts, Cu and some trace metals are essential to living organisms for their normal physiological activities, but accumulation in higher concentrations becomes toxic to most life forms [26, 27]. In this study, the Cu acts as a micronutrient at small concentrations in the soil for the activities of luminescent marine bacteria (*Photobacterium phosphoreum*), and the other metals have a toxic effect on the bacteria.

In the sections of $T_{\text{Cd}}$, $T_{\text{Pb}}$, $T_{\text{Cu}}$, and $T_{\text{Co}}$, the sign of the coefficients describe the interactions between the heavy metals. The positive coefficients indicate the synergistic effect, while the negative coefficients indicate the antagonistic effect. The combined effects of the metals were found to be antagonistic for Co-Zn, Co-Cu, Co-Cd, Cu-Pb, Cd-Sb, Pb-Zn, and Pb-Cd, and synergistic for Co-Ni, Co-Cr, Co-Sb, Co-Pb, Cu-Sb, Cu-Cd, Cd-Ni, Cd-Fe, Pb-Cr and Pb-Sb, revealing a complex pattern of possible interactions. There is a complex pattern of possible interactions between the heavy metals, and other studies have shown that heavy metals can affect biotoxicity by the interactions reported as antagonism, synergism and additivity [10-12].

Furthermore, the biotoxicity of soils to aquatic organisms could also be related to the soil properties (pH, Org C and Fe$_{ox}$) and to the reactivity of potentially toxic elements in soils [28], therefore, more research is required to reveal the interactions of heavy metals to the biotoxicity in the actual soil environment.

### 4 CONCLUSIONS

The following conclusions may be drawn based on the MPCR analysis of the toxicity test data presented in this paper:

1. The MPCR can be used for modelling the interactions between heavy metals in the biotoxicity analysis of oilfield soil.
2. According to the MPCR model, the Cu acts as a micronutrient at small concentrations in soil for the activities of luminescent marine bacteria (*Photobacterium phosphoreum*), and the other nine metals have a toxic effect on the bacteria.
3. The potential interactions of Co with Zn, Cu, Cd, and Cu with Pb, and Cd with Sb, and Pb with Zn, Cd can be described as antagonistic, while the interactions of Co with Ni, Cr, Sb, Pb, and Cu with Sb, Cd, and Cd with Ni, Fe, and Pb with Cr, Sb can be described as synergistic.

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### REFERENCES


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DEGRADATION OF ATRAZINE IN WATER BY GAMMA-RAY IRRADIATION

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ABSTRACT
Atrazine degradation values and the degradation process by gamma-ray irradiation were investigated. The proposed degradation mechanism and change of acute toxicity were further examined. Atrazine concentration decreased with increase of irradiation dose, and 99% removal of atrazine at the initial concentration of 0.04 mmol L\(^{-1}\) was achieved when 0.8 kGy was selected as the irradiation dose. The process could be depicted by first order reaction kinetics. The contribution to atrazine degradation by the radicals was in the order of \(\cdot\text{OH} > \text{eaq}^- > \cdot\text{H}\), and the degradation process was mainly caused by the reaction of atrazine with \(\cdot\text{OH}\). The quantum efficiency ratio of \(\cdot\text{OH}, \text{eaq}^-\) and \(\cdot\text{H}\) was calculated to be 5:1:1. The removal efficiency decreased with increase of atrazine initial concentration at the same irradiation dose. \(\text{H}_2\text{O}_2, \text{HCO}_3^-\), \(\text{NO}_3^-\), \(\text{NO}_2^-\) and humic acid as additives reduced the degradation value. Furthermore, the increase of \(\text{H}_2\text{O}_2, \text{NO}_3^-\) and \(\text{NO}_2^-\) would result in the decrease of removal efficiencies. The concentration of \(\text{Cl}^-\) increased with increase of irradiation dose, and loss of chlorine atoms was realized on atrazine. A remarkable reduction of atrazine was observed by gamma-ray irradiation, but brought to a toxicity increase, and more toxic substances were produced.

KEYWORDS: Degradation; atrazine; gamma-ray irradiation; mechanism; toxicity

1 INTRODUCTION
Atrazine (2-chloro-4-(ethylamino)-6-isopropylamino-s-triazine) has become the most widely used herbicide in agricultural and forestry applications since it was introduced in the 1950s [1]. Atrazine shows a relatively high persistence in soils and aquatic system; as a result, it is the most commonly detected herbicide in surface and ground water [2]. Atrazine is one of the most common pollutants in US ground and surface waters, as well as the most common pesticide pollutant of UK ground waters [3, 4]. US EPA has set the drinking water limit at 3 µg L\(^{-1}\).

* Corresponding author
MS, and the effects of additives on the degradation process. Furthermore, the changes of acute toxicity were tested after gamma-ray irradiation.

2 MATERIALS AND METHODS

2.1. Materials

Atrazine (97.4% purity) was gained from Sigma-Aldrich and methanol used in the analysis was of HPLC-grade. The bacterium *Photobacterium phosphoreum* T3 (*P. phosphoreum*) was provided as freeze-dried powder (0.5 g each bottle) by the Institute of Soil Science, Chinese Academy Sciences, Nanjing, P.R. China. The other reagents were all of analytical grade.

2.2. Sample preparation

Atrazine solutions at different initial concentrations were prepared, and different additives of different concentrations were added into 0.04 mM atrazine solution to test their effects on the degradation process. In order to clarify which radical played the key role in atrazine degradation, 2-propanol and tert-butanol as radical scavengers were added into atrazine solution at the initial concentration of 0.04 mM, and nitrogen in high purity was bubbled into these samples for 2 min to ensure that the solutions were saturated with it. The initial concentrations of both scavengers were 60 mM. The samples (25 ml each) were placed in 50-ml air-tight glass vessels.

2.3. Irradiation process

Gamma-ray was obtained from 60Co source, and the samples were placed in radiation field to a specific distance from the source with the dose rate of 27 Gy min⁻¹. Two controls were tested. The doses that samples absorbed were determined with a silver dichromate dosimeter [18].

2.4. Analytical methods

The concentration of atrazine was measured using a HPLC system (Agilent, USA, 1200 Series) equipped with Hypersil ODS HPLC column (250 mm×4.6 mm i.d., 5 µm, Agilent, USA). The measurement was performed in a methanol/water (60: 40, v/v) phase at a flow-rate of 1.0 ml min⁻¹. The determination wavelength for atrazine was set at 220 nm, and the column temperature was kept at 30 °C. Formation of Cl⁻ ions was followed by LC-IC (ICS-2000, anions column ASII-HC, 250 mm long). The eluent for Cl⁻ ions was KOH (30 mM), and the flow-rate was 1.0 ml min⁻¹. The degradation value for each sample was calculated from the following Equation (1):

$$\eta = \frac{C_0 - C_D}{C_0} \times 100\%$$

where, $\eta$ was the degradation efficiency of atrazine (%); $C_D$ was the residual concentration of atrazine after gamma-ray irradiation (mM); $C_0$ was the initial concentration of atrazine before gamma-ray irradiation (mM).

The identification of atrazine and its resulting by-products was first performed by LC-MS (Thermo Quest LCQ Duo, USA) with a Hypersil ODS HPLC column (250 mm×4.6 mm i.d., 5 µm, Agilent, USA). A solution aliquot (10 µl), after gamma-ray irradiation, was injected automatically into the LC-MS system. The flow-rate was 0.2 ml min⁻¹. The other LC conditions were the same as used in determining atrazine concentration. MS conditions were as follows: the atmospheric pressure chemical ionization interface was selected. The capillary temperature was set to 150 °C with a voltage of 10.00. The spectra were gained in the positive scan mode, over the m/z range 50-600.

2.5. Analysis of acute toxicity

Samples with the initial concentration of 0.04 mM before and after gamma-ray irradiation were selected to do this test. The bacterium was pre-reactivated in 1 ml 2.5% NaCl solution and kept in the ice-water bath. 0.2 ml of each treated sample and 10 µl reactivated bacterium was added to 2 ml 3% NaCl solution. The bioluminescence, indicating a toxic effect by atrazine, was measured by a toxicity analyzer (model DXY-2, Institute of Soil Science, Chinese Academy of Sciences, Nanjing, P.R. China) after a 15-min exposure at 15±1 °C. Luminescence inhibition percentage was used to express the effect degree of atrazine on *P. phosphoreum*.

3 RESULTS AND DISCUSSION

3.1. Analysis of atrazine degradation process by gamma-ray Irradiation

Figure 1 illustrates change of $\eta$ versus irradiation dose after gamma-ray irradiation. The results showed that atrazine concentration decreased with increase of irradiation dose, and the degradation rates decreased with the increasing of initial concentration; 99% removal of atrazine at the initial concentration of 0.04 mM was realized when 0.8 kGy was selected as the irradiation dose. By plotting the natural logarithm of $C_0/C_D$ as a function of

![FIGURE 1 - $\eta$ at different initial concentrations as a function of gamma-ray irradiation dose.](image)
irradiation dose, a linear relationship could be derived (not shown here). This result indicated that degradation of atrazine by γ-irradiation followed a pseudo first-order with respect to irradiation dose, and could be described by Equation (2) [19]:

$$\ln \left( \frac{C_0}{C_D} \right) = k D$$  \hspace{1cm} (2)

where, $k$ was the degradation rate constant (kGy$^{-1}$) and $D$ was the irradiation dose (kGy). Through linear regression analysis, the corresponding parameters are listed in Table 1.

**TABLE 1 - The parameters of atrazine degradation through linear regression analysis at different initial concentrations by gamma-ray irradiation.**

<table>
<thead>
<tr>
<th>Initial concentration (mM)</th>
<th>$C_0^*$ (mM)</th>
<th>$k$ (kGy$^{-1}$)</th>
<th>$R$</th>
</tr>
</thead>
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<td>3.144±0.3585</td>
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</table>

$C_0^*$ denoted the initial concentrations predicted.

When the initial concentration of atrazine was 0.02 mM, the degradation rate constant was 5.3422 kGy$^{-1}$. However, when the initial concentrations were 0.04 and 0.06 mM, the degradation rate constants were 4.6960 and 3.1446 kGy$^{-1}$, respectively. This indicated that initial concentration greatly affected the degradation of atrazine by gamma-ray irradiation.

During gamma-ray irradiation, water radiolysis happened within a very short time. Three reactive species, such as $\cdot$H, $e_{aq}^-$, and OH, as well as the less reactive species H$_2$O$^-$, which could be seen in Equation (3) (the numbers in the brackets present the amount of the produced radicals (100 eV energy)$^{-1}$) [20], were produced.

$$\text{H}_2\text{O} \rightarrow e_{aq}^- (2.6) + \cdot \text{H} (0.55) + \cdot \text{OH} (2.7) + \text{H}_2 (0.45) + \text{H}_2\text{O}_2 (0.71) + \text{H}_3\text{O}^+ (2.6)$$ \hspace{1cm} (3)

As shown in Equation (3), $e_{aq}^-$, H and OH were the most active species responsible for atrazine degradation. To further explore the role of $e_{aq}^-$, H and OH and their reactivities, two radical scavengers, 2-propanol and tert-butanol, were added prior to γ-irradiation.

H and OH could be scavenged by 2-propanol: OH + 2-propanol $\rightarrow$ (CH$_3$)$_2$COH+H$_2$O (rate constant was 1.9×10$^6$ M$^{-1}$S$^{-1}$) and H + 2-propanol $\rightarrow$ (CH$_3$)$_2$CO+H$_2$ (rate constant was 7.4×10$^5$ M$^{-1}$S$^{-1}$). However, tert-butanol could only scavenge OH by the reaction: OH+ tert-butanol $\rightarrow$ ·CH$_2$C(CH$_3$)$_2$OHxH$_2$O (rate constant was 6.0×10$^5$ M$^{-1}$S$^{-1}$) [21]. Since (CH$_3$)$_2$COH and CH$_2$C(CH$_3$)OH were inert radicals, $e_{aq}^-$ played the predominant roles when tert-butanol was added, and $e_{aq}^-$ played the crucial role when 2-propanol was added [22].

Figure 2 shows the change of ln ($C_0/C_D$) versus irradiation dose in the presence and absence of 2-propanol and tert-butanol. The results demonstrated that atrazine concentration decreased with increase of irradiation dose. At the same irradiation dose, the degradation value of the sample when 2-propanol and tert-butanol were not added was higher than that when 2-propanol and tert-butanol were added. This indicated that the degradation process was inhibited when 2-propanol and tert-butanol were added. The values of $k$ in the three cases could be calculated through the linear regression analysis and they were $k_{2\text{-propanol}} = 0.2368\pm 0.0065$ kGy$^{-1}$, $k_{\text{tert-butanol}} = 0.2611\pm 0.0194$ kGy$^{-1}$, and $k_{\text{blank}} = 5.6824\pm 0.1998$ kGy$^{-1}$, respectively. Thus, the apparent degradation rate constant ratios of OH, $e_{aq}^-$ and H could be calculated as follows:

$$k_{\text{OH}} : k_{e_{aq}^-} : k_H = (k_{\text{Blank}} - k_{\text{tert-butanol}}) : k_{2\text{-propanol}} : (k_{\text{tert-butanol}} - k_{\text{2-propanol}}) = 5.4213 : 0.2368 : 0.0243 = 223 : 10 : 1$$

The result showed that OH played the most important role in atrazine degradation process, and almost all atrazine was removed by OH, and $e_{aq}^-$ and H just played a minor role. Consequently, the quantum efficiency ratios of OH, $e_{aq}^-$ and H for the degradation of atrazine could be calculated as follows:

$$\beta_{\text{OH}} : \beta_{e_{aq}^-} : \beta_H = \frac{\Delta C_{\text{OH}}}{n_{\text{OH}}} : \frac{\Delta C_{e_{aq}^-}}{n_{e_{aq}^-}} : \frac{\Delta C_H}{n_H}$$

$$= \frac{\Delta C_{\text{OH}}}{G_{\text{OH}}} \cdot \frac{\Delta C_{e_{aq}^-}}{G_{e_{aq}^-}} \cdot \frac{\Delta C_H}{G_H}$$

$$= \frac{n_{\text{Blank}} - n_{\text{tert-butanol}}}{G_{\text{OH}}} \cdot \frac{n_{2\text{-propanol}}}{G_{e_{aq}^-}} \cdot \frac{n_{\text{tert-butanol}} - n_{2\text{-propanol}}}{G_H}$$

$$= 99 \cdot 20.78 \cdot 17.69 \div 2.7 \cdot 0.6 = 5 : 1 : 1$$

where, $\beta$ was the quantum efficiency; $\Delta c$ was the reduction of atrazine concentration after 0.8-kGy irradiation; $V$ was the volume of the solutions, and $n$ was the amount of the active species generated during the irradiation.
3.2. Effects of humic acid, HCO$_3^-$, H$_2$O$_2$, NO$_3^-$ and NO$_2^-$ on atrazine degradation by gamma-ray irradiation

Figure 3 shows the effects of humic acid and 0.24 mM HCO$_3^-$ on atrazine degradation by gamma-ray irradiation. The results showed that the degradation value in the presence or absence of humic acid and HCO$_3^-$ was improved with increasing irradiation dose. As shown in Fig. 3, at the same irradiation dose, the removal value was lower in the presence of humic acid and HCO$_3^-$ than that in the absence of them. When 10 mg L$^{-1}$ humic acid and 0.24 mM HCO$_3^-$ were added, the degradation rate constants were 4.0340 and 4.0877 kGy$^{-1}$, respectively. The results indicated that atrazine degradation process was inhibited when humic acid and HCO$_3^-$ were added. But the effects were not very apparent. As we knew, •OH played the most important role, and almost all atrazine was removed by it, based on the analysis of atrazine degradation process. Humic acid and HCO$_3^-$ affected the removal process through the reactions between OH with them. It might be the reason that OH could be scavenged by humic acid. HCO$_3^-$ could also react with OH with the rate constant of 8.5×10$^6$ M$^{-1}$·s$^{-1}$ [21].

\[
\text{OH} + \text{H}_2\text{O}_2 \rightarrow \text{HO}_2^+ + \text{H}_2\text{O}
\]

FIGURE 3 - Effects of humic acid and HCO$_3^-$ on atrazine degradation by gamma-ray irradiation.

H$_2$O$_2$ as an effective oxidant was widely used in the field of water treatment, and NO$_3^-$ and NO$_2^-$ were the most common anions in nature water-bodies. In order to test their effects on atrazine degradation process, the experiments were conducted. Figure 4 shows the effects of H$_2$O$_2$, NO$_3^-$ and NO$_2^-$ on atrazine degradation by gamma-ray irradiation. The removal value was lower in the presence of H$_2$O$_2$, NO$_3^-$ and NO$_2^-$ than that in the absence of them, at the same irradiation dose. Furthermore, the increase of these additives would result in the decrease of the degradation efficiency. It might be the reason that the amount of active species that reacted with atrazine was affected when H$_2$O$_2$, NO$_3^-$ and NO$_2^-$ were added. The hydroxyl radicals generated in water radiolysis process reacted with NO$_2^-$, and a local excess of H$_2$O$_2$, produced hydroperoxyl radicals (HO$_2^*$):

\[
\text{OH} + \text{H}_2\text{O}_2 \rightarrow \text{HO}_2^+ + \text{H}_2\text{O}
\]

and $e_{aq}^-$ was quickly cleared by NO$_3^-$ and NO$_2^-$ [23, 24].

FIGURE 4 - Effects of H$_2$O$_2$, NO$_3^-$ and NO$_2^-$ on atrazine degradation by gamma-ray irradiation.
3.3. Degradation mechanism of atrazine by gamma-ray irradiation

Figure 5 shows the HPLC chromatograms of atrazine solution before and after gamma-ray irradiation. It could be seen from Fig. 5 that the retention time of all degradation products was shorter than that of atrazine. It indicated that all products are more polar than the parent compound. At the same time, Cl⁻ ions concentration of atrazine solution after gamma-ray irradiation was tested, and the removal value of chlorine is shown in Fig. 6.

The removal values of atrazine and chlorine after gamma-ray irradiation (Fig. 6) evidenced that the removal value of chlorine was becoming higher with increase of irradiation dose. The concentration of Cl⁻ ions was 0.73 mg L⁻¹ when 0.8 kGy was selected as the irradiation dose, and only a loss of 52% chlorine atoms was realized on atrazine. So, not more than 52% of atrazine was removed by loss of chlorine atoms.

In the present work, the mechanism of atrazine degradation by gamma-ray irradiation was of particular care in the identification of resulting by-products by LC-MS. After LC-MS analysis, mass spectra of atrazine and degradation products are shown in Table 2.

According to the results of LC-MS analysis, several main degradation products were found to be the dealkylation product (R1: 2-chloro-4-(ethylamino)-6-amino-s-triazine) and alkylic-oxidation product (R3: 2-chloro-4-acetamido-6-amino-s-triazine) underlining that the dealkylation and alkylic-oxidation of atrazine were two proposed mechanisms for atrazine degradation. An attack of the hydroxyl group on N-adjacent carbon atom might result in an alkylic-oxidation (alkylamino lateral chain oxidation) process which is proven by identification of R2 (2-chloro-4-acetamido-6-(isopropylamino)-s-triazine) [25]. The formation of R4 (2-hydroxy-4-(isopropylamino)-6-(ethylamino)-s-triazine) could result either from homolytic cleavage of the C-Cl bond, followed by electron transfer from the carbon to the chlorine radicals processed by the carbocation reaction with water, or the heterolytic cleavage of the excited state atrazine molecule which is also favoured by water [26].

Based on the identification of intermediate products and the above analysis, the corresponding degradation pathway of atrazine by gamma-ray irradiation was proposed in Fig. 7.
3.4. Change of acute toxicity of atrazine solution after gamma-ray irradiation

While our results demonstrated that gamma-ray irradiation degradation of atrazine is rapid, it is critical to establish biological activity of the resulting treated solution or the individual breakdown products. In general, gamma-ray radiation leads to a complex mixture of products in low overall yields. It is a daunting task to assess all products individual biological activities. We chose to use the photo-bacterium assay to assess the biological activity of the gamma-treated solutions at various doses, and results are shown in Fig. 8.

It could be concluded that acute toxicity of atrazine solution increased after gamma-ray irradiation. Before gamma-ray irradiation, luminescence inhibition percentage of atrazine solution was only 20.2%. But after 0.05-kGy irradiation, luminescence inhibition percentage was high reaching 52.9%. In the period of 0.05 kGy- 0.2 kGy, luminescence inhibition percentage was becoming lower with increase of irradiation dose, and acute toxicity became lower. When 0.2 kGy was selected as irradiation dose, luminescence inhibition percentage was reduced to 27.6%. In the period of 0.2 kGy- 0.8 kGy, change of acute toxicity was not remarkable and luminescence inhibition percentage was 34.6% when 0.8 kGy of radiation dose was selected. The results showed that atrazine solution became more toxic after gamma-ray irradiation than before, and more toxic substances were produced.

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HEAVY METALS CONCENTRATION AND METALLOTHIONEIN CONTENT IN RESIDENT AND CAGED MUSSELS *Mytilus galloprovincialis* FROM RIJEKA BAY, CROATIA

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ABSTRACT

Concentration of lead, cadmium, mercury, arsenic, copper, zinc, chromium, nickel, manganese and iron and metallothionein content were examined in resident and caged mussels (*Mytilus galloprovincialis*) at five sites along Rijeka Bay, a coastal ecosystem highly susceptible to urban and industrial pollution. Higher concentration of metals was found in resident mussels, with the highest level in comparison to control site Lim detected for lead, copper and chromium. Caged mussels from all sites displayed increasing level of metals with respect to control site Lim after one month of exposure. Significantly elevated metallothionein content was found in resident mussels at all sites except Kraljevica shipyard, and in caged mussels at sites Luka Rijeka harbour and urban area Bakar. PCA analysis revealed that mussels resident to urban area of river Rječina mouth, Rijeka harbour and Treći Maj shipyard were clearly separated from Kraljevica shipyard due to different pattern of metal bioaccumulation and metallothionein content. Resident mussels from urban area of Bakar and caged mussels were less influenced by metal load.

KEYWORDS: Mussels, *Mytilus galloprovincialis*, Metal exposure, Metallothionein content, PCA analysis.

1 INTRODUCTION

To evaluate the risk for integrity of marine ecosystem, with respect to increasing trends in urbanization and industrialization along the coastal zones, the marine monitoring programs have been widely implemented since the late 70-ties [1]. The health status of coastal areas is assessed by chemical analysis of biota, water and sediment and by monitoring of biological effect of contaminants [2].

Among them, metals are considered as potentially highly toxic and capable to induce a whole range of deleterious processes ultimately decreasing the quality of marine ecosystem.

Bivalve mussels *Mytilus galloprovincialis* are bioindicator organisms frequently used for monitoring the quality of coastal waters [3] and are commonly employed for assessment of heavy metal pollution [4-8]. Exposure of a variety of marine organisms, including mussels, mainly to cadmium, copper and zinc, is associated with induction of metallothioneins, low molecular weight, cysteine rich cytosolic polypeptides involved in homeostasis of essential and detoxification of non-essential metals [9-11]. Metallothionein content determination is employed for assessment of adverse biological effects of metals pollution [12-15].

In this study, heavy metal concentration and metallothionein content were determined in resident and caged mussels from Rijeka Bay, to evaluate the extent of metal pollution and to provide the baseline data for future monitoring of the anthropogenic impact in this economically important region of the north-eastern Adriatic. Rijeka Bay is surrounded by many land-based sources of contamination spread along the coastline related to heavy industries and urban wastewater. Consequently, a wide range of metals are released directly into the sea, by flows through the coastal waters and rivers, or from distant sources by atmospheric transport as evidenced by recent report on sediment metal concentration [16, 17].

2 MATERIALS AND METHODS

Five sampling sites along Rijeka Bay selected for the study were located in the proximity to discharge points of urban sewage and industrial outlets and within the zone of intensive marine traffic (Figure 1 and Table 1).

The study was carried out in April 2006, in the period of lowest influence of temperature and reproductive processes on the metal accumulation and metallothionein induction [18-19]. Mediterranean mussels (*Mytilus galloprovincialis* Lam.) of shell length 5-6 cm, and weight 15 – 20 g, were purchased from aquaculture farm within the
inner part of Lim Bay (site Lim) on the north eastern part of Adriatic Sea. In accordance with previous biomonitoring studies [13], site Lim was considered as control site, since it is far from urban and industrial influence. Mussels were transported in wet and cool plastic containers to selected sites within two hours from collection. Sets of randomly chosen animals were placed in synthetic mesh and immersed close to the mussels resident to sampling site. After one month of exposure, caged mussels as well as mussels resident to investigated sites, were simultaneously collected, transported to the laboratory as described above, and immediately processed. Water temperature, pH, salinity were determined using Mettler Toledo - SevenMulti® meter, whereas dissolved oxygen (DO) was measured using Winkler method (Table 1).

Following collection, the soft tissue of thirty mussels was dissected with a clean scalpel blade, pooled, freeze-dried and homogenized according to AOAC Official Method 937.07 [20]. Aliquots (1g) of mussels’ tissue sample were added with 5 ml of 65 % HNO₃ and subsequently digested in a microwave oven (MLS 1200 MEGA, “exhaust module” EM-45/A, Milestone, Italy) according to standardized procedure (HRN EN 14084). The digested samples were then diluted 5 times (v/v) with Mili-Q water. Lead, cadmium, arsenic, copper, chromium, nickel, manganese and iron were analysed by atomic absorption spectrophotometry (Perkin Elmer, Analyst 600) in a graphite oven with autosampler (Perkin Elmer AS 800). Zinc was analyzed using air acetylene flame atomic absorption spectrophotometer (Perkin Elmer, AS 200) and mercury was analyzed with cold-vapour flow injection Mercury System (Perkin Elmer, FIAS 400) supplemented with Autosampler (Perkin Elmer, AS 90). Metallothionein content was determined using spectrophotometric method based on the estimation of the sulfhydryl residue content [21]. Five pools were prepared from three digestive glands (fifteen mussels in total per site) and homogenized in three volumes of ice-cold 20 mM Tris–HCl buffer, pH 8.6, containing 0.5 M sucrose, 0.006 mM leupeptin, 0.5 mM PMSF and 0.01% β-mercaptoethanol using Teflon Potter homogenizer. The homogenate was centrifuged at 15000g for 30 min (+4°C) and the pellet discarded. Aliquots of 1 ml of supernatant were added with 1.05 ml of cold ethanol (−20°C) and the pellet discarded. Aliquots of 1 ml of supernatant were added with 1.05 ml of cold ethanol (−20°C) and 80 µl chloroform, and centrifuged at 6000g for 10 min. Metallothioneins present in the supernatant were collected by precipitation with three volumes of cold ethanol (−20°C), kept at −20°C for 1 h and centrifuged at 6000g for 10 min. The pellets were then dissolved with 87% ethanol and 1% chloroform in 0.5 M sucrose homogenizing buffer and
dried. The metallothionein content was evaluated by colorimetric method using Ellman’s reagent (5,5′-dithiobis 2-nitrobenzoic acid, DTNB) [22]. Pellets were re-suspended in 300 µl of solution containing 5 mM Tris–HCl buffer with 1 mM EDTA at room temperature. Aliquots of 4.2 ml containing 0.2 M Na-phosphate buffer pH 8.0 and 0.43 mM DTNB were added to the suspension of pellets. Following 30 minutes of incubation and final centrifugation of samples at 6000g for 10 min, the absorbance of the supernatant was measured in triplicates at 412 nm. Metallothionein content was calculated using reduced glutathione (GSH) as reference standard [21] and expressed as micrograms of metallothionein per gram of wet weight tissue.

Statistical differences in metallothionein content between sampling sites were established by Wilcoxon test. Mean values of the metallothionein content and chemical data were used for the correlation analysis using Spearman’s test. A value of p<0.05 was considered significant. Principal component analysis (PCA) was applied using data on metal concentrations and metallothionein content in the tissues of resident and caged mussels from all sites, and control site of origin. The data used for PCA analysis were logarithmically transformed.

3 RESULTS AND DISCUSSION

Concentrations of ten metals (lead, cadmium, mercury, arsenic, copper, zinc, chromium, nickel, manganese and iron) in resident and caged mussels from all sites and from control site Lim are given in Figure 2. Generally, both resident and caged mussels displayed elevated concentration of metals with respect to mussels from control site Lim. Differences in the accumulation pattern of metals were observed between resident and caged mussels.

Lead, copper and chromium generally displayed the most notable increase both in resident and caged mussels in comparison to control site Lim.

The concentrations of lead found in the tissues of resident mussels were near or above 6 mg/kg d.w. with exception of urban site UR. As for the caged mussels, the highest concentration was found in LR harbour (4.3 mg/kg d.w.). Levels of lead above 5 mg/kg d.w. were recently found within Rijeka Bay by other authors [23, 24] indicating an ongoing trend in the input of this metal possibly due to nearby intensive anthropogenic activities. Contamination of sediment with lead recently found within the zone investigated in this study was also strongly related to anthropogenic input, mainly in relation to port activities and oil refinery [16-17]. In general, lead concentrations reported in this study fall within the range of values previously found in polluted hot spots along the eastern Adriatic coast [5-6,24] and some Mediterranean areas [15]. However, they were lower than those recently reported for sites under urban and industrial impact along the south eastern Adriatic coastal zone [25].

Resident mussels displayed concentration of copper between 1.3 and 8.2 mg/kg d.w. with exception of KR shipyard where notably higher level (22 mg/kg d.w.) was detected. The observed accumulation of copper could be associated with the influx of copper-based antifouling paints. Similarly high copper concentrations were previously observed along the eastern Adriatic coast in the vicinity of harbours and urbanised areas [5-6] although in highly polluted environment even higher levels were occasionally found [6,15]. With exception of KR shipyard, the values reported herein for copper concentrations in the tissues of mussels are in the range of those recorded in the south eastern Adriatic coast and the Mediterranean [15, 25, 26] within coastal zones characterised by different level of anthropogenic impact.

Chromium concentration in resident and caged mussels ranged from 0.59 to 1.61 mg/kg d.w. and from 0.41 to 0.78 mg/kg d.w., respectively. The lowest level was found at urban site BK, and highest level at urban site UR, for both populations of mussels. Generally higher level of chromium in comparison to control site Lim was found particularly in the tissues of resident mussels. Nevertheless, it should be taken into account that the concentrations found at the investigated sites did not exceed the range of values determined along the eastern Adriatic coast irrespective on the pollution impact [5, 6, 27].

The concentrations of mercury, iron, cadmium, zinc and nickel in the tissues of both resident and caged mussels at all sites generally displayed lower degree of accumulation in comparison to control site Lim.

Values for mercury concentration varied between 0.12 and 0.79 mg/kg d.w. Values close to or above 0.25 mg/kg d.w. that were detected in resident mussels from LR harbour, TM and KR shipyards and caged mussels from LR harbour and KR shipyard corresponded to those recently detected in the close vicinity to a former chlor-alkali plant [28]. Besides, mercury concentration reported for mussels resident to KR shipyard exceeded those previously reported for highly polluted harbours of Barcelona and Genoa [15] and for the region within Spanish coast known for mercury exploitation and production [4]. Probable contamination of mussels from LR harbour and TM shipyard is in accordance with particularly high mercury enrichment of sediments found at these locations [16-17]. However, even higher concentrations of mercury in the tissues of mussels resident to polluted marine environment were detected in the south eastern part of the Adriatic coast [25, 29].

The concentrations of cadmium ranged from 0.63 to 1.75 mg/kg d.w. Values above 1.2 mg/kg d.w. that were found in the tissues of resident mussels at almost all locations could be attributed to cadmium pollution [6]. Furthermore, nearly as high level of cadmium was found in the tissues of caged mussels from TM and KR shipyard. In agreement with our results, concentration of cadmium above 1.5 mg/kg d.w. were recently found at site LR.
harbour and TM shipyard [23] suggesting relatively steady input of this metal over the last few years. The cadmium concentrations reported herein were lower than those detected in mussels from south eastern Adriatic coast [29]. On the other hand, values for cadmium were above those recorded in Barcelona harbour [15] and some sites within urbanized and industrialized areas of Spanish coast [4].
Zinc concentrations were in the range between 117.1 and 270.6 mg/kg d.w. with maximum values found in resident mussels from sites TM shipyard and LR harbour. These values were slightly above the typical level established for this metal in other monitoring studies of eastern Adriatic coast [5,6,27] and Mediterranean coastal regions [30].

The range of iron concentrations reported in this study (116.15-418.8 mg/kg d.w.) corresponds to those detected along the eastern Adriatic coast [27]. In particular, the maximum values that were found in the tissues of resident mussels from urban site UR and caged mussels from LR harbour were also previously observed at sites in the vicinity to heavy industries and discharge of untreated wastewater [25,27].

Nickel concentrations varied from 0.47 to 2.78 mg/kg d.w. with the highest values found in resident mussels from LR harbour, urban area UR, TM shipyard and caged mussels from KR shipyard (above 2 mg/kg d.w.). These values were either in agreement [27] or far below those recorded at sites highly influenced by anthropogenic activity along the eastern Adriatic coast [25,29].

Finally, the lowest degree of accumulation with respect to control site Lim was found for arsenic and manganese.

With exception of mussels caged at LR harbour, the concentration of arsenic was above 24 mg/kg d.w. both in resident and caged populations. In addition, arsenic level in the tissues of control mussels from Lim was nearly 27 mg/kg d.w. These values were relatively high when compared to the data reported for unpolluted to highly polluted locations of the eastern Adriatic coast [24,29,31].

Manganese concentrations ranged between 4.4 and 11.2 mg/kg d.w. with the highest values (above 8 mg/kg d.w.) found in resident mussels at sites LR harbour and TM shipyard. However, these maximums couldn’t be indicative of manganese pollution considering wide range of values for this metal reported previously for both unpolluted and highly polluted sites along eastern Adriatic coast [6,29].

Resident mussels exhibited lower concentrations of metals than mussels caged at the same site for copper at urban area UR, LR harbour and TM shipyard, cadmium at TM shipyard, arsenic at KR shipyard, nickel at urban site BK and KR shipyard, manganese at urban site UR and iron at LR harbour (Figure 2). This result could indicate adaptation of resident mussels to long-term exposure to above metals at those sites [32,33]. However, more detailed research of temporal trends in metal accumulation is needed to support this hypothesis.

A strong and significant (p<0.05) positive correlation between Hg and Cu (R = 0.83), lead and copper (R = 0.94) and zinc and manganese (R = 0.83) was revealed from correlation matrix of metals in the tissues of the resident mussels (Table 2). Caged mussels displayed significant (p<0.05) correlations between lead and copper (R = 0.94) and mercury and zinc (R = 0.83) and iron and chromium (R = 0.89). Significant positive correlation between copper and lead and mercury and zinc was also reported previously [6] suggesting similar accumulation and elimination process for these metals.

Metallothionein content in the digestive gland of resident and caged mussels is presented in Table 3. In general, the metallothionein content of both resident and caged mussels was higher with respect to control site. Significant increase was detected in the tissues of resident mussels from all sites except in KR shipyard and caged mussels from urban area BK and LR harbour. The increased metallothionein content in the digestive gland of mussels indicates the biological response to metal exposure. In agreement, elevated metallothionein content in relation to metal exposure was reported in other biomonitoring studies [34-36]. Positive correlation between metallothionein content in the tissues of resident mussels and metal concentration was found for zinc (R = 0.83, p<0.05) (Table 2). As for the caged mussels, significant correlation was detected only for copper (R = 0.89; p<0.05). The induction of metallothionein with increasing zinc and copper concentrations in the tissues of mussels was found previously [37-38] in line with the role of this protein in the metabolism as well as detoxification of the excess amount of these essential metals. Elevated metallothionein content could be also attributed to cadmium accumulation in the tissues of mussels. Cadmium is a toxic non-essential metal most often associated with metallothionein induction in mussels [7,18]. However, the clear relationship of each metal and induction of metallothionein is difficult to establish since complex interactions between different metals and other pollutants normally occur in the realistic environmental conditions [39-41]. In addition, the specificities of metal sequestration mechanisms, scavenging of metallothionein-metal complexes and metallothionein turnover are still unknown for most toxic metals [41].

Metallothioneins could be also induced under oxidative stress conditions generated by pro-oxidant activity of organic compounds [42]. As for common marine contaminants such as PAHs, higher concentrations were detected at the sites investigated in the current study (personal communication). However, the clear relationship of PAH’s and metallothionein content in the tissues of mussels remains to be clarified. In fact, benzo(a)pyrene (BaP), one of the most toxic of the class of PAHs, failed to induce metallothionein synthesis in mussels M. galloprovincialis [43] and clams Ruditapes philippinarum [44]. However, in the latter study it was implied that BaP metabolites increase the toxicity of metals since the induction of metallothioneins was less pronounced upon exposure to mixture of cadmium and BaP, in comparison to cadmium exposure only.

It is well known that seasonal fluctuations of metal concentrations in the tissues of mussels related to abiotic and biotic factors could have significant influence on metallothionein induction in the tissues of bivalves [18,19,23,36,42]. This study was conducted in spring, to avoid the influence of winter and summer temperature extremes typical for Northern Adriatic, on metallothioneins induc-
TABLE 2 - Spearman correlation coefficients between MT content and Pb, Cd, Hg, As, Cu, Zn, Cr, Ni, Mn and Fe concentrations in resident and caged mussels. Correlations significant at p<0.05 are indicated by asterisk (*).

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TABLE 3 - Metallothionein content (µg/g WW) in the digestive gland of resident and caged mussels from five sites along Rijeka Bay (TM, LR, UR, BK, KR) and control site (LIM). Data are expressed as mean ± S.D (N=5). Significant difference (p< 0.05) with respect to control site LIM is indicated by asterisk (*).

<table>
<thead>
<tr>
<th>Sampling sites</th>
<th>Resident</th>
<th>Caged</th>
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<tbody>
<tr>
<td>LIM</td>
<td>153 ± 1.3</td>
<td>153 ± 1.3</td>
</tr>
<tr>
<td>TM</td>
<td>206 ± 7.9*</td>
<td>158 ± 11.2</td>
</tr>
<tr>
<td>LR</td>
<td>219 ± 7.3*</td>
<td>198 ± 18.6*</td>
</tr>
<tr>
<td>UR</td>
<td>193 ± 9.2*</td>
<td>147 ± 15.0</td>
</tr>
<tr>
<td>BK</td>
<td>184 ± 17.2*</td>
<td>172 ± 6.7*</td>
</tr>
<tr>
<td>KR</td>
<td>161 ± 20.3</td>
<td>161 ± 3.7</td>
</tr>
</tbody>
</table>

tion, as suggested previously [19]. In addition, large fluctuations were recorded in the salinity level between sampling sites. Previous studies revealed considerable negative correlation of salinity and metallothionein level in the digestive gland of mussels [18,45]. Thus, it should be taken in consideration that significantly higher metallothionein content recorded at urbanised sites BK and UR could be also linked to lower salinity (13.38 and 16.40 ‰, respectively), rather than to metal bioavailability alone.

Finally, gradual accumulation of nutrients during the pre-spawning season in summer and autumn could introduce significant variation of metal concentrations and metallothionein content in the digestive gland due to “biological dilution” effect [18]. Moreover, it has been previously reported that mussels display the maximum content of metallothionein in digestive gland in late winter and spring [18,36]. This could explain the elevated values of metallothionein obtained in the current study (above 150 µg/g w.w.) in comparison to other field studies along the eastern Adriatic coast [18,46].

Metallothionein content in control mussels at site Lim and resident mussels from KR shipyard characterized by substantial input of copper, a known inducer of this protein [7], were nearly equal. Previous laboratory experiments showed that despite significant accumulation of copper in the tissues of mussels no induction of metallothionein was detected suggesting that basal level of this proteins could be sufficient for copper detoxification [47]. On the other hand, no metallothionein increase at KR shipyard could also indicate that the critical level of copper in the tissues could be reached and exceeded due to exposure to relatively high copper concentration, and mussels were not able to cope anymore with metal stress by de novo synthesis of metallothionein [48-50]. However, the underlying regulation mechanisms of these molecular events are currently not well understood.

In this study, the response to metal exposure was analysed in resident and caged mussels. Generally higher concentration of metals and metallothionein content was detected in resident mussels at most of the sampling sites. This could be a result of cumulative effect of contaminants as already observed when mussels resident to inves-
tigated area were employed in coastal monitoring programmes [51-53]. Caged mussels were exposed for one month, before metal accumulation in the tissues could reach maximum level. In fact, maximum load of metals in the tissues of mussels and adaptation to environmental conditions by reaching steady state equilibrium between metal uptake and excretion is typically observed after three months of exposure in marine environment [32]. However, despite relatively short time of exposure, the increase of metallothionein content at sites BK and LR was observed indicating a rapid response to stress conditions. Thus, it seems that application of caged mussels from the same origin, as an alternative approach used to minimise the effect of innate factors (sex, age, reproductive stage), and the influence of adaptation to local conditions on biomarker response [51-54] could be applicable for examination of response following short-term exposure to metal stress. Furthermore, our results support the simultaneous use of resident and caged mussels to provide better picture of metal bioavailability in marine environment, as previously suggested [32].

In order to synthesize the results and illustrate the differences between resident and caged mussels, the Principal Component Analysis was conducted on the metal concentration and metallothionein content for all sampling sites and control (Figure 3a, 3b).

The first and the second principal components explain 65% of the total variability. Resident mussels from LR harbour, urban area UR, and TM shipyard were distinctly separated in the negative part of first component that was characterized by high loadings of MT, Cd, Zn and Cr (Table 4).

TABLE 4 - PCA results: Table of correlations between variables and principal components (PC's). Values in bold represent correlations that are statistically significant (p<0.05).

<table>
<thead>
<tr>
<th></th>
<th>PC 1</th>
<th>PC 2</th>
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<td>0.38</td>
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<td>Cd</td>
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</tr>
<tr>
<td>Hg</td>
<td>-0.57</td>
<td>0.66</td>
<td>0.19</td>
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<tr>
<td>As</td>
<td>-0.06</td>
<td>-0.51</td>
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<tr>
<td>Cu</td>
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<td>0.91</td>
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<tr>
<td>Zn</td>
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<td>0.02</td>
<td>-0.03</td>
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<tr>
<td>Cr</td>
<td>-0.95</td>
<td>-0.06</td>
<td>-0.14</td>
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<tr>
<td>Ni</td>
<td>-0.63</td>
<td>-0.50</td>
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<tr>
<td>Mn</td>
<td>-0.59</td>
<td>-0.45</td>
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<tr>
<td>Fe</td>
<td>-0.65</td>
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</table>

Resident mussels from LR harbour and most notably KR shipyard were positioned in the positive part of PC2, which takes into account the high level of Cu and Hg. In comparison to mussels from above sites, resident mussels from urban site BK and caged mussels from KR and TM shipyards and urban area UR were less influenced by metal input.

Finally, caged mussels from urban area BK were positioned closer to control site Lim indicating lower anthropogenic impact. Multivariate analysis of the overall data clearly visualized differences in the distribution of resident and caged mussels in the PCA ordination plot, in relation to metal bioaccumulation level and metallothionein induction over short and long term exposure period.

4 CONCLUSION

The comparison of the overall results provided within the framework of this study with the most recent data available in the literature, revealed that metal concentrations found in mussels from Rijeka Bay are consistent with the range of values typically found at sites with different level of anthropogenic activity along the eastern Adriatic coast. Lead, cadmium, mercury and arsenic concentration in Rijeka Bay displayed levels that were comparable to those previously determined within coastal areas in the close vicinity to heavy industries and/or urban wastewater discharge. With exception of concentrations of copper at site KR shipyard, iron at urban site UR and LR harbour and
zinc at TM shipyard and LR harbour, the values for other metals analysed in this study, correspond to those previously determined for coastal zones of eastern Adriatic characterised by low or moderate anthropogenic influence. Elevated metallothionein content in resident and caged mussels indicates the physiological adaptation to metal exposure in polluted environment.

A site-specific pattern of metal accumulation and metallothionein content, as well as differences between resident and caged mussels within the studied area were detected. The present study shows that the combined use of resident and caged mussels for detection of metal accumulation and metallothionein induction could improve our knowledge of the impact of metal contamination in marine environment. This study also provided valuable baseline data to support future risk assessment efforts in the coastal area of Rijeka Bay challenged by anthropogenic pressure.

ACKNOWLEDGEMENTS

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OZONOLYSIS OF β-PINENE AT CONDITIONS RELEVANT FOR INDOOR ENVIRONMENTS: INFLUENCE OF TEMPERATURE ON THE COMPOSITION OF SECONDARY ORGANIC AEROSOL

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ABSTRACT

Ubiquitous use of fragrances and air fresheners in the indoor environment may pose health implications for consumers because of the reaction of terpenes with ozone, which generates not only harmful gas phase products, such as formaldehyde, but also secondary organic aerosol (SOA) consisting of nanometer-sized particles. In outdoor air, SOA formation is strongly linked to biogenic emissions and may play a role in climate change. In order to gain information relevant for the modelling of nano-particle formation indoors as well as outdoors, the composition of SOA from β-pinene (170-400 ppbV) and ozone (60-120 ppbV) was studied over a broad temperature range (278-307 K). Within less than 5 min of reaction time a nucleation burst of SOA particles appeared in all experiments (5-7 x 10^5 particles per cm^3) measurable down to a diameter of 6 nm. As the reactions proceeded, the nano-particles grew in diameter by condensation and coagulation and reached the size of 400-600 nm within 30 min. This caused a steady increase in the particle mass concentration of up to several hundreds µg/m^3. SOA formation tended to decrease with temperature in the order of 1.3% per degree K. Thirteen reaction products were quantified in SOA, most of which being multifunctional carboxylic acids and carbonyls. Cis-pinic acid was the most abundant compound followed by isomers of hydroxypinonic acid, pinolic acid, and pinaketone (non-pinone). The SOA composition varied with temperature consistent with a Clausius-Clapeyron type temperature dependence of the reaction products’ partition coefficients.

KEYWORDS: Submicron particulate matter, multifunctional carboxylic acids, carbonyls, LC-MS.

1 INTRODUCTION

It has long been recognised that atmospheric oxidation of terpenes leads to formation of secondary organic aerosol (SOA) [1]. The global emission of terpenes has been estimated in the order of 40-50 Tg/year, which can produce SOA in quantities large enough to significantly influence the radiative transfer of the Earth’s atmosphere by light scattering, absorption and by serving as condensation nuclei for the formation of cloud droplets [2]. It is much more recent that scientific attention has been drawn to the impact of ozone-initiated chemistry on indoor air quality and human health (see review by Weschler, 2011 [3]). A series of early studies from the group of Wolkoff have pointed out that reaction products of terpenes with O_3 may be responsible for reported symptoms of health impact (see e.g. [4]) and it is commonly agreed today, that nanometre-sized SOA and its constituents can pose health problems in the indoor environment [5].

Ozone enters buildings in concentrations up to more than 50 ppbV [6] as a result of infiltration of outdoor air and ventilation, but may also be emitted directly indoors from photocopiers, printers and air cleaning devices [7,8]. Terpenes are ubiquitous in the indoor environment and derive from cleaning and household products, air fresheners, adhesives, paints, toiletries, houseplants, wood-based construction materials, and furnishing [9-13].

Previous work on indoor formation of SOA from the reactions of ozone with terpenes has mainly been focused on limonene [14-20] and to some extent α-pinene and linalool [21-26]. Although β-pinene is found in the indoor environment emitted from wood-based products, floor wax, and cleaning agents [3], little attention has been given to this compound with respect to formation of SOA. To our knowledge, no data has been published till date, on the influence of temperature on the formation of reaction products from ozonolysis of β-pinene. Such information is necessary for a good understanding of particle formation and related health impacts and useful for parameterization of air chemistry models.

* Corresponding author
2 MATERIALS AND METHODS

A series of experiments were carried out in which β-pinene was oxidized by ozone at different temperatures followed by analysis of the evolved SOA mass fraction and composition. A generally accepted short hand nomenclature [27] for the β-pinene reaction products are used throughout the paper (names and structures in Table 1).

2.1 Reaction conditions

In order to acquire results easily transferable to air chemistry box-models smog chamber experiments were carried out in the static mode with terpene concentrations in excess of ozone concentrations, which is typical for indoor air environments. A broad temperature range was investigated (278-307 K) comprising expected extremes of indoor environments. The reaction pressure followed the atmospheric conditions.

The experiments were carried out indoors in a 4 m³ Teflon® bag inflated by clean air generator (AADCO 737), which produced air with very low concentrations of organic and inorganic chemical compounds (Σ< 1 ppbV) and particles (<100 particles/cm³) at a relative humidity (RH) of 17.5 (±2.5) %. Although indoor air humidity can typically be higher it was decided to avoid addition of water vapour as this has been demonstrated to complicate the study of SOA formation in ozonolysis reactions [28-30]. The air temperature in the reaction bag was kept constant (±1 K) during each experiment by regulating the room temperature. For each experiment, when the bag was almost completely inflated, β-pinene (99% pure, Fluka; Milan, Italy) was injected into an evaporation chamber upstream of the Teflon® bag and the chamber was purged into the bag for 20 min to produce a target mixing ratio of β-pinene around 200 ppbV. After filling, the β-pinene concentration was allowed to stabilize (complete mixing), the actual β-pinene concentration was determined by gas chromatography (170-400 ppbV) and a low volume of ozone-enriched air (generated by silent discharge) was pumped into the Teflon® bag to produce a target concentration of 80 ppbV (monitored range: 60-120 ppbV). The ozone addition lasted less than five minutes, during which the air in the bag was stirred by tapping of the walls of the bag. During this short period, evidence of incomplete mixing was visible on the ozone monitor, which is assumed insignificant compared to the 3 to 4 hours duration of the experiments. The assumption of a well-mixed air mass in the reaction bag was ascertained in preliminary tests by frequently changing position of the inlet of a scanning mobility particle sizing instrument (SMPS) and the ozone monitor inlet, which produced insignificant changes (<5%) of the measured concentrations.

Between experiments the reaction bag was kept inflated overnight with “zero air” containing one ppmV of ozone. This served to deplete the walls for reactive compounds and as a check for leaks. Before use the bag was emptied and purged twice with “zero air”. Occasional samples of the gas-phase were collected onto Tenax® for confirmative analysis by gas chromatography-MS. These analyses proved the purity of the “zero air” and the utilized β-pinene (<0.2 ppbV of any other terpene and oxidation product).

2.2 Monitoring of reactants and SOA evolution

2.2.1 Ozone and SOA concentrations

Ozone was monitored online by UV photometry (Dasibi 1108) and β-pinene was measured every 12 min by gas chromatography (GC) with photoionization detection (SyntechSpectras GC855). Preliminary experiments with an ozone scrubber [31] inserted into the sampling line to minimize sampling artefacts for terpenes [32] verified that the hold-up time in the sample loop was sufficiently short and the ozone degrading potential of the metal surface of the sample loop was sufficiently high to preserve >90% of the β-pinene concentration.

Particle number and size distributions were measured by SMPS with a 31-bin group differential mobility analyzer (6 nm to 600 nm size range) coupled with a TSI Condensation Nuclei Counter (CNC Model 3010). The SMPS system scanned the size range every 4.5 minutes. The number-size distributions were converted to aerosol volume distributions assuming a spherical shape of the SOA particles and the total aerosol volume was calculated as the sum of the binned volumes.

2.2.2 Uncertainty

Compared to large smog chambers the surface to volume ratio of the reaction bag utilized for the present experiments is large and a substantial deposition to the wall of the reaction bag can be expected for reactants, reaction products and particles. Preliminary experiments were conducted to estimate the extent of the wall-loss, in which freshly prepared SOA particles from the β-pinene/ozone reaction were let into the reaction bag in the absence of ozone. During our main experiments it was unavoidable that the reaction bag deflated from the prolonged sampling of air for analysis, by which the surface area to volume ratio increased with time. These experiments demonstrated that the wall-loss of particles increased as the experiments proceeded and reached maximum values above 50% after (see example in Fig. 1). Wall-loss of SOA particles is not the only error-source for small reaction chambers. The adsorption/re-desorption of terpene may happen from beginning to end of an experiment, with potential overestimation of aerosol yields at the experiments proceed as a consequence.

Sampling of SOA by drawing the air from the reaction bag through filters may produce artefacts from adsorption of gas phase products to the filters and/or evaporation of volatile compound from the sampled SOA induced by the low pressure necessary to draw the air. These artefacts are expected to depend on the reaction temperature, and it is not possible to estimate the extent of their impact on the aerosol yield and on the particle composition.
All these error-sources must be taken into consideration when interpreting the results.

2.3 Analysis of stable β-Pinene reaction products

2.3.1 Sample preparation

SOA was sampled at the end of each experiment for later analyses by high performance liquid chromatography-mass spectrometry (LC-MS). Terpenes are known to undergo rearrangement reactions on acidic surfaces [31, 32]. This may also be true for terpene ozonolysis products. Thus, the use of glass or quartz fibre material was avoided. Before and during the experiments, SOA was collected on Teflon® filters (25 mm diameter; 0.5 µm pore size; Fluorelpore membranes from Millipore, Rome, Italy). The filters were placed in a metal holder through which 200 L air was drawn from the reaction bag with a flow rate of 8-12 l/min. Preliminary tests with these conditions had proved the absence of particle breakthrough. One filter sample was collected before β-pinene and ozone were mixed, and the remaining samples were collected during the experiment every 45 min, starting 30 min after the reaction was started. After sampling, the filters were stored in 2-3 mL extraction fluid (methanol with 0.1% NH₄OH) in sealed glass vials at 5 °C until analysis by LC-MS. The samples were prepared for injection by removing the extracted filters, rinsing with methanol, and eliminating NH₃ by gentle reducing the volume of the extracts to 250 µl in a N₂ flow followed by the addition of 750 µl of 17.5 mM acetic acid as buffer. In line with previous findings in our laboratory [33] the recovery of the combined procedure for extraction and sample preparation was found to be 79±10% with lowest values for the most volatile reaction products.

Terpenes are known to rapidly react with ozone not only in the gas-phase but also in the adsorbed state during sampling [34] thereby forming artefact reaction products on the adsorbent [31-35]. β-Pinene is one of the least reactive terpenes and previous experiments in our laboratory [36] have verified that only insignificant yields of artefact reaction products are formed for this compound in the adsorbed state.

2.3.2 LC-MS

The analysis followed the procedures described in details elsewhere [37]. In brief, the products were quantified using a Finnigan MAT LCQ ion-trap mass spectrometer by negative ion, pneumatically assisted electron-spray LC-MS (carboxylic acids) and positive ion atmospheric pressure ionization (non-carboxylic acids) [37]. For identification chromatographic and mass spectrometric data were also available for 10-hydroxy-pinonaldehyde, 9-hydroxy-norpinonaldehyde, 10-hydroxy-pinonic acid and 9-hydroxy-norpinalic acid, prepared in a crude mixture by ozonolysis of myrtenol. The [M-H]⁻ ions were monitored for carboxylic acids and the [M+H]⁺ ions for neutral products and authentic standards of pinic acid (>99%), pinonic acid (>99%), pinonaldehyde (90%), nopinone (pinaketone; >99%), 2-hydroxy-3-pinanone (>99%) were used as calibrants. For products which authentic standards were not available, surrogates were used assuming equimolar response factors of the chromatographic peak areas (pinic acid for norpinic acid; pinonic acid for norpinolic acid, pininic acid and norpininic acid; pinonaldehyde for hydroxy-pinonaldehyde; hydroxy-norpinonaldehyde, norpinicaldehyde, and hydroxy-norpinicaldehyde; 2-hydroxy-3-pinanone for 3-hydroxy-pina ketone and 3-oxo-pinaketone). The inaccuracy in the quantitation with surrogate standards varies from target compound to target compound and is expected to be below 50% based upon a previously published study of the variation of ESI and APCI response factors within polar terpenoids [37].

Chemical structures were proposed for all major peaks in the chromatograms by comparing mass spectral data and retention times with those of an authentic standard and with those of ozone/terpene reaction products identified previously in our laboratory [33, 36-38].

More than 30 experiments were carried out including preliminary tests. 15 experiments produced a complete set of concentration data for reactants and stable reaction products in SOA. The results of these experiments are discussed in the following.

3 RESULTS AND DISCUSSION

The temporal evolution was similar for all experiments (Fig. 1): After a short initial period of oscillation following the addition of ozone to the reaction bag, the β-pinene and ozone concentrations continued a steady decline to 40-60% of the initial values until the experiment was interrupted after 3-4 hours. During the first minutes, reaction products were only present in the gas-phase. However, within less than 5 min sufficiently high gas-phase concentrations of reaction products had build up to induce a nucleation burst of SOA particles (5-7 x 10⁵ particles per cm³) observable above the 6 nm size range

![FIGURE 1 - Typical evolution of a SOA experiment for the concentrations of β-pinene (black bullets); ozone (gray bullets); aerosol volume (open bullets); wall-loss corrected aerosol volume (open rhombs). T = 293 K.](image-url)
(data not shown). As the reaction proceeded and condensable reaction products were formed, these particles grew in diameter by condensation and coagulation and reached a median diameter of 400-600 nm within 30 min corresponding to a steady increase in the aerosol particle volume concentration to several hundred µm^3/cc.

When interpreting the results it is important to note that the ozonolysis of β-pinene produces OH radicals at a molar yield around 0.35 [39]. It is possible to scavenge the OH by conducting the SOA experiments in presence of high concentrations of a compound that does not react significantly with ozone but does so with OH (like e.g.

### TABLE 1 - Retention time, molecular weight key ion masses, and structure of the reaction products identified and quantified in SOA from ozonolysis of β-pinene. The average concentration (C±± SD) for all experiments (all temperatures) is also shown.

<table>
<thead>
<tr>
<th>Retention Time (min)</th>
<th>Mw</th>
<th>ESI- m/z</th>
<th>APCI+ m/z</th>
<th>Mean Ci (µg m^-3)</th>
<th>Name</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>158</td>
<td>157 217</td>
<td>-</td>
<td>0.4 ± 0.1</td>
<td>Norpinolic acid*</td>
<td>[Structure]</td>
</tr>
<tr>
<td>2.5</td>
<td>200</td>
<td>199 259</td>
<td>201</td>
<td>2.2 ± 0.5</td>
<td>Hydroxy-pinonic acid isomer</td>
<td>[Structure]</td>
</tr>
<tr>
<td>2.7</td>
<td>172</td>
<td>171 231</td>
<td>173</td>
<td>6 ± 4</td>
<td>Pinolic acid**</td>
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<tr>
<td>3.7</td>
<td>186</td>
<td>185 Weak</td>
<td>187</td>
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<tr>
<td>4.7</td>
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<td>171 231</td>
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<td>169 229</td>
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<td>2 ± 2</td>
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<td>[Structure]</td>
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<td>9.6</td>
<td>182</td>
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<td>183</td>
<td>0.1 ± 0.5</td>
<td>Keto-pinonaldehyde*</td>
<td>[Structure]</td>
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<tr>
<td>9.6</td>
<td>200</td>
<td>199 259</td>
<td>201</td>
<td>6 ± 4</td>
<td>10-hydroxy-pinonic acid**</td>
<td>[Structure]</td>
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<td>9.9</td>
<td>138</td>
<td>-</td>
<td>139</td>
<td>5 ± 2</td>
<td>Pinaketone**</td>
<td>[Structure]</td>
</tr>
</tbody>
</table>

**Products found in all samples with concentrations above quantification limit. The identification of pinolic acid is tentative *Products only detected in the most concentrated samples (close to the quantification limit). 1Alternatively this may be identified as hydroxy-norpinonic acid. 2Alternatively this may be identified as dihydroxy-norpinonaldehyde or hydroxy-pina-ketone.
cyclohexane). In real indoor environments, no scavenger would exist at sufficiently high concentrations to consume OH. Thus, a scavenger was not utilized in the present experiments. This means, that SOA produced in our experiments may derive from reactions with both ozone and hydroxyl radicals.

3.1 Reaction products

All together 13 different reaction products were detected and quantified in the formed SOA (Table 1). The major compounds, observed in all SOA samples, were cis-pinonic acid (mean concentration after 120 min of reaction time, C_{mean} \sim 20 \mu g/m³), pinolic acid (C_{mean} \sim 6 \mu g/m³), two isomers of hydroxy-pinonic acid (C_{mean} \sim 8 (2+6) \mu g/m³), and pinaketone (C_{mean} \sim 5 \mu g/m³), pininic acid (C_{mean} \sim 2 \mu g/m³), two isomers of hydroxy-pininal acid (C_{mean} \sim 2 \mu g/m³), hydroxy-norpinalic acid/hydroxy-norpinonic acid (C_{mean} \sim 1 \mu g/m³), and norpinic acid (C_{mean} \sim 1 \mu g/m³). Minor compounds, only observed in the most concentrated SOA samples, were dihydroxy-pinonaldehyde (C_{mean} \sim 1 \mu g/m³), hydroxy-pinonaldehyde / dihydroxy-norpinonaldehyde / hydroxy-pinolketone (C_{mean} \sim 1 \mu g/m³), hydroxy-pinaketone acid (C_{mean} \sim 0.3 \mu g/m³), and ketopinonaldehyde (C_{mean} \sim 1 \mu g/m³).

3.2 Mass balance

The detected products on the filters accounted on average for \sim 10% of the reacted β-pinene mass, which is higher than the 2-5% reported in previous studies for the pure ozone/β-pinene reaction [36, 40] and may be explained by a higher partition into the particle phase due to lower reaction temperatures in our experiments. It has been shown [40] that the volatile β-pinene reaction products, such as formaldehyde and pinaketone (nopinone), are retained in the gas phase and that these products may account for more than 30% of the reacted β-pinene, which means that a considerable part of the reacted β-pinene is unaccounted for in our chemical analysis. Besides mass lost to the walls by deposition of SOA particles and adsorption of reactant and reaction products, unidentified compounds, such as peroxides, hydroperoxides and epoxides [41], may also account for the remainder of the reacted β-pinene. The majority of the detected SOA products are multifunctional carboxylic acids and carboxyls, which can undergo condensation reactions – once they are partitioned into SOA - to form high molecular weight (HMW) compounds on dimeric and oligomeric forms (see review by Kroll and Seinfeld [41]). Such HMW compounds may account for the remaining SOA fraction together with absorbed water. However, they can only be analysed with advanced mass spectrometric techniques not available for the present study. Nevertheless, the monomeric forms of terpene oxidation products govern the early steps of SOA formation and information on their formation is essential for aerosol modelling.

Almost all of the observed products have been identified previously in SOA formed by oxidation of β-pinene with ozone or OH and plausible reaction mechanisms have been proposed [44, 38]. The tentative identification of pinolic acid, a hydroxy-pinonic acid isomer, and hydroxy-pinonaldehyde / dihydroxy-norpinonaldehyde / hydroxy-pinolketone, which have not been reported before, will be discussed in details in the following.

3.3. Compound with the molecular weight (MW) 186.

Three compounds were detected with MW = 186. Reaction mechanistic considerations let to the proposal of three possible structures. One (RT = 6.15) was identified as cis-pinonic acid by use of an authentic standard. The cis-isomer was unequivocally confirmed by the lack of an acetic acid adduct in the ESI(-) mass spectrum [31]. Pinic acid was first compound identified as a possible precursor for SOA in ozonolysis of α-pinene and β-pinene in 1998-1999 [41-42] and as a possible precursor for SOA from the oxidation of these terpenes by the OH radical in 2001 [38]. Since then, an overwhelming amount of studies have been dedicated to the role of pinic acid in SOA nucleation and formation and today it is utilized in source apportioniment of ambient PM as a tracer compound [53 and references cited herein]. The key participation of pinic acid in SOA formation and it's suitability as tracer compound were confirmed in the present study. In all the experiments in which the time evolution was studied, SOA formation evolved in tight synchronization with the particle-bound pinic acid concentration - independent of the reaction temperature and start concentration of β-pinene (see examples in Fig. 2). With pinic acid’s tendency to form dimers with vapor pressures below 1 x 10⁻⁶ torr [43], the bulk of pinic acid may be assumed to be in the particulate phase at the concentration regime of our experiment. If this assumption holds, a plot of the particle-bound pinic acid molar mixing ratio vs. reacted β-pinene molar mixing ratio should show up as a straight line, which was actually the case for our data (Fig. 3). The molar yield of pinic acid was thus estimated as the slope of this line resulting in an average value for all experiments of 0.33±0.003 (10 % higher if corrected for the analytical recovery). Only a weak variation was observed for the yield during the time evolution of an experiment and the influence of the experimental temperature was not statistically significant (p>0.05).

The mass spectrometric data for the second compound with M = 186 (RT = 3.7 min) is consistent with hydroxy-norpinalic acid with its quasi-molecular ion with ESI(+) at 185 m/z, its acetic acid adduct and its [M-H+2CH3OH+H2O]⁺ adduct. The MS ion pattern of this peak could be mistaken for the trans-pinic acid, but in the chromatogram of a pure standard trans-pinic acid appears with a retention time half a minute later than cis-pinic acid. The APCl(+) spectrum contained a signal at [M+H]⁺ which indicates that the compound contains either an alcohol, aldehyde or a ketone group besides the carboxylic acid. Hydroxypinalic acid has previously been reported from the β-pinene ozone reaction [41] with a plausible reaction mechanism. However, hydroxy-norpinalic acid is another
FIGURE 2 - The particle-bound pinic acid concentration and SOA volumes vs. reaction time at 281 K (β-pinene start conc. = 189 ppbV; Top) and 303 K (β-pinene start conc. = 298 ppbV Bottom).

FIGURE 3 - Particle-bound pinic acid mixing ratio (ppbV) vs. reacted β-pinene (ppbV).

MW186 compound that contains the functional groups fitting with the mass spectral data previously reported from the oxidation of β-pinene with OH with a plausible reaction mechanism [38]. Thus, based on the data at hand we are not able to positively distinguish one from the other.

The third compound with M = 186 (RT = 4.7 min) was detected with APCI(+) and gave no signal with ESI(-) source, which shows it is not a carboxylic acid. In the literature no products have been reported with this MW 186 that is not a carboxylic acid. The a formula C_{10}H_{18}O_{5} fits with this mass. Potential candidates and could be dihydroxy-norpinonaldehyde, hydroxy-pinolaldehyde or hydroxy-pinolketone. However for these products is it difficult to suggest a plausible formation mechanism even if sequential reactions with ozone and OH are taken into account.

3.4 Acidic compound with the molecular weight 172.

The second most abundant product was tentatively identified as pinolic acid based on the ESI(-) mass spectrum that contains the masses 171 m/z, 231 m/z and 253 m/z corresponding to the negative quasi-molecular ion and the two solvent adducts +60 and +82. This compound also gave a response with APCI(+) (see Table 1), which means that besides the carboxylic acid the compound contains a carbonylic group or a hydroxyl group. However, a plausible reaction mechanism for the formation of pinolic acid has not yet been proposed. In SOA formed by photo-oxidation of α-pinene a major product with the molar weight 172 has been identified as terpenylic acid [54] and it should be taken into consideration as a possible candidate compound although the very long retention time for terpenylic acid reported by Clayes et al. [54] speaks against this in our experiments. It is possible to suggest a mechanism, by which terpenylic acid may be formed from the oxidation of β-pinene - a mechanism similar to the one proposed for the case of α-pinene. However, such a mechanism involves many steps and the formation of a stable intermediate product that have not yet been confirmed. The ambiguity in the identification of the discussed product calls for more experiment evidence.

3.5 Compounds with the molecular weight 200

Three compounds with MW 200 were determined, one of which (RT = 9.6 min) was identified as 10-hydroxy pinonic acid by comparison of retention time and mass spectral data from previous experiments with hydroxyl radical reaction of β-pinene [38]. The second (RT = 2.5 min) was tentatively identified as an isomer of hydroxy-pinonic acid based on the [M+H]^+ signal in APCI(+) and the ESI(-) mass spectrum with a very intense 259 m/z ion (acetic acid adduct), a very small 199 m/z signal, and the absence of the [M-H+2CH_{3}OH+H_{2}O]^- adduct. However, a plausible reaction mechanism has not yet been proposed. The third (RT = 6.1) did not give a signal with ESI(-), which rules out a carboxylic acid. Such a compound (C_{10}H_{18}O_{5}) has not yet been reported in SOA from oxidation of any
SCHEME 1 - Proposed reaction mechanism for formation of an isomeric form of hydroxy-pinonic acid based on oxidation of \( \alpha \)-pinene with ozone followed by OH.

terpene. We suggest that the product could be the isomeric form of dihydroxy-pinonaldehyde shown in Table 1 with a plausible reaction mechanism shown in Scheme 1.

3.6 Influence of temperature on SOA formation and composition

The reaction rate for the pure \( \beta \)-pinene/ozone reaction is known to increase with temperature \((k = (1.7(\pm 0.3)) \times 10^{-15} \times \exp[(1297 \pm 75)/T] \text{cm}^3 \text{mol}^{-1} \text{s}^{-1};[45])\). Although, this tendency may be slightly counteracted by the evolved OH radicals \((k = (1.5(\pm 0.3)) \times 10^{-11} \times \exp[(467 \pm 50)/T] \text{cm}^3 \text{mol}^{-1} \text{s}^{-1};[46])\) it can be expected that the mixed ozone/OH oxidation of \( \beta \)-pinene in the present experiments would speed up with temperature. The OH radical concentration cannot be measured due to lack of specialized instrumentation that works at the experimental conditions used. However a raw estimate of the apparent rate constant for the mixed ozone/OH oxidation ignoring OH and assuming second order reaction kinetics with quasi-constant ozone concentrations in infinitesimal time steps showed that an increase by 10 K speed-up the reactions by approximately a factor 1.5. In other words increasing temperature augment the formation of reaction products available for SOA formation. Counteracting to this, is the influence of temperature on condensation/partition of the reaction products onto SOA particles, which is known to diminish with increasing temperature. Thus, is not possible a priori to predict the overall influence of temperature on SOA formation.

As explained under Materials and Methods the focus of the present investigation was on the chemical composition of SOA and the utilized experimental set-up was not optimized for an accurate quantitation of the absolute SOA yield. However, if it can be assumed that the experimental uncertainties do not vary significantly from experiment to experiment, the relative impact of temperature on the SOA yield can be estimated as the formed SOA relative to the consumed \( \beta \)-terpene. In Fig. 4 the tendency of an increasing formation of SOA by a decreasing temperature is shown, which is in the order of 1.3% per degree K. In other words for SOA formation physics beats chemistry in our experimental set-up (279-307 K; 17.5 ±2.5 % RH).

![Graph showing aerosol mass yield as a function of temperature](image)

**FIGURE 4** - Aerosol mass yield (measured at SOA mass concentrations of 200 \( \mu g/m^3 \) assuming a density of 1 g/cm\(^3\)) as function of temperature.

Under dry conditions (<10% RH) published results on \( \beta \)-pinene ozonolysis (ozone in excess of \( \beta \)-pinene) are consistent with an overall trend of increasing SOA formation with decreasing temperature. The magnitude of the published temperature effect increases with decreasing SOA concentration regimes and range from 1-7% per degree K [28] to 5-8% per degree K [47]. Similar trends have been observed for ozonolysis of \( \alpha \)-pinene (6-8 % per degree K) and limonene (2-4% per degree K) and seemed independent of humidity or the use of OH scavengers [48].

Under humid conditions (26-68% RH) examples of the inverse effect of temperature have been reported [28]. Experiments with mixtures of terpenes released to a simulated indoor environment from cleaning products at 80%
RH have confirmed the tendency of increasing SOA formation with decreasing temperature down to a point, where emission rates of terpenes out of the cleaning product became a limiting factor [49] and similar results have been found for terpenes from car air fresheners [50].

**3.7 SOA composition**

The relative mass contribution of the β-pinene/ozone(OH) reaction products (100% = sum of all detected compounds) vs. temperature. It should be noted that the identification of pinolic acid is tentative.

![SOA composition](image.png)

**FIGURE 5 - SOA composition after 120 min reaction time: Relative mass contribution of the β-pinene/ozone(OH) reaction products (100% = sum of all detected compounds) vs. temperature.**

Under conditions relevant to indoor environments, the oxidation of β-pinene by ozone leads to a significant formation of secondary organic aerosol consisting of nanosize particles. The formation decreased by increasing temperature in the order of 1.5% per degree K for the set-up used in the present investigation (279–307 K; 17.5±2.5 %RH). At all studied temperatures, cis-pinic acid was the most abundant compound in SOA. However, strong effects of temperature were observed on the chemical composition of the particles - a finding that must be taken into consideration when modelling these processes. The methodology described in the present paper will form the basis of the design of future experiments to investigate consumer products’ potential for health impact.

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TREATMENT OF STABILIZATION POND EFFlUENTS
USING HORIZONTAL SUBSURFACE FLOW
CONSTRUCTED WETLAND PROCESS

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Harran University, Engineering Faculty, Department of Environmental Engineering, Sanliurfa, Turkey.

ABSTRACT

Stabilization ponds are commonly used for wastewater treatment purposes in rural small regions due to low-cost and easy operation. During spring and summer, algae bloom in the lagoon is inevitable, which may cause low effluent quality. As a consequence, algae decomposition can deplete oxygen in the receiving environment. Therefore, high algae and chemical oxygen demand (COD) concentrations in the effluent of stabilization ponds restrict its discharge and use for irrigation purposes. In this context, this study aims at evaluating the efficiency of a low-cost horizontal sub-surface flow constructed wetland process to improve the quality of stabilization pond effluent. The pilot scale constructed wetland removed around 56±15% total COD, 40±13% soluble COD, 90% suspended and volatile suspended solids and 57±22% total nitrogen from stabilization pond effluent. The pollutant concentrations in constructed wetlands effluent were always below the values given in Turkish wastewater discharge guidelines. Therefore, the constructed wetland systems are effective for polishing the stabilization pond effluents.

KEYWORDS: Stabilization ponds, Constructed wetland, Suspended solids, Wastewater treatment

1 INTRODUCTION

The treatment of domestic wastewaters in stabilization ponds have been increasing in South East part of Turkey. The simplicity in construction and operation of stabilization ponds have made it popular. Other factors include low operational costs, a capability of withstanding both organic and hydraulic shock loadings. Moreover, there is no need for continuous sludge handling and disposal facilities [1]. However, there have been several complaints from plant operators about low effluent quality and difficulties for keeping effluent discharge limits especially during spring and summer time. High temperatures and sun lights in the presence of nutrients may lead to excessive algal growth and subsequently high suspended solids in stabilization pond effluents. This is the main disadvantage of stabilization ponds in wastewater treatment due to strict discharge standards on effluent COD and suspended solid concentrations. Large quantities of algae in stabilization pond’s effluent may cause problems during irrigation, especially in the case of drip irrigation. Hence, an effective method for algae removal from stabilization pond effluents is necessary for an effective wastewater reuse.

In order to overcome this problem, different techniques for the removal of algae from the effluent of stabilization ponds have been tested. Sand filtration [2], gravel media filtration [3], trickling filters, and constructed wetlands [4]. One of the sustainable treatment alternatives is constructed wetlands since they are efficient, easy to run, ecologically friendly and low cost wastewater treatment systems for the treatment of both domestic and industrial wastewaters. Horizontal subsurface flow (HSSF) constructed wetlands are designed to create subsurface flow through a permeable medium, keeping the water being treated below the surface, thereby helping to avoid the development of odors and other nuisance problems [5].

Research has shown that constructed wetlands can have a positive influence on improving the quality of the effluent from stabilization ponds [6-9].

An experimental pilot scale treatment plant combining an activated sludge, a trickling filter, a 5 stage Bardenpho, a stabilization pond and an horizontal subsurface flow constructed wetland had been built in the Harran University Osmanbey Campus. One goal of the project was to define the most appropriate system for small communities. By using effluents of the stabilization pond, this paper presents the study of the effectiveness of horizontal subsurface flow constructed wetland in improving the stabilization pond effluents over a period of 10 months.
2 MATERIAL AND METHODS

2.1 Experimental Set-up

The study was performed on the full-scale plant at Harran University, in the south east part of Turkey. The treatment facility was built in 2008. There are five different biological treatment processes in the plant, stabilization pond, wetland, trickling filter, activated sludge and bardenpho processes. The plant was designed to serve total 2500 PE. Each process in the plant can treat 500 PE wastewater, equally. Flow sheet of the Harran University Osmanbey Campus wastewater treatment plant can be seen in Fig. 1.

Stabilization pond in the plant has dimensions of 1m x 18m x 48m. A pilot scale horizontal subsurface constructed wetland (Fig. 2) has been positioned after the full-scale stabilization pond. It was made of galvanized metal sheet with the dimensions of 2 m length, 2 m width and 1 m in height (4m² of surface area). The support media for the system consisted of 50 cm of natural gravels (8–12mm diameter) and 20cm of soil. Soil was used in order for plants to grow. It was planted with *Phragmites australis* at a density of 4 rhizomes/m². A peristaltic pump has been used to feed the wetland with stabilization pond effluents. Hydraulic loading was changed between 40-65 L/m².day.

The average climate of the study area can be seen in Table 1. The study was started on the 1st of August. The first 90 days were hot and sunny, and the following 120 days were cold and rainy, and the remaining 90 days were average.

2.2 Monitoring and measurements

The performance of the pilot-scale wetland with respect to total suspended solids (TSS), volatile suspended solids (VSS), chemical oxygen demand (COD), temperature, dissolved oxygen (DO), total nitrogen (TN), and ammonium...
FIGURE 2 - Horizontal flow constructed wetland for the polishing of stabilization pond effluent. Before (a) and after (b) the harvested plant.

TABLE 1 - Climatic conditions of the study area (Average data from 1975-2008).

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nitrogen (NH₄-N), nitrate nitrogen (NO₃-N) removals were determined. The influent and effluent water samples of the wetlands were collected periodically to evaluate the treatment performances three times a week. COD, VSS, TSS, NH₄-N analyses were carried out using the procedure described in the Standard Methods [10]. Nitrite and nitrate were measured on syringe filtered samples using ion chromatography, Schimadzu, Prominence HIC-NS. TN analyses were conducted on filtered samples using TOC analyzer (Shimadzu, Japan). DO and pH were measured in place using a portable pH meter (HACK). For each parameter, samples were analyzed at least in duplicates. When the standard deviation was larger than the size of the plotting symbol, the standard deviation is shown by +/- error bars.

3 RESULTS AND DISCUSSION

3.1 pH, Temperature and DO

Fig. 3 shows the relations between temperature and pH. As it is expected, pH values increases with algae activity due to the fact that algae use CO₂ as carbon source (Reaction 1). Algae population decreases with temperature and thus pH in stabilization ponds decreases in cold temperatures.

\[
\text{CO}_2 + 2\text{H}_2\text{O} \rightarrow \text{CH}_2\text{O} + \text{H}_2\text{O} + \text{O}_2 \quad \text{(Reaction 1)}
\]

Due to algae blooms, high pH (around 10) and dissolved oxygen (around 10 mg/L) occurred in the effluent of the stabilization pond. When the wetland was fed with this wastewater, pH and dissolved oxygen decreased in the treated effluent, which can be explained with Reaction 2. Dissolved oxygen is used during aerobic biological oxidation occurring in the wetland and CO₂ is produced simultaneously, which lowers pH, and dissolved oxygen concentration.

\[
\text{CH}_2\text{O} + \text{O}_2 \rightarrow \text{CO}_2 + \text{H}_2\text{O} \quad \text{(Reaction 2)}
\]

A buffering of the pH values for more than two pH units was measured in the wetland. There is a close link between high lagoon values and a high buffering effect of the wetland independent of the season.

3.2 COD Removal

Effluents of stabilization ponds may contain algae at high concentration. This could increase the organic content of the effluent; hence exhibit high COD and BOD values. COD removal efficiencies of the wetland can be seen in Fig. 4 and 5. COD concentrations in the final effluent were below the regulation limit. Turkish regulations for discharge in receiving environment with effluent concentrations should be lower than 120 mg/L for COD and 50 mg/L for BOD₅.

COD removal efficiency has increased with time. After three months of start-up, stable removal has been observed (Fig. 4). As can be seen from the Fig. 5, total and soluble COD removal efficiencies of the system reached up to 90%. This is in agreement with the previous results reported by Tsalkatidou et al. [8]. However, better performance was achieved than the systems of Kimwaga et al. [6] and Torrens et al. [9].
3.3 Suspended Solid Removal

Subsurface wetlands act like horizontal gravel filters and thereby provide opportunities for TSS (total suspended solids) separations by gravity sedimentation, straining and physical capture, and adsorption on biomass film attached to gravel and root systems [11].

The main aim of this study was to remove algae by wetlands and consequently reduce suspended solids and organic matter. Suspended solids were first removed by screening and organic fraction was degraded by aerobic and facultative microorganisms for assimilative and dissimilative processes. Over 90% suspended and volatile suspended solids were removed by the wetland (Fig. 6). Expected high suspended solids removal was obtained as previous studies [6, 7, 8, 9]. The effluent of the wetland can easily meet the criteria required by Turkish water authorities (150 mg/L for total suspended solids).

Nitrogen Removal

Total nitrogen removal in the wetland can be seen in Fig. 6. Nitrogen is removed by two mechanisms; namely plant uptake by roots as nutrients and nitrification and denitrification process. Average %60 total nitrogen removals were observed.

FIGURE 3 - Influent and effluent temperature, pH, and oxygen concentration variations.

FIGURE 4 - Constructed wetland influent and effluent variations of soluble COD, total COD, suspended solids and volatile suspended solids concentrations.

FIGURE 5 - Total and soluble COD removal efficiencies of constructed wetland throughout the study
4 CONCLUSION

The study has proved that subsurface constructed wetlands can be used for polishing stabilization pond effluent. It was demonstrated that the wetland has a good efficiencies at retaining algae, and completing organic matter degradation from the pond effluent as very high COD and SS reductions were achieved even in winter periods.

TSS concentrations in the final effluent were quite below the suggested value by the Turkish regulation of 150 mg/L. It is assumed that the effluent can be reused without causing plugging in irrigation systems. To conclude, the effluent of the system can be reused without further treatment for irrigation purposes in agriculture after completing full analysis whether the irrigation water is suitable with regard to plant and soil toxicity and final product is safe for human consumption.

ACKNOWLEDGEMENTS

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REFERENCES


DEGRADATION OF REACTIVE RED 198 DYE BY CATALYTIC OZONATION USING PUMICE AND COPPER COATED PUMICE

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³Department of Environmental Health Engineering, Faculty of Health, Hamadan University of Medical Sciences, Hamadan, Iran

ABSTRACT

Due to environmental and aesthetical problems of dyes, in this study, we tried to use catalytic ozonation by focusing on a different aspect of pumice stone as a catalyst and a media for immobilization of metal catalyst to remove reactive red 198 dye.

The heterogeneous catalytic ozonation experiments were carried out in a semi-batch reactor to determine the effect of pH, contact time, initial concentration of dye, catalyst dosage, radical scavenger and COD removal. The pumice granules were modified by CuSO₄. X-ray Diffraction, X-ray Fluorescence and Scanning Electron Microscope analyzes were used to investigate the structural and morphological character of pumice and copper coated pumice. Also, pHZPC of both catalysts were determined.

The results showed that the removal efficiency of dye has been increased by increasing pH, contact time and catalyst dosages. The optimum conditions with both processes were determined at reaction time 15 min, pH 8, copper coated pumice dosages 10 g/L and pumice 20 g/L. Furthermore, the presence of radical scavenger in catalytic ozonation process didn’t have noticeable effect on oxidation efficiency in comparison with single ozonation. Compared with single ozonation, catalytic ozonation with both pumice and copper coated pumice have shown higher efficiency but the modified pumice was more efficient than raw pumice.

These results suggest that copper coated pumice is an effective catalyst in ozonation and may be used to develop a simple and efficient method in dye removal from aqueous solution.

KEYWORDS: catalytic ozonation, copper, pumice, radical scavenger, reactive red 198 dye

1 INTRODUCTION

Dyes are one of the synthetic organic pollutants released from different industries such as textile, food industries, paper production and so on [1]. The dye molecules are toxic and refractory substances, so their discharge to water resources leads to serious health, ecological, environmental problems [2, 3].

Due to the complexity of dye molecular structure, all of the conventional methods used to remove dyes such as adsorption, coagulation and flocculation, chemical precipitation were not suitable enough [4, 5]. Recently, advanced oxidation processes (AOPs) are considered as effective methods to remove non-biodegradable and toxic organic compounds[6]. Ozonation is one of the most common of these processes [7]. Sole ozonation process (SOP) has some imperfections such as low ability to react with some organic compounds, low solubility in water, and incomplete oxidation of refractory organic compounds [8].

Therefore, the combination of ozone with other compounds called Catalytic Ozonation Processes (COP) has been widely used [9]. COP is based on higher degradation of ozone molecule through catalysts activity which can be done in two heterogeneous and homogeneous forms even though heterogeneous systems are more preferable[8, 10].

Therefore, in this research, pumice stone was used as a media to immobilize the catalyst to destroy reactive red 198 dye.

Pumice stone is a light, highly porous volcanic stone which has been widely used in different fields such as agricultural fields, textile, chemistry industries [11-13]. But, in this research, a different aspect of pumice as a catalyst and a support for copper metal immobilization as a catalyst was investigated to trigger the free radicals production.

So the main objective of this research was to investigate the effect of different parameters on sole ozonation process (SOP), catalytic ozonation process by original pumice (COP) and catalytic ozonation process with copper coated pumice (COP-MOD) to remove reactive red 198 dye.
2 MATERIALS AND METHODS

2.1. Collection, Preparation and Characterization of Catalyst and Chemicals

Pumice stone was collected from Tikmeh Dash region of Azarbayejan province in Iran. The samples were crushed and sieved into 20-40 mesh (0.4-0.8 mm). Pumice samples were coated by CuSO4 5H2O (1N) solution according to Kitis et al and Rezaee et al methods [14, 15] with some modification. They were washed by distilled water several times, then dried and pretreated in HCl (1M) solution for 24 hr and then the samples were rinsed out with distilled water and put in distilled water for 24 hr. Then they were washed by acetone solution and dried at 110°C for 14 hr. A stock solution of CuSO4 5H2O (1N) was prepared and added to dried samples. pH of mixture was adjusted on 9.5 by NaOH (3N) solution during mixing and then boiled for 30 min. In the next step, boiled mixture was placed stable for 72 hr and dried at 105°C for 14 hr. Finally, dried mixture was rinsed out with distilled water several times, dried at 110°C for 14 hr and stored in a container for next uses.

Reactive red 198 and chemical reagents used in this study were obtained from Merck (Hohenbrunn, Germany). 1 M HCl or NaOH was used to adjust the pH of the solution. The pH of the solution was controlled by pH meter (Suntex model sp-701, Taiwan). Distillated water was prepared by Fater Electronic water distiller model 2104 (Tehran, Iran). For all batch experiments, glassware and bottles were washed and rinsed with HNO3 before the use and then by distilled water. The chemical characteristics of selected dye are listed in Table 1.

<table>
<thead>
<tr>
<th>Chemical characteristics of RR198 dye</th>
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</thead>
<tbody>
<tr>
<td>Chemical formula</td>
</tr>
<tr>
<td>Class</td>
</tr>
<tr>
<td>Molecular weight</td>
</tr>
<tr>
<td>Optimum wavelength (λmax)</td>
</tr>
<tr>
<td>Molecular structure</td>
</tr>
</tbody>
</table>

2.2. Ozone Pilot

Ozone gas generated from air by an ARDA ozone generator (Model COG-1A, France, 5 g/h) and continuously conducted by silicon pipes and diffused using a disperser into 500 ml glass cylindrical reactor. The reactor was filled separately with synthetic solutions and ozonizer was turned on. The experiments were carried out at 20±1°C. A magnetic stirrer steadily mixed the solution in the reactor to achieve complete homogeneity. At the end of the predetermined reaction time, the samples were analyzed for residual color. In all experiments, the ozone concentration was trapped and determined 0.54 mg/min by potassium iodide (KI) standard titrimetry method [16]. The schematic diagram of the used AOP system was shown in Figure 1.

2.3. Set-up and Procedures

Ozonation and catalytic ozonation experiments were carried out in a pyrex 500 mL semi-batch reactor (DURAN®-Germany) to determine the effect of pH (2, 8 and 10), contact time (2.5 to 30 min), initial concentration of dye (100 to 500 mg/L), catalyst dosages (4 to 30 g/L), radical scavenger and COD removal. Several experiments were carried out to investigate the dye removal efficiency in both sole and catalytic ozonation which are given in Table 2.

Unknown concentrations of dye were determined using a Hatch-DR 5000 UV-Vis spectrophotometer. pH was determined using an electrode (Sartorius Professional meter PP-50). The COD measurement was based on standard method of open reflex [15]. The pHzpc of pumice and copper coated pumice samples were determined according to
TABLE 2 - Experimental conditions

<table>
<thead>
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<th>Experiment</th>
</tr>
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<tr>
<td>2</td>
<td>Effect of pumice dosage</td>
</tr>
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<td>3</td>
<td>Effect of initial concentration of dye</td>
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<td>Effect of Radical Scavenger</td>
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<table>
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<th>C&lt;sub&gt;modified pumice&lt;/sub&gt; g/L</th>
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<td>10°</td>
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<td>2.5-30</td>
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<tr>
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<td></td>
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<td>4-30°</td>
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<tr>
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<td>2°</td>
<td>10°</td>
<td>8</td>
<td>2.5-30</td>
</tr>
</tbody>
</table>

a) The same experiments were carried out for sole ozonation
b) Optimum pH in which the maximum dye removal was obtained in catalytic ozonation

Dastgheib et al. [17]. Each experiment was repeated 3 times and the average value was considered for more validation. Scanning Electron Microscope (SEM), X-ray Diffraction (XRD), X-ray Fluorescence (XRF) and BET were used to determine the structural properties, chemical compounds, specific surface area and morphological characteristics of pumice and copper coated pumice

### 3 RESULTS AND DISCUSSION

#### 3.1. Characterization of raw and modified pumice

Chemical compounds of pumice were determined by XRF analysis. Pumice structure was mainly composed of different metal oxides such as SiO<sub>2</sub> (65.22%), Alumina (15.47%), LOI (8.07%), K<sub>2</sub>O (2.68%), Fe<sub>2</sub>O<sub>3</sub> (2.61%), CaO (2.51%) and Na<sub>2</sub>O (1.81%).

The XRD patterns of pumice and copper coated pumice indicated that SiO<sub>2</sub> as main compound in pumice structure and also shows copper peak in copper coated pumice structure. The SEM micrograph of the pumice before and after modification is shown in Figure 2 (a-b). Belsorp software was used to determine the specific surface area which was 9 and 13.7 m<sup>2</sup>/g and total pore volume was 0.05 cm<sup>3</sup>/g for both raw and coated pumice respectively.

Also pH<sub>ZPC</sub> (pH at which both positive and negative charges come to a balance) as an effective parameter for catalysts was determined 7.5 and 7.16 for both pumice and coated pumice respectively. pH<sub>ZPC</sub> is one of the most important surface properties of catalysts [6]. Naturally, surface charge of catalyst can be either positive or negative which depends on surface characteristics, surface functional groups and chemical compounds of catalyst structure [7]. The surface charge of catalyst will be positive at pH values below pH<sub>ZPC</sub> and negative at pH values above pH<sub>ZPC</sub> [15]. This characteristic can widely be used in interpreting the heterogeneous catalytic ozonation mechanism. At pH values below and above pH<sub>ZPC</sub>, hydroxyl groups existing on metal oxides surface are in protonated and deprotonated forms, respectively [7]. (Eqs.1 and 2)

\[
S - OH + H^+ \leftrightarrow S - OH_2^+ \quad (1)
\]

\[
S - OH + OH^- \leftrightarrow S - O^- + H_2O \quad (2)
\]

Protonated and deprotonated of these functional groups can cause pumice to act as a lewis base or acid. This phenomenon can affect pumice performance as a catalyst [7].

#### 3.2. Effect of pH and Contact time

Since the pH value play a significant role in catalytic ozonation performance, the effect of pH solution was studied at pH values ranging from 2 to 10, under constant conditions (as defined in Table 2) in both sole and catalyst ozonation. As shown in Figure 3, in SOP, dye removal increased from 30% to 52.6% when the pH increased from 2 to 10. Also in COP and COP-MOD, increasing the pH from 2 to 10 showed 45% to 72% and 69% to 97% increase in removal efficiency, respectively.
According to Figure 3, with increasing pH values to alkaline region, the degradation rate of dye increased. This can be due to dye structure, catalyst structure and also catalyst pHzpc. As told before, pHzpc of pumice and copper coated pumice was 7.5 and 7.16 respectively. Investigations show that at pH values above pHzpc, functional groups existing on pumice are degraded and their nucleophilic property increase so can promote ozone reaction rate [7]. Electron which is released from these acidic functional groups can transfer to ozone molecule, so can progress the reaction and cause increase in ozone degradation into free radicals more rapidly [7, 15]. This electron transfer cause ozonide radical production and radical chain production goes on. On the other hand, concentration of OH ion increases with increasing pH values. This ion is one of the factors which acts as an initiator in ozone degradation and improves the ozone degradation rate [18].

As seen, at all pH values the dye removal efficiency in both COPs is higher than what is in SOP. The most removal efficacy achieved in the first 15 minutes of the ozonation. In catalytic ozonation processes adsorption, direct and indirect oxidation mechanisms are involved in dye removal in acidic conditions [8]. Hydroxyl radicals are not produced at pH values below 3 and do not have any effect on ozone degradation. Some researches show that, this increase in dye removal in acidic conditions is due to the production of non-hydroxyl radicals [7]. Additionally, another interpretation for this increase under acidic condition is due to adsorption of ozone and hydroxyl ion on catalyst surface [8]. This ozone adsorption and degradation cause increase in non-hydroxyl radical production and consequently results in dye removal [8].

Therefore, dye removal mechanism at pH values below pHzpc, is assumed to be due to adsorption of ozone and hydroxyl ion on liquid layer near catalyst surface, on the other hand, at pH values above pHzpc, surface functional groups which are located on the metal oxides degraded and acted as lewis acid, the reaction between ozone and these surface functional groups end in production of hydroxyl radicals. It can be comprehended that catalyst surface play the main role in progressing the reactions and formation of different radicals [8]. Mousavi and Mahmoudi [1] found a direct relation between pH and dye removal and reported that the degradation mechanism at alkaline pH is based on indirect ozonation. Valdes et al. [19] investigated the heterogeneous catalytic ozonation of benzothiazole promoted by volcanic sand and concluded that increasing the pH cause increase in benzothiazole removal efficiency and this increase is due to ozone interaction with lewis acid sites on metal oxides.

### 3.3. Effect of Catalyst dosages

To investigate the effect of catalyst dosage on dye removal, experiments were carried out in the presence of various pumice dosages (4-30 g/l) at pH 8 and constant 15 min reaction time. The initial dye concentration was 100 mg/l. As shown in Figure 4, with increasing the catalyst dosages from 4 to 30 g/l, dye removal efficiency increased from 35% to 56.5% and 70% to 90% for original pumice and copper coated pumice, respectively. Increase of catalyst dosages cause increase in catalyst surface area and of active sites which result in highly ozone adsorption, the greater ozone adsorption on catalyst surface, the greater ozone decomposition on pumice surface which end in radical concentration on catalyst surface and bulk solution [1].

Several investigations show the positive effect of metal based catalysts on catalytic ozonation efficiency. Mousavi and Mahmoudi [1] investigated the catalytic ozonation of RR198 dye using MgO nanocrystal and have showed the increase in MgO dosage cause increase in RR198 removal. Valdes et al. [19] determined that increase in catalyst dosage accelerates the benzothiazole oxidation. Although the optimum dosage of catalyst is strongly depends on used metal-based catalyst, contaminant type and reaction con-
conditions [20], the optimum dosages for raw and copper coated pumice were determined 20 and 10 g/l, respectively.

In this research, degradation kinetics of dye in sole and catalytic ozonation was investigated. The results show that dye degradation in both sole and catalytic ozonation was fitted to first order kinetics. Degradation of dye is related to homogeneous and heterogeneous reactions. This degradation is due to direct reactions with ozone molecule and indirect reactions with produced radicals. Both direct and indirect reactions take place in bulk solutions and pumice surface. These reactions can be shown as below [8, 9]:

\[
R = \frac{-d[C_{dye}]}{dt} = r_{dye}\text{hom} + r_{dye}\text{hetero} = (k_{dye}\text{hom} + k_{dye}\text{hetero})C_{dye} \tag{3}
\]

In this equation, \(K_{dye}\text{hom}\) represents the dye degradation rate constant in homogeneous condition or bulk solution which shows as below:

\[
k_{dye}\text{hom} = k_{1}^{dye}C_{O_3} + k_{2}^{dye}C_{OH} \tag{4}
\]

Also, \(k_{dye}\text{hetero}\) shows the dye degradation rate constant in heterogeneous condition (conditions which reactions take place on catalyst surface)

\[
k_{dye}\text{hetero} = k_{3}^{dye}C_{O_3} + k_{4}^{dye}C_{OH} \tag{5}
\]

Assuming \(C_{dye0} = C_{dye}\), the Eq.1 can be written as follows:

\[
\ln \frac{C_{dye}}{C_{dye0}} = -(k_{dye}\text{hom} + k_{dye}\text{hetero})C_{A-S}t = -K_{overall}^{dye}t \tag{6}
\]

In this equation, \(k\) is defined as:

\[
K_{overall}^{dye} = K_{dye}\text{hom} + K_{dye}\text{hetero}C_{A-S} \tag{7}
\]

\(C_{dye0}\) and \(C_{dye}\) are dye concentration at time zero and any time, respectively. \(k_{1}\), \(k_{2}\), \(k_{3}\) and \(k_{4}\) are dye degradation rate constants which stand for homogeneous reaction with ozone and hydroxyl radicals and heterogeneous reaction with ozone and hydroxyl radicals, respectively. Furthermore, \(C_{O_3}\) and \(C_{A-S}\) represent radical hydroxyl concentration and pumice active site concentration, respectively. \(K_{overall}^{dye}\) is obtained by drawing \(\ln \left( \frac{C}{C_{0}} \right)\) vs time.

The results show that by increasing pH from 2 to 8, \(K_{overall}^{dye}\) increased from 0.0552 to 0.1582 min\(^{-1}\) in catalytic ozonation and from 0.022 to 0.0382 min\(^{-1}\) in sole ozonation so it can be found that by adding copper coated pumice, dye degradation rate will be 2.5 and 4.14 times more than sole ozonation process in acidic and alkaline condition, respectively.

### 3.4. Effect of Initial Dye Concentration

To determine the effect of initial concentration of dye on degradation process, experiments were carried out under constant conditions defined in Table 2, but the initial concentration range varied from 100 to 500 mg/L. Increasing the initial concentration of dye from 100 mg/L to 500 mg/L end in a slight decrease in dye removal efficiency. As shown in Figure 5, the degradation rate of RR198 decreased from 45 % to 25 % when no catalyst was added to reaction tank (SOP) and from 64% to 48% in catalytic ozonation with raw pumice and from 96.8 % to 79.6 % with copper coated pumice.

Color concentration increasing will increase the molecules of the color in reaction tank. On the other hand, with fixed amount of reactive radicals produced as the result of ozone decomposition in the catalyst surface, color degra-
oration in higher concentrations will progress slowly. This problem can be solved by increasing the dosage of ozonation either by increasing the volume or the time of ozonation.

3.5. Effect of Radical Scavenger

Radical scavengers are one of the most important problems in AOPs. These radical consuming or interfering agents such as sulphate, carbonate, chloride and nitrate can reduce AOPs efficacy [21, 22]. To determine the sole and catalytic ozonation mechanism, 1.2 g/l tert-butanol (TBA) was added to 100 mg/l dye solution and removal efficiency was determined in 30 minutes reaction time at pH 8, respectively. As shown in Figure 6, adding tert-butanol decrease the removal efficiency from 45% to 30% in sole ozonation but in catalytic ozonation the removal efficiency decreased from 64% to 60% and 96.8% to 94% in the presence of raw and copper coated pumice, respectively.

As seen, catalytic ozonation is less affected than sole ozonation in the presence of tert-butanol. This may be due to the degradation of functional groups on pumice surface which increase the nucleophilic properties and cause them to act as lewis acid [19]. On the other hand the resonance structure of ozone molecule (the more electron density on one of its oxygen atoms) shows an intense affinity to react with active sites on catalyst surface [18]. Increasing the pH values above pHzpc, ozone molecule reacts with acidic functional groups which act as lewis acid, so the ozone degradation rate and radical production increase [8].

Interfering agents act as lewis base and compete with ozone molecule in reacting with functional groups. In this competition, ozone molecule act better than these scavengers, so the catalytic ozonation won't be affected by these agents [8].

The other explanation for not being affected of catalytic ozonation by these agents is taking placing of some reactions on catalyst surface and production of some other reactive radicals except hydroxyl radical which not react with tert-butanol [19]. In some other researches which conducted about sole ozonation processes, radical scavengers such as tert-butanol and acetic acid can stop the radical production chain and reduce the removal efficiency drastically. Valdes et al. [7] investigated the effect of acetic acid as radical scavenger on SOP and COP promoted by natural zeolite and volcanic sand. 50%, 38% and 14% reduction in zone decay rate constants observed in SOP, COP with zeolite and volcanic sand, respectively. But Moussavi et al. [20] obtained different results. They reported that COP strongly affected by tert-butanol and indirect oxidation was determined as the main mechanism.

FIGURE 5 - The effect of initial dye concentration on RR198 degradation in SOP and COPs (pH 8.0, raw pumice 20 g/L and copper coated pumice 10 g/L, contact time 30 min.)

FIGURE 6 - Effect of radical scavenger on RR198 dye removal in SOP and COPs (raw pumice 20 g/L and copper coated pumice 10 g/L, C dye 100 mg/L, pH 8.0, contact time 30 min.)
3.6. Effect of Sole and Catalytic Ozonation on COD Removal

Chemical oxygen demand (COD) is one of the common indexes for determining of organic compounds amounts. To evaluate the effect of catalytic ozonation on COD removal, experiments were carried out in constant conditions (as defined in Table 2). The obtained results (Figure 7) show that COD removal efficiency increased from 20% in sole ozonation to 25% in catalytic ozonation using pumice and finally to 50% in catalytic ozonation using copper coated pumice.

On the other hand, these results show that coating the pumice with copper can increase its catalytic activity. As it can be seen, catalytic ozonation with copper coated pumice cause more dye degradability and mineralization, whereas common oxidants such as ozone do not show this efficiency or in some cases increase COD. Due to the importance of COD index to determine of industrial effluent discharge standards, combining of this process with biological processes can meet the institutional regulations.

Qu and Zheng [23] investigated the effect of catalytic ozonation with activated carbon fiber to remove phenolic wastewater. They observed that this combined process could increase the yield of the oxidation process significantly for phenol and COD removal. Dong and He [24] investigated the effect of natural mineral brucite on catalytic ozonation of Brilliant red X-3B dye, their findings show that catalytic ozonation with brucite can remove dye and COD up to 32.5% and 89%, respectively.

4 CONCLUSION

The results obtained from this research show that catalytic ozonation with both catalysts can remove dye much more efficient than single ozonation process. Due to its structure, pumice stone has shown a great ability as a catalyst and a media to immobilization of metal catalyst. Considering to obtained results from this research and some other factors such as low cost, abundance and special structure of pumice, it can be used to remove pollutant from aqueous solutions.

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MICROBIAL AND ENZYME PROPERTIES OF ACIDIC RED SOILS UNDER ALUMINUM STRESS

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ABSTRACT

Information relating to the effects of Al3+ on soil microbial and enzyme properties is lacking in the studies on acidic red soils. In the present study, under the stress of available Al of Chingkang mountains red soil, such effects were measured by the changes of microbial numbers, enzyme activities (URE and ACP) and microbial biomass carbon (Cmic). The results showed that the microbial numbers, Cmic, ACP and URE activities in the Al-treated samples were significantly lower (p<0.05) than those in control, and clear differences between agricultural soil (AGR soil) and forest soil (FOR soil) were found. The average number of bacteria and fungi was significantly inhibited with an addition of Al3+ higher than 80 mg kg\(^{-1}\) (for bacteria) and 160 mg kg\(^{-1}\) (for fungi), and the decrease in the number of fungus was more prominent in AGR soil than in FOR soil. URE activities decreased significantly only in the early incubation days and were obviously suppressed at the higher level of Al3+ (240 mg kg\(^{-1}\)). AGR soil had a lower effect on pH and ACP activities in the case of Al3+ additions than FOR soil. Moreover, a negative correlation was found between monomeric Al concentration and microbial numbers, enzyme activities and soil Cmic.

KEYWORDS: Acidic red soil; Aluminum stress; Microbial numbers; Enzyme activities; Microbial biomass carbon

1 INTRODUCTION

Aluminum (Al) is the most abundant trivalent element in soils and immobilized in all kinds of mineral forms at mild acid or neutral pHs [1]. As the pH decreases, this element becomes more soluble in the soil solution and more toxic to the growth of plants [2, 3] and microorganisms[4,5]. Red soil, which is acidic in reaction, contains a high level of exchangeable Al and low content of organic matter, covers extensive areas of the humid regions in China. In these areas, Al toxicity is considered as the major yield-limiting factor on crop production [1]. Due to acid deposition and strong rain leach, the concentration of exchangeable Al in these areas is becoming higher and higher in recent years [6, 7]. However, up until now, it has been an unsolved problem to clearly distinguish between pH- and Al-effects [7-9]. Many studies are focused on the toxic effects of pH on the soil microbial ecosystem [10-12] and the mechanisms of Al3+ toxicity and Al3+ tolerance of plants [13,14], but the effects of Al stress on soil microorganisms have mostly been ignored [15]. Especially, the information about the effects of Al3+ on soil microbial and enzyme properties is lacking in the studies on acidic red soil.

The microbial biomass and activities of natural and/or cultivated soils might be influenced by abiotic properties of soils such as water/air ratio of soil atmosphere, temperature, and concentrations of nutrients and pollutants. The interactions of these attributes along with human induced disturbances would alter the soil ecological processes [16, 17]. Soil microbial biomass is considered to be a transformation agent of soil organic materials and a labile pool for plant nutrients. Hence, the change of soil microbial biomass could lead to some changes in the rate of nutrient cycling and the size of nutrient pool [18]. Soil enzyme activities are known as sensors towards any natural and anthropogenic disturbance occurring in the soil ecosystem. Among the different enzymes in soils, urease (URE) and acid phosphatase (ACP) play important roles in the transformation of different soil nutrients [19, 20].

In this study, the investigation on the effects of Al3+ on microbial and enzyme properties in simulative experiment conditions was carried out by the real-time monitoring, analysis for the relations among these soil parameters and the comparison between forest soil (FOR soil) and agricultural soil (AGR soil) in response to Al3+ stress. The objective was to understand the changes of soil biological properties in response to Al3+ stress.

2 MATERIALS AND METHODS

2.1 Soil characteristics

The soil samples represent a typical forest acidic red soil collected from the middle section of the Lohsiao
mountain range (FOR soil) and one adjacent agricultural field (AGR soil) less than 200 m apart. Both sites are located in the Chingkang Mountain in Jiangxi province, Eastern China (27° 06’ N, 115°01’ E, 83 m altitude; an area of continental subtropical monsoon with mild climate, abundant rainfall and four distinct seasons). FOR soil has been isolated from anthropogenic intervention for more than 100 years. It is mainly occupied by misiducional tree species and shrubs. AGR soil has been in continuous cultivation of single-cropped Rice under conventional tillage for more than 10 years, and always uses the farmyard manure as the fertilizer. The experimental soil samples obtained from the soil at a depth of 2-20 cm. when brought into the lab, the soil was firstly air-dried, ground and passed through a 2 mm mesh. All soil samples were then homogenized by thorough mixing and kept in a refrigerator at 4°C until the start of incubation. Some important soil properties are shown in Table 1. The methods used to determine the soil properties were described by Wiatrowska et al. [21].

<table>
<thead>
<tr>
<th>Parameter</th>
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</tr>
<tr>
<td>AP (mg/kg⁻¹)</td>
<td>4.05</td>
<td>58.7</td>
</tr>
<tr>
<td>AK (mg/kg⁻¹)</td>
<td>48.5</td>
<td>198.5</td>
</tr>
<tr>
<td>EC (dS/m⁻¹)</td>
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</tr>
<tr>
<td>Monomeric Al (µmol/L)</td>
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<td>6.8</td>
</tr>
</tbody>
</table>

OM: organic matter; AN: available N; AP: available P; AK: available K; EC: Electrical conductivity.

2.2 Experimental design

The soil samples, 100g (dry soil) in each glass conical flask (250 mL), were kept in a climate-control chamber at 28°C. Water was added to get maintained soil moisture raised to 60% of water holding capacity. After 1 week, Aluminum was added to soil as an aqueous mixture of the salt AlCl₃•6H₂O. The concentrations of Al³⁺ were 0, 80, 160, 240 and 320 mg kg⁻¹ of dry weight soil, respectively. All the tested soil samples were designated F₀ (FOR soil, 0 mg/kg Al³⁺, control), F₁ (FOR soil, 80 mg/kg Al³⁺), F₂ (FOR soil, 160 mg/kg Al³⁺), F₃ (FOR soil, 240 mg/kg Al³⁺), F₄ (FOR soil, 320 mg/kg Al³⁺) and A₀ (AGR soil, 0 mg/kg Al³⁺, control), A₁ (AGR soil, 80 mg/kg Al³⁺), A₂ (AGR soil, 160 mg/kg Al³⁺), A₃ (AGR soil, 240 mg/kg Al³⁺), A₄ (AGR soil, 320 mg/kg Al³⁺). After thorough mixing, the treated soils were incubated in the climate-control chamber at 28°C and maintained the same moisture content during the experimental period. All the tests were conducted in triplicate. Samples from each flask were collected for analysis at 1, 2, 4, 6, 8, 10, 12 weeks, respectively.

2.3 Measurements of soil pH and monomeric Al

Soil pH was measured with a glass electrode by using a 1:2.5 soil-to-water ratio after shaking the samples to the equilibrium for approximately 30 min. The concentration of monomeric Al in soil solution was estimated by using the pyrocatechol violet (PCV) colorimetric method described by Zheng et al. [3]. The reaction solution consisted of the sample solutions (2 mL) + 0.8 mL deionized water + 0.2 mL 0.0375% PCV. After mixing, 1.0 mL of 50 mM Mes was added, mixed, and adjusted to pH 6.2. 15 min later, the absorbance at 590 nm was recorded.

2.4 Enumeration of culturable microbes

The total numbers of culturable bacteria and fungi were determined as colony forming units (CFUs) on agar plates by dilution plate methods. Briefly, 0.1 mL of each serial dilution of the sample suspension was spread over an agar (2%) plate with beef extract peptone medium (0.05% beef extract, 0.05% peptone, 0.03% NaCl (w/v)) containing 0.05% fungicidine for culturing bacteria, and Martin’s medium (1% glucose, 0.5% peptone, 0.1% K₂HPO₄, 0.05% MgSO₄·7H₂O and 0.003% (w/v) Rose Bengal) containing 0.003% of streptomycin for culturing fungi. Six continuous 10-fold dilutions of each sample were prepared starting with 9.0 mL sterilized phosphate buffered saline and 1.0 g of soil sample. All the plates were incubated at 30°C in the dark until colonies appeared (2 days for bacteria and 3 days for fungi).

2.5 Soil enzyme assays

Soil urease (URE; urea amidohydrolase, EC 3.5.1.5) activity was determined by the method of Kandeler [22]. Briefly, 5 g of air-dried soil was mixed with 1.5 mL methylvanzenene, 10 mL buffer (citric acid and potassium hydrate) with pH 6.7 and 5 mL 10% (w/v) urea solution in a reaction flask and incubated at 37°C for 24 h. The indophenol colorimetric method measured the NH₄⁺ released by urease enzymatic hydrolysis of urea and the indophenol was determined colorimetrically at 578 nm. This method is based on the determination of the NH₄⁺ released (expressed as µg NH₄-N g⁻¹ h⁻¹).

The activity of acid phosphatase (ACP; orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) was determined using the substrates p-nitrophenyl phosphate in modified universal buffer (MUB: 12.1 g Tris amino-methane, 11.6 g maleic acid, 14 g citric acid, 6.3 g boric acid in 488 mL sodium hydroxide and diluted to 1:1 with water) adjusted to pH 6.5. The p-nitrophenol (PNP) in the filtrate was determined colorimetrically at 410 nm after 1 h incubation with p-nitrophenyl phosphate. Results were expressed as 1 µg PNP g⁻¹ soil h⁻¹ at 37°C [23].

2.6 Soil microbial biomass carbon

Soil Cmic was determined by fumigation extraction method followed by determination of K₂SO₄-extractable C [24]. The soil Cmic was calculated according to the equation: Cmic= Ec/EKC, where Ec is the difference between the extractable C from fumigated and non-fumigated samples, and KEc=0.38 [25].

2.7 Statistical analysis

All treatments and assays were carried out in triplicate. All data were analyzed using the SPSS 17.0 statistics.
software for Windows. The differences in all measurements were compared using a one-way analysis of variance (ANOVA) followed by a least significant difference (LSD, \( p \leq 0.05 \)) test. Bivariate correlation analysis was performed to determine the association between tested parameters.

### 3 RESULTS

#### 3.1 Soil pH and monomeric Al

Soil pH significantly decreased when Al\(^{3+}\) was added into AGR soil, and the same result was only found in FOR soil at low Al\(^{3+}\) treatment concentration (as shown in Fig. 1). When the treatment concentration was greater than 160 mg kg\(^{-1}\), the pH had no significant change in FOR soil. Meanwhile, no significant difference was found at different incubation periods under the same Al\(^{3+}\) treatment concentration in both soils. Almost for all the treatments, the monomeric Al content decreased during the first 6 weeks of incubation and returned gradually to the initial value in the next 6 weeks. In the FOR soils, the monomeric Al content decreased 8.7\(\pm\)3.2\% after 6 weeks. Similarly, the monomeric Al content in the AGR soils decreased 15.7\(\pm\)3.3\% after 6 weeks (Fig. 1).

#### 3.2 Microbial numbers

Fig. 2 showed the results of the quantitative analysis of bacteria and fungi. In the present study, the number of bacteria significantly decreased (\( p < 0.05 \)) in the Al-treated samples compared to the control. This decrease in the number of bacteria was markedly high in FOR soil (from 13.3\% to 85.6\%) and AGR soil (from 11.3\% to 83.4\%) samples. The viable count of fungi also showed a significant decrease (from 9.2\% to 74.5\% in FOR soil and from 4.6\% to 85.2\% in AGR soil) in the Al-treated soil samples. The average number of bacteria was significantly inhibited, as Al\(^{3+}\) concentration was above 80 mg kg\(^{-1}\). It was the same with fungi only as Al\(^{3+}\) concentration is above 160 mg kg\(^{-1}\). In addition, the average numbers of bacteria and fungi increased significantly under lower Al-treated concentrations in both soils after 4 weeks of incubation, while the phenomenon disappeared at high Al-treated concentrations in full incubation time (Fig. 2).

#### 3.3 Soil enzyme activities

The effects of Al\(^{3+}\) on soil URE and ACP activities are shown in Fig. 3. There were significant effects on the enzyme activities from the addition of Al\(^{3+}\) in the two soils. The treatment of Al\(^{3+}\) with high concentrations may have unrecoverable effects on the enzyme activities. The

![FIGURE 1 - Effects of Al stress on soil pH and monomeric Al concentration in FOR and AGR soils. Data are the means of three replicates. Error bars indicate the standard deviations. Different letters above or below standard error bars indicated significant difference at \( p < 0.05 \).](image-url)
FIGURE 2 - Changes of colony forming units (CFUs) for bacteria and fungi as affected by Al application in FOR and AGR soils. Data are the means of three replicates. Error bars indicate the standard deviations. Different letters above or below standard error bars indicated significant difference at $P<0.05$.

FIGURE 3 - Inhibitory effects of Al$^{3+}$ on ACP and URE activities in different incubation periods in FOR and AGR soils. Data are the means of three replicates. The error bars indicate the standard deviation. Different letters above or below standard error bars indicated significant difference at $P<0.05$. 
ANOVA showed that ACP enzyme activity in the Al-treated samples was significantly lower (p<0.05) than those in the control. The ACP inhibition rate got increased with Al$^{3+}$ concentration increasing. Similarly, the inhibition extent was also obvious between different incubation periods and varied as the incubation proceeded, the highest rate was detected in the samples of 4 weeks. In the FOR soil, ACP activity had a decrease of 19.4% to 63.9% and the highest inhibition rate was observed in F$_3$ treatments. In the AGR soil, ACP activity decreased significantly (p<0.05) as the levels of Al$^{3+}$ increased and ranged from 12.1% to 65.5%. For all treatments, ACP values were heavily inhibited in the first 4 weeks, followed by the reduction of the inhibition during incubation time.

URE activity of the treated samples decreased significantly (p<0.05) with Al$^{3+}$ level increasing (Fig. 3). In FOR soil, URE activity dropped from 4.7% to 69.7%. Similarly, the inhibition rate varied as the incubation proceeded and the highest rate was detected in the samples at 4 weeks. Like ACP, the URE activity was strongly inhibited up to 4 weeks and then increased with incubation time at low Al-treated concentration. In AGR soil, the extent of inhibition was higher than that in FOR soil but increased with Al$^{3+}$ level increasing. Table 2 shows the Pearson’s correlation coefficients between Al$^{3+}$ level and two enzyme activities (ACP and URE). There was significant negative correlation between the enzyme activity and Al$^{3+}$ concentration (Table 2).

3.4 Soil microbial biomass carbon

The values of soil microbial biomass carbon (C$_{mic}$) in the Al-treated and control samples in different incubation periods were shown in Fig. 4. C$_{mic}$ of treated samples decreased significantly (p<0.05) with the level of Al$^{3+}$ concentration. The effects of Al$^{3+}$ on C$_{mic}$ were highly varied in the treated samples during incubation under low Al-treated concentration, irrespective of the soil type. In Al-treated samples, C$_{mic}$ decreased greatly in the 2-week AGR samples and 4-week FOR samples, ranging from 68.2% to 16.8% and 70.4% to 24.8%, respectively. The prolonged and restrictive effect indicated that less microbial biomass was produced per unit substrate input in the presence of Al$^{3+}$. A little smaller decrease in microbial biomass was observed in AGR soil than in FOR soil. Like enzyme activities, C$_{mic}$ showed significant negative correlation with Al$^{3+}$ level (Table 2).

![FIGURE 4 - Microbial biomass carbon in Al$^{3+}$ treated soils at different incubation periods in FOR and AGR soils. Data are the means of three replicates. The error bars indicate the standard deviation. Different letters above or below standard error bars indicated significant difference at $P<0.05$.](image)

| TABLE 2 - Correlation coefficients among microbial and enzyme properties |
|---|---|---|---|---|
| Soil samples | Monomeric Al | pH | URE | ACP | C$_{mic}$ |
| FOR soil | | | | | |
| Al$^{3+}$ | -0.955** | -0.934** | -0.934** | -0.916** |
| pH | -0.955** | 1 | 0.886** | 0.958** | 0.834** |
| URE | -0.934** | 0.886** | 1 | 0.955** | 0.980** |
| ACP | -0.934** | 0.958** | 0.955** | 1 | 0.857** |
| C$_{mic}$ | -0.916** | 0.834** | 0.980** | 0.857** | 1 |
| AGR soil | | | | | |
| Al$^{3+}$ | -0.982** | -0.982** | -0.873** | -0.891** | -0.966** |
| pH | -0.982** | 1 | -0.392* | 0.879** | 0.957** |
| URE | -0.873** | -0.392* | 1 | 0.990** | 0.956** |
| ACP | -0.891** | 0.879** | 0.990** | 1 | 0.971** |
| C$_{mic}$ | -0.966** | 0.957** | 0.956** | 0.971** | 1 |

**all the coefficients are significant at the 0.01 significant level (2-tailed). *all the coefficients are significant at the 0.05 significant level (2-tailed)**
4 DISCUSSIONS

Many studies showed that acid deposition led to a reduction of soil pH and thus to a mobilization of potential toxic Al species [1]. Al\(^{3+}\) led to a significant decrease in soil pH, but no significant difference was found in two soils at different incubation periods under the same Al-treated concentration in our study. The concentration of monomeric Al also changed significantly in response to Al\(^{3+}\) addition in two soils (Fig. 1). All the results supported the view reported by Illmer and Mutschlechner [26] that there is an obvious correlation between soil pH and Al\(^{3+}\) concentration. However, the range of the pH value and monomeric Al in AGR soil was higher than in FOR soil, which strongly proved that the AGR soil has a greater sensitivity to Al stress than the FOR soil.

As is well-known, Al\(^{3+}\) toxicity had many effects on the growth of many plants [27, 28]. However, the effects of Al stress on soil micro-organisms are lacking [29]. Our findings were that Al\(^{3+}\) stress significantly decreased the numbers of microbes, and that the higher the concentration of the Al\(^{3+}\) that was added to the soil, the lower the CFU counts of the microbes that were ascertained (Fig. 2). Some similar effects were also observed by Keyser [30] and Wood [31] in earlier experiments. Meanwhile, clear differences could be found between bacteria and fungi in the two soils. In general, the average number of bacteria significantly was inhibited at the Al\(^{3+}\) concentration higher than 80 mg g\(^{-1}\), and the average number of fungi significantly was inhibited at the concentration higher than 160 mg g\(^{-1}\). In addition, the number of fungus decreased more prominently in AGR soil than in FOR soil, which indicated that the microbial characteristics of the bacteria were more sensitive than fungi to Al stress, and FOR soil maybe contain some high tolerance fungi by further isolation and genus identification.

Soil urease and phosphatase play a major role in the mineralization of organic N and P in soil. These enzymes are frequently used for determining the influence of the various pollutants on the microbiological quality of soil [32, 33]. In our study, ACP and URE activities were inhibited by the application of Al\(^{3+}\) in both AGR and FOR soils (Fig. 3). This was the first report on the effect of Al on soil enzyme activities in acidic red soils. Such a result was consistent with many studies in which heavy metals significantly suppressed soil enzyme activities [34, 35]. Compared to the FOR soil, the enzyme activities in the AGR soil had higher inhibition rate during the incubation time, which may further indicate the AGR soil was more sensitive to Al stress. This can be related to the results of the long-time cultivated soil with low resistance to external interference.

The soil C\(_{\text{mic}}\) in this study was significantly reduced in the Al\(^{3+}\) added soils compared to the control, and a significant negative relationship was found between soil C\(_{\text{mic}}\) and soil monomeric Al concentrations (Fig. 4, Table 2). This observation is consistent with previous study of Illmer et al. [36], who found that Al\(^{3+}\) concentration was the main inhibiting factor for the microbial biomass. Moreover, the soil samples subject to short-term Al stress, even at lowest levels of exposure in this study, were not able to maintain the same overall biomass as in the control soil samples. This could be related to the results of the changes in microbial population sizes, and low level of Al exposure would result in immediate death of cells due to disruption of essential functions [37]. In additional, the significant relationship between monomeric Al concentration and soil C\(_{\text{mic}}\), strongly suggests although not conclusively, the accumulation of Al due to the acidic deposition would cause a reduction of soil C being incorporated into microbial biomass and thus decrease soil organic matter quality, in agreement with the other results of the measured microbial indicators [38, 39]. It is possible that the monomeric Al concentration could provide a useful estimate of the potentially ecotoxicological effect, with regard to soil microbiological processes in the acidic red soils that have received acidic rains input.

5 CONCLUSIONS

In summary, our results suggest that the addition of Al\(^{3+}\) have changed the soil microbial and enzyme properties in acidic red soils. The extent of inhibition increases significantly with the level of Al\(^{3+}\) increasing, and varies with the incubation periods. Negative correlations are found between Al\(^{3+}\) concentration and microbial numbers, enzyme activities and soil C\(_{\text{mic}}\). Moreover, the responses of enzyme activities and microbial numbers are closely related to soil types because AGR soil shows more sensitivity to Al stress than FOR soil. These results help to select the Al-resistant microorganisms in the acidic red soil. Also, to some extent, this study may provide some scientific basis for the evaluation on the potential risks of the increasing level of Al\(^{3+}\) due to the serious acid deposition in red soil.

ACKNOWLEDGEMENTS

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ADSORPTION OF PSEUDOMONAS AERUGINOSA FROM AIR BY CLINOPTILOLITE: MODELING, ISOTHERM AND KINETIC

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ABSTRACT

The aim of this research was to study the Pseudomonas aeruginosa adsorption from air by clinoptilolite using response surface method (RSM). The obtained data were employed to assess the effects of clinoptilolite dose (g), air flow rate (L/min) and initial microbe concentration (CFU/mL) on the efficiency of clinoptilolite for Pseudomonas aeruginosa adsorption. The results showed that maximum adsorption efficiency was achieved at the following optimum conditions: an initial microbe concentration of 2000000 (CFU/mL), a clinoptilolite dose of 3 (g) and an airflow rate of 1.5 (L/min). Statistical checks indicated that the model was acceptable for representing the experimental data. The Langmuir, Freundlich and Dubinin–Radushkevick isotherm models were used to describe Pseudomonas aeruginosa adsorption onto clinoptilolite. In accordance with the obtained correlation coefficients, the adsorption data showed that the Freundlich model was the most suitable for modeling Pseudomonas aeruginosa adsorption onto clinoptilolite (R² = 0.948). The results indicated that the pseudo-second order kinetic model was the best of the kinetic models (R² = 0.999).

KEYWORDS: Adsorption; Pseudomonas aeruginosa; clinoptilolite; RSM; Kinetic; Isotherm

1. INTRODUCTION

Bio-aerosols are airborne particles that are living (bacteria, viruses and fungi) or originate from living organisms. Bio-aerosols are ubiquitous, highly variable, complex, natural or man-made in origin. The sampling and analysis of airborne microorganisms has received attention in recent years due to concerns with contamination in indoor environments, the occurrence of associated health effects, including infectious diseases, acute toxic effects, allergies and cancer [1]. The suspended bacterial particles in an aerosol usually range in size from 1 mm (single cells) to 10 mm (multiple cells). Hence, indoor air pollution is estimated to be a cause of several health-related issues and of reduced work productivity. The accurate estimation of the quantity and types of airborne microorganisms is important because these values can be used both as an index for the cleanliness of the environment and as an index in relation to human health [2]. To effectively mitigate the threat of a biological agent release, effective methods for adsorbing airborne microbes are necessary [3]. Although different types of adsorbent materials have been used for contaminant removal which activated carbon is the most widely used adsorbent [4]. Scientists have been primarily interested in cheap materials such as zeolite and local soils that represent low-cost and readily available absorbents [5]. It has recently been suggested that clinoptilolite might represent a low-cost adsorbent could be used as an adsorbent [6]. Clinoptilolite is a natural zeolite comprising a microporous arrangement of silica and alumina tetrahedral with the complex formula, \( \text{(Na,K,Ca)}_{2-3} \text{Al}_2(\text{Al,Si})_2\text{Si}_{12} \text{O}_{36} \cdot 12\text{H}_2\text{O} \). It forms as white to reddish crystals with a Moh’s hardness of 3.5 to 4 and a specific gravity of 2.1 to 2.2 and commonly occurs as a devitrification product of volcanic glass shards in tuff and as vesicle fillings in basalts, andesites and rhyolites. It has interesting and potentially useful properties, such as a large surface area (200-1000 m²/g), hydrophobic or hydrophilic properties that create electrostatic interactions, different ion-exchanged forms, and exert mechanical and chemical resistances [7]. Pseudomonas aeruginosa was used in this work as a bacterial model. Cultured Pseudomonas spp. is the most widely used microorganism among those that have been employed for environmental studies due to its nonpathogenicity, availability, and high growth rate in media [8]. In most of the earlier reported batch mode studies, the effects of individual parameters were reported while maintaining other process parameters constant at unspecified levels. This approach does not depict the combined effect of all process parameters. This approach, proceeding one parameter at a time, is time-consuming and requires a number of experiments to determine optimum levels, which may be unreliable. These limitations of the classical method can be eliminated by optimizing all process parameters collectively through statis-
tical experimental design, for example, by using response surface methodology [9]. RSM provides an effective tool for investigating the aspects affecting a desired response if there are many factors and interactions present in the experiment. When determining a suitable polynomial equation for describing response, RSM can be employed to optimize the process [10]. The present work focuses on the effects of operating parameters such as clinoptilolite dose, microbial concentrations and air volume to investigate and optimize the conditions for \textit{Pseudomonas aeruginosa} adsorption from contaminated air using clinoptilolite. To the best of our knowledge, there are no existing reports that have investigated \textit{Pseudomonas aeruginosa} adsorption from air by clinoptilolite.

2. MATERIALS AND METHODS

2.1. Microorganisms and growth conditions

\textit{Pseudomonas aeruginosa} was cultivated in nutrient broth overnight at 37°C. The bacterium was dissolved in 0.5 M McFarland solution with a sterile loop until the O.D reached 0.08 -0.1 at 620 nm as measured by spectrophotometer, which implies that the cell count reached a minimum of 10^8 CFU/mL. McFarland tubes were used for preparation of the bacterial solution. First, 0.5 mL of 0.048 M BaCl_{2} was added to 99.5 mL of 0.18 M H_{2}SO_{4} (1% v/v) with constant stirring. The McFarland tubes were slowly mixed to ensure that bacteria were evenly suspended. Using matched cuvettes with a 1 cm light path and water as a blank standard, the absorbance was measured in a spectrophotometer at a wavelength of 625 nm. The control standard was distributed into screw cap tubes of the same size and volume as those used to prepare the test inoculums. The tubes were sealed tightly to prevent sample loss by evaporation and protected from light at room temperature. Standards can be stored for up to 6 months, after which time they should be discarded [11].

2.2. Experimental system

The system set-up contained a glassware column (8 cm length and 1.2 cm diameter) with inflow and outflow ports at 2 and 7 cm from the bottom, respectively (Fig.1). Bacterial solution was placed into a 12 mL plastic nebulizer (3000 L/min, 50 w, Germany) to convert it into bioaerosol. Tygon lab tubes were used for connections (1/4" ID, 3/8" OD, 1/16" Wall thickness, 25 psi at 70°F Max psi). All the equipment was sterilized by a solution of 70% alcohol, 5% HCl, UV light and autoclaving (121°C bar pressure) prior to and after usage. A rotameter (SKC, USA) was used to regulate bacterial aerosol flow. Variables defined by the software were tested within the system. The tygon tube connected to the outflow of the adsorbent column was held over cetrimide agar for 5 minutes. Then, the plates were put in an incubator for 24 h at 37°C. After this time, the number of colony forming units (CFU) was counted. Finally, the removal efficiency of \textit{Pseudomonas aeruginosa} by means of clinoptilolite was calculated as below:

\[
\text{Removal efficiency (\%) = } \left(1 - \frac{C_f}{C_i}\right) \times 100 \tag{1}
\]

Where \(C_i\) and \(C_f\) are the initial and final microbial concentrations (CFU/mL), respectively [12].

![FIGURE 1 - A schematic flow diagram of the experimental reactor: 1) Air source; 2) Tygon tube; 3) Nebulizer; 4) Reactor; 5) Clinoptilolite; 6) Impactor; 7) Microbial plate](image)

<table>
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<th>Variable</th>
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<th>Low factorial (-1)</th>
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<th>High axial (+2)</th>
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2.3. Experimental design and optimization

In this study, the effects of operating parameters were optimized using response surface methodology (RSM). Central composite design (CCD) was used for the RSM in the experimental design; CCD is well-suited for fitting a quadratic surface and usually works well for process optimization. The CCD is an effective design method that is ideal for sequential experimentation and allows a reasonable amount of information for testing lack of fit while not involving an unusually large number of design points [13]. Therefore, face-centered CCD with three factors was applied using Design-Expert 7.0 To evaluate the influence of operating parameters on *Pseudomonas aeruginosa* adsorption onto clinoptilolite, three main factors were chosen: clinoptilolite dosage (1-4 g), microbial concentrations (10^3-10^7 CFU/mL) and air flow rate (0.5-5 L/min), as shown in Table 1. A total of 20 experiments were carried out in this study. It should be considered that preliminary experiments were carried out prior to the 20 performed experiments to determine the extreme values.

3. RESULTS AND DISCUSSION

3.1. Kinetics of adsorption

The kinetics of adsorption describes the adsorption rate of the pollutant onto the adsorbent and governs the equilibrium time [14]. The kinetic data of the adsorption of *Pseudomonas aeruginosa* onto clinoptilolite was treated with Morris–Weber, Lagergren and pseudo-second order equations. To investigate the change in the concentration of adsorbate in terms of shaking time, the kinetic data of *Pseudomonas aeruginosa* adsorption onto clinoptilolite was subjected to the Morris–Weber Eq. (2) [15]:

\[
q_t = K_{id}(t)^{0.5} + C
\]  

(2)

where \( q_t \) (CFU/g) is the is the amount of adsorbed *Pseudomonas aeruginosa* at time \( t \), and \( K_{id} \) is the value of rate constant of intraparticle transport. The pseudo-first order model of Lagergren is described by the equation below, and it is used to determine the rate constant of *Pseudomonas aeruginosa* adsorption onto clinoptilolite.

\[
\log(q_e - q_t) = \log(q_e) - \left( \frac{K_1}{2.303} \right) t
\]  

(3)

Here, \( q_e \) is the amount of adsorbed *Pseudomonas aeruginosa* at equilibrium and \( K_1 \) is the first order rate constant. The kinetic data of *Pseudomonas aeruginosa* adsorption onto clinoptilolite was subjected to the linear form of a pseudo-second order model as described in the following equation:

\[
\frac{t}{q_t} = \frac{1}{(K_2 q_m^2)} + \frac{t}{q_m}
\]  

(4)

where \( k_2 \) (g/CFU.min) is the rate constant. If the pseudo-second order kinetic equation is applicable to the adsorption process, a plot of \( t/q \) versus \( t \) should provide a straight line. The kinetic parameters and correlation coefficients of the kinetic models are shown in Table 2. For the adsorption of *Pseudomonas aeruginosa* onto clinoptilolite, the correlation coefficient of the pseudo-second order model \( (R^2 = 0.999) \) was the highest, which suggested that the pseudo-second order model best fit the experimental data.

3.2. Adsorption isotherm

Adsorption data are usually described using adsorption isotherms which relate the equilibrium uptake \( (q_e) \) to the equilibrium concentration in the solution \( (C_e) \). The Langmuir isotherm model is valid for monolayer adsorption onto surfaces containing restricted numbers of similar adsorption sites. Eq. (5) illustrates the linear form of the Langmuir isotherm model:

\[
\frac{C_e}{q_e} = \frac{1}{b_{LM}} + \frac{C_e}{q_m}
\]  

(5)

Here, \( q_e \) (CFU/g) is the amount of *Pseudomonas aeruginosa* adsorbed per unit weight of adsorbent and \( C_e \) (CFU/L) is the concentration of free *Pseudomonas aeruginosa*. \( q_m \) (CFU/g) is the amount of adsorbate (*Pseudomonas aeruginosa*) per unit weight of adsorbent that is required to form a monolayer on the surface and \( K_L \) (L/CFU) is related to the affinity of the binding sites. The equilibrium data were analyzed using the linearized form of the Langmuir model. The \( K_L \) and maximum adsorption capacity \( (q_m) \) were calculated from the slope and intercept of the plot of \( C_e/q_e \) versus \( C_e \). The results showed that the values of \( q_m \) and \( K_L \) for *Pseudomonas aeruginosa* adsorption onto clinoptilolite are 1250000 CFU/g and 0.0001 L/CFU, respectively, with a correlation coefficient \( (R^2) \) of 0.808.

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<th>Parameters</th>
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</table>

TABLE 2 - Constants of kinetic equations for *Pseudomonas aeruginosa* adsorption by clinoptilolite
The adsorption data have been subjected to analysis with Freundlich adsorption isotherms. The linearized form of the Freundlich model is illustrated in the following equation:

\[ \log q_e = \log K_F + \frac{1}{n} \log C_e \]  

(6)

Here, \( n \) and \( K_F \) are the Freundlich constants and are related to the adsorption intensity and the adsorption capacity, respectively. A plot of \( \log q_e \) versus \( \log C_e \) would exhibit a straight line with a slope of \( 1/n \) and an intercept of \( \log K_F \). The results indicate that the values of \( n \) and \( K_F \) are 2 and 5407.543, respectively, with a correlation coefficient (R²) of 0.948.

The Dubinin–Radushkevich (D–R) isotherm was used to determine the nature of the adsorption process. The linearized form of this model is defined as the equation below:

\[ \ln(q_e) = \ln(q_m) - \beta \varepsilon^2 \]  

(7)

Here, \( \beta \) is the activity coefficient related to the mean adsorption energy and \( \varepsilon \) is the Polanyi potential that can be derived from the equation below:

\[ \varepsilon = \frac{R T}{\beta} \ln \left( 1 + \frac{1}{q_e} \right) \]  

(8)

Here, \( R \) is the gas constant (8.314 J/mol K) and \( T \) is the absolute temperature (K). According to the plot of \( \ln(q_e) \) versus \( \varepsilon^2 \), the values of \( \beta \) and \( q_m \) will be determined by the slope and intercept of the linearized plot. The adsorption data showed that the Freundlich model was more suitable than the Langmuir model for describing the adsorption of \textit{Pseudomonas aeruginosa} onto clinoptilolite in agreement to the obtained results by other performed investigations.

### 3.3. Development of a regression model equation

Prior knowledge and understanding of the process and the process variables under investigation are necessary to achieve a realistic model. The variables in question include the initial microbe concentration (CFU/mL), the adsorbent dosage (g) and the air flow rate (L/min). These variables were chosen to obtain optimum levels using CCD under response surface methodology. The experimental range and levels of the independent test variables investigated in this study are shown in Table 1. Twenty experiments were augmented with six replications conducted at the mean values for each variable, to evaluate the error for each variable. Experiments varying the initial microbe concentration (CFU/mL), adsorbent dose (g) and airflow rate (L/min) at five values were carried out to locate the maximum \textit{Pseudomonas aeruginosa} adsorption as the system response in batch experiments. The five coded levels of each parameter are \(-\alpha, -1, 0, +1, +\alpha\). The codes were calculated as functions of the range of each factor, as shown in Table 3. The design matrix and the correlated results of RSM experiments to determine the effects of the three independent variables are shown in Table 4. The final empirical model based on the coded factors for \textit{Pseudomonas aeruginosa} adsorption (Y) is given in Eq. (9).

\[ Y = 88.93 - 2.76A + 5.99B - 2.19C + 0.31A \times B - 0.31A \times C + 0.31B + 0.65A^2 - 2.18B^2 + 1.46C^2 \]  

(9)

### TABLE 3 - Relationship between the coded and actual values of a factor

<table>
<thead>
<tr>
<th>Code</th>
<th>Actual Value of factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-\alpha)</td>
<td>(X_{\text{max}})</td>
</tr>
<tr>
<td>(-1)</td>
<td>(X_{\text{max}})</td>
</tr>
<tr>
<td>0</td>
<td>(X_{\text{max}})</td>
</tr>
<tr>
<td>(+1)</td>
<td>(X_{\text{max}})</td>
</tr>
<tr>
<td>(+\alpha)</td>
<td>(X_{\text{max}})</td>
</tr>
</tbody>
</table>

### TABLE 4 - Experimental design matrix and results

<table>
<thead>
<tr>
<th>Run</th>
<th>Initial microbe (CFU/mL)</th>
<th>Clinoptilolite dosage (g)</th>
<th>Air flow rate (L/min)</th>
<th>Removal Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
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<td>1.41</td>
<td>89</td>
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<td>4</td>
<td>7973220</td>
<td>3.39</td>
<td>1.41</td>
<td>95</td>
</tr>
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<td>2027780</td>
<td>1.61</td>
<td>4.09</td>
<td>83</td>
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<td>6</td>
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<td>2.75</td>
<td>90</td>
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</table>
TABLE 5 - Analysis of variance (ANOVA) for response surface quadratic model for *Pseudomonas aeruginosa* adsorption onto clinoptilolite.

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of squares</th>
<th>Degree of freedom</th>
<th>Mean square</th>
<th>F-value</th>
<th>Prob&gt;F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>779.6258</td>
<td>9</td>
<td>86.62509</td>
<td>27.11594</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>A-initial microbe (CFU/mL)</td>
<td>103.9695</td>
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<td>103.9695</td>
<td>32.5452</td>
<td>0.0002</td>
</tr>
<tr>
<td>B-bone char dosage (g)</td>
<td>490.71</td>
<td>1</td>
<td>490.71</td>
<td>153.6052</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>C-Air flow rate (L/min)</td>
<td>65.66073</td>
<td>1</td>
<td>65.66073</td>
<td>20.55354</td>
<td>0.0011</td>
</tr>
<tr>
<td>AB</td>
<td>0.78125</td>
<td>1</td>
<td>0.78125</td>
<td>0.244552</td>
<td>0.6316</td>
</tr>
<tr>
<td>AC</td>
<td>0.78125</td>
<td>1</td>
<td>0.78125</td>
<td>0.244552</td>
<td>0.6316</td>
</tr>
<tr>
<td>BC</td>
<td>0.78125</td>
<td>1</td>
<td>0.78125</td>
<td>0.244552</td>
<td>0.6316</td>
</tr>
<tr>
<td>A^2</td>
<td>6.035966</td>
<td>1</td>
<td>6.035966</td>
<td>1.889416</td>
<td>0.1993</td>
</tr>
<tr>
<td>B^2</td>
<td>68.56689</td>
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<td>68.56689</td>
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</tr>
<tr>
<td>C^2</td>
<td>30.73376</td>
<td>1</td>
<td>30.73376</td>
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<td>0.0112</td>
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<tr>
<td>Residual</td>
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<td>3.194619</td>
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<td>Total</td>
<td>811.572</td>
<td>19</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Analysis of variance (ANOVA) is required to test the significance of the obtained model. The ANOVA for the reduced quadratic model is shown in Table 5. The statistical significance of the quadratic model was evaluated by the analysis of variance (ANOVA), prob> F (0.0001), which shows that the second-order quadratic model is significant at less than 0.05% (at a 95% confidence interval).

3.4. Statistical analysis

The results of the second-order response surface model based on the analysis of variance (ANOVA) are shown in Table 5. The models were checked using a numerical method employing the correlation coefficient (R^2) and the adjusted R^2; variance was then calculated as shown in Eqs. (10) and (11). The correlation coefficient (R^2) indicates how much of the observed variability in the obtained data was accounted for by the model (for a good statistical model, the R^2 value should be in the range of 0 - 1.0, and the nearer to 1.0 the value, the more fit the model is deemed to be), while R^2_adj modifies R^2 by taking into account the number of covariates or predictors in the model.

\[
R^2_{adj} = 1 - \frac{n-1}{(n-p)(1-R^2)}
\]  
\[
R^2 = 1 - \frac{SS_{residual}}{SS_{model} + SS_{residual}}
\]

Here, SS, n and p are the sum of the squares, the number of experiments and the number of predictors in the model, respectively. Our results prove that the experimental values are in good agreement with the predicted values because there is not a great difference between R^2 and R^2_adj. The values of R^2 and R^2_adj were found to be 0.96 and 0.92, respectively. Additionally, the relatively low value for the coefficient of variance (CV = 2.01%) indicates the precision and reliability of the experiments that were performed. Moreover, the ANOVA in Table 5 shows the results of the lack of fit test for the models. The lack of fit test describes the variation in the data around the fitted model. If the model does not fit the data well, the lack of fit will be significant. The large p

values of 0.1 for the lack of fit test of the *Pseudomonas aeruginosa* adsorption model (the p value of the lack of fit is 0.1011) illustrate that the lack of fit was not significant; this implies that the models sufficiently describe the obtained experimental data.

3.4. Interactive influences of independent variables on *Pseudomonas aeruginosa* adsorption

3.4.1. Effect of the adsorbent dosage and the initial adsorbate concentration

The optimum parameters for maximum *Pseudomonas aeruginosa* adsorption (%) were found to be higher adsorbent dosages and lower initial *Pseudomonas aeruginosa* concentrations. Increasing adsorbent dosage can be achieved by increasing the adsorbent surface area and the number of available adsorption sites for *Pseudomonas aeruginosa* uptake. Therefore, the number of sites available for adsorption increases the adsorbent dosage. The maximum adsorption capacity of clinoptilolite for *Pseudomonas aeruginosa* was 96% at an initial microbe concentration of 200,000 CFU/mL and an adsorbent dosage of 3 g.

3.4.2. Effect of initial microbe concentration and air flow rate

The adsorption increased with a decrease in the initial airflow rate from 1.4 to 4.1 (L/min) and an increase in the initial microbe concentration from 2027780 to 7973220 CFU/mL. The maximum adsorption capacity of clinoptilolite for *Pseudomonas aeruginosa* was higher than 96% at a constant adsorbent dosage of 3 g.

3.4.3. Effect of adsorbent dosage and airflow rate

The percentage of *Pseudomonas aeruginosa* adsorption increased with an increase in the amount of adsorbent from 2 to 3 g. Increasing adsorbent dosage corresponds to increasing the adsorbent surface area and the availability of binding sites. Under these conditions, the maximum adsorption capacity of clinoptilolite for *Pseudomonas aeruginosa* was higher than 97%. Finding the optimum process parameters to maximize the adsorption of *Pseudomonas aeruginosa* onto clinoptilolite by means of a developed mathematical model was the aim of this study. A quadratic model equation was used within the studied experimental
The optimum adsorption conditions were an initial microbe concentration of 2207246 CFU/mL, an adsorbent dosage of 3 g and an airflow rate of 1.5 L/min. Verification of the determined conditions for Pseudomonas aeruginosa removal was performed by carrying out experiments in shake flasks under the conditions predicted by the model. The experimental values were found to be close to the predicted values, successfully validating the model. Five experimental combinations were used to validate the statistical model. The results of this analysis indicated that the experimental values were in good agreement with the predicted values (Table 6). Under optimal conditions, a Pseudomonas aeruginosa removal of 99.5%, which is in agreement with the predicted value of 98.12%, was obtained by the studied model. The residuals from the least squares fit play an important role in judging the adequacy of the model.

4. CONCLUSIONS

In this study, the adsorption of airborne Pseudomonas aeruginosaby clinoptilolite was optimized using RSM. The results indicated that according to this model the optimization process was successful. The optimum parameters for Pseudomonas aeruginosaremoval by clinoptilolite were as follows: an initial microbe concentration of 2000000 (CFU/mL), a clinoptilolite dosage of 3 (g) and an airflow rate of 1.5(L/min), as determined by RSM. The percentage removal of Pseudomonas aeruginosaafter parameter optimization was 99.5%, while the predicted value was 98.12%. The results of a confirmation experiment were in agreement with the value predicted by the model. The obtained results demonstrate that to obtain a maximum amount of removal by clinoptilolite was optimized using RSM. The authors thank Tarbiat Modares University for financial support.

TABLE 6 - Model validation.

<table>
<thead>
<tr>
<th>Initial microbe (CFU/mL)</th>
<th>Clinoptilolite dosage (g)</th>
<th>Air flow rate (L/min)</th>
<th>Adsorption (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2207246</td>
<td>3</td>
<td>1.5</td>
<td>98.1</td>
</tr>
<tr>
<td>2712087</td>
<td>3</td>
<td>1.6</td>
<td>97.2</td>
</tr>
<tr>
<td>2069199</td>
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<td>2.0</td>
<td>97.2</td>
</tr>
<tr>
<td>2869549</td>
<td>3</td>
<td>1.5</td>
<td>97.7</td>
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<tr>
<td>2098095</td>
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GENOTOXIC EFFECTS OF LOW DOSES OF PESTICIDES ON MONONUCLEAR LEUKOCYTE ISOLATED FROM HIGHER CELLS

Murat Dikilitas¹*, Abdurrahim Kocyigit², Hasan Bilinc² and Abdullah Taskin²

¹Harran University, Faculty of Agriculture, Department of Plant Protection, 63300, S. Urfa, Turkey
²Harran University, Faculty of Medicine, Department of Clinical Biochemistry, 63300, S. Urfa, Turkey

ABSTRACT

Although it is known that the advised or lower than advised doses of eco-friendly pesticides cause a genotoxic effect on non targeted higher cells as well as on targeted organisms, their very low or ultra low doses, however, have not been tested with an efficient method. The single cell gel electrophoresis (SCGE) or comet assay was used to detect the DNA damages on higher cells in vitro exposed to insecticides such as dimethoate (100-, 50-, 10 µg ml⁻¹), methyl parathion (90-, 45-, 5 µg ml⁻¹), alphacypermethrin (25-, 15-, 5 µg ml⁻¹) and dichlorvos (100-, 50-, 10 µg ml⁻¹) in which the concentration of each insecticide was designated as “very low volume” (vlv), “very very low volume” (vvlv) and “ultra low volume” (ulv) doses, respectively. The induction of DNA damage was evident on human peripheral lymphocytes (PBL) after 1 h treatment in vitro with the above insecticides. The results showed that the “vlv” and “vvlv” doses of methyl parathion (LD50=3 µg ml⁻¹), alphacypermethrin (LD50=57 µg ml⁻¹) and dichlorvos (LD50=50 µg ml⁻¹) caused significantly higher DNA damages in treated groups than those of their controls; on the other hand, dimethoate (LD50=387 µg ml⁻¹) caused a significant damage in blood cells only in “vlv” dose. DNA damage was not detected in “ulv” doses of all insecticides. It is probable that the “vlv” or “vvlv” doses of highly toxic chemicals expressing DNA damages on blood cells might be related with their very low LD50 values, which may play an important role on the impact of environmental pollution and human health even with their very low doses. It would also be beneficial to determine the genotoxic effect of “ulv” doses of all insecticides along with the non-harmful doses of dimethoate with extended incubation period if time has potential to create extensive DNA damages.

KEYWORDS: Alkaline single cell gel electrophoresis, comet analysis, DNA damage, eco-friendly pesticides.

1 INTRODUCTION

In recent years, eco-friendly pesticides have replaced the agrochemicals that were used for a long time for the same purpose. Although they have a lesser degree of impact on environment, their active substances are also carcinogenic and therefore, it is unquestionable that their overdose applications cause lung cancer [1], bladder cancer [2], pancreatic cancer [3] and even Parkinson’s disease [4]. In our previous study, we reported that the use of environmentally friendly pesticides such as dimethoate, methyl parathion and alphacypermethrin had detrimental and significant genotoxic effects on non target organisms when they were applied at advised or lower than advised doses in in vitro conditions [5]. However, their negative effects on human health and environment should be questioned even when they are applied with very low doses. When pesticides applied, they generate toxicity in the target organisms basically through oxidative imbalances or genotoxic effects [6]. Their mode of action and toxicity mechanisms as well as effects on target organisms was explained in the study of Dikilitas and Kocyigit [5]. Widespread use of these chemicals, even they are claimed to be safe, exerts remarkable stress on human health and causes environmental pollution. Even if it is claimed that their decomposition is quick and the lower doses than the applied doses are not harmful to the non-target organisms such as birds, fish, and crop plants, their very low doses, however, should be thoroughly evaluated [7]. Although understanding of the biological effects of the eco-friendly pesticides has increased, however, very few reports have been made determining the existence of DNA damages at low doses [5, 8]. Our main target, therefore, is to find out whether the prolonged or repeated exposure to these pesticides might result in the same effects as that of advised doses at acute exposure. For this, we studied the genotoxicity of “vlv- very low volume”, “vvlv- very very low volume” and “ulv- ultra low volume” doses of the pesticides on human peripheral blood lymphocytes through the results of DNA fragments. A highly regarded genotoxicity test, comet assay, was employed for measuring the damage in individual cells. The assay is rapid and sensitive and has been widely used in genotoxicity tests [9-13].
**2 MATERIALS AND METHODS**

2.1 Chemicals

The pesticides were purchased from the local markets and used in our study. Their active ingredients were named instead of trade names. Dimethoate (O,O-dimethyl S-methylcarbamoylmethyl phosphorodithioate), methyl parathion (O,O-dimethyl O-P-nitrophenyl thiophosphate), alphacypermethrin [(S)-alpha-cyano-3-phenoxybenzyl-(IR-3R) plus (R)-alpha-cyano-3-phenoxybenzyl-(1S-3S)-3 (2,2 dichlorovinyl)-2,2-dimethylcyclopropane carboxylate] and dichlorvos (O,O-dimethyl-2,2-dichloro-vinyl phosphate) were purchased from the local pesticide markets. Other chemicals used in the comet assay were purchased from Sigma (www.sigma.com) unless otherwise stated. The chemicals used here were of analytical reagent grade quality form.

2.2 Sample preparation

The peripheral blood samples (total 6 ml) obtained from a healthy subject who did not have any backgrounds of any diseases such as diabetes mellitus, coronary artery disease, malignancy, systemic or local infection, hypertension, acute-chronic liver diseases, renal dysfunction, anemia and non smoking habit, which might negatively affect the condition of DNA. The blood samples were placed into tubes with heparin (50 U mol⁻¹ sodium heparin) and processed within 2h. An amount of 1 ml heparinised blood was carefully layered over 1 ml Histopaque 1077 (Sigma) for mononuclear leukocyte isolation and centrifuged for 35 min at 500 g. The resulting pellets were resuspended in phosphate buffered saline (PBS) and then collected by 15 min centrifugation at 4°C. Membrane integrity of mononuclear leukocyte was determined by an automatic cell counter (Abbott 3700, USA). Membrane preparations in the comet assay were purchased from Sigma (www.sigma.com) unless otherwise stated. The chemicals used here were of analytical reagent grade quality form.

2.3 Comet Assay

2.3.1 Slide preparation

After incubation period, the comet assay was performed as described by Kocyigit et al. [11] with the following modifications: 10 µl of fresh mononuclear leucocyte cells (around 20,000 cells) were mixed with 80 µl of 0.7% low-melting agarose (LMP) in PBS at 37 °C.

After removing cover slips, the slides were submersed in freshly prepared cold (4 °C) lysing solution (2.5 M NaCl, 100 mM EDTA-2Na; 10 mM Tris-HCl, pH 10 - 10.5; 1% Triton X-100 and 10% DMSO added just before use) for at least 1 h. Then, 90 µl of the mixture was layered onto slides that had been previously coated with thin layers of 1% normal melting point (NMP) agarose (60 °C) and immediately covered with a coverslip at 4 °C for at least 5 min to allow the agarose to solidify.

2.4 Electrophoresis and expression of DNA damage

Slides were then placed in freshly prepared cold alkaline electrophoresis buffer (0.3 mol l⁻¹ NaOH, and 1 mmol l⁻¹ Na₂EDTA, pH=13) for 25 min to allow DNA unwinding and expression of alkaline-labile sites as DNA strand breaks. Electrophoresis was then conducted at a current of 25V/300 mA for 25 min at 4 °C. All procedures were conducted under minimal illumination and the electrophoresis tank was covered with a black paper in order to avoid additional DNA damage due to stray light. After electrophoresis, the slides were neutralized (0.4 mol l⁻¹ Tris–HCl, pH 7.5) for 5 min. The dried microscope slides were stained with ethidium bromide (2 µg ml⁻¹ in distilled buffer were added to top up the total volume to 1 ml. The concentrations of each insecticide such as dimethoate (100-, 50-, 10 µg ml⁻¹), methyl parathion (90-, 45-, 5 µg ml⁻¹), alphacypermethrin (25-, 15-, 5 µg ml⁻¹) and dichlorvos (100-, 50-, 10 µg ml⁻¹) were arranged as “very low volume” (vlv), “very very low volume” (vvlv) and “ultra low volume” (ulv) doses, respectively. The cells were then incubated for 1 h at 37°C in an incubator together with the control samples to see the effect of prolonged exposure of pesticides in low concentrations. Each treatment consisted of 5 replicates.

**TABLE 1 - DNA damage parameters for the performed comet assay [14].**

<table>
<thead>
<tr>
<th>DNA damage parameters</th>
<th>Explanation and calculation of each parameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Head</td>
<td>Length of Head DNA</td>
</tr>
<tr>
<td>L-Tail</td>
<td>Length of Tail DNA</td>
</tr>
<tr>
<td>L-Comet</td>
<td>Length of DNA (Whole)</td>
</tr>
<tr>
<td>DNA head (DNA-H)</td>
<td>sum of intensities of all points of the head</td>
</tr>
<tr>
<td>DNA tail (DNA-T)</td>
<td>sum of intensities of all points of the tail</td>
</tr>
<tr>
<td>Percent tailDNA</td>
<td>% DNA-T = 100 DNA-T/(DNA-H + DNA-T).</td>
</tr>
<tr>
<td>(% DNA-T)</td>
<td></td>
</tr>
<tr>
<td>Percent head DNA</td>
<td>% DNA-H = 100-% DNA-T</td>
</tr>
<tr>
<td>(% DNA-H)</td>
<td></td>
</tr>
<tr>
<td>Tail moment (TM)</td>
<td>the product of the tail length and percent tail DNA, TM = TL x (% DNA-T)</td>
</tr>
<tr>
<td>Olive tail moment (OTM)</td>
<td>the product of the distance (in x direction) between the center of gravity of the head (CGH) and the center of gravity of the tail (CGT) and percent tail DNA, OTM=(CGT-CGH) X Tail%DNA/100</td>
</tr>
</tbody>
</table>
with the same letters in the columns are pesticides when compared to their advised doses. *LV* = very low volume, **vvlv** = very very low volume and *ulv* = ultra low volume.

Parameters

- Acute oral effect on rats. ** Concentrations used in this study were selected as the one quarter-, one eighth and ultra low concentrations of the

2.6 Data analysis

The values were expressed as mean ± SE. The comparisons of parameters were performed using One-Way ANOVA test. The experiment was conducted with 5 replications in each dose concentration. A *p* value less than 0.05 were accepted as significant. Data were analyzed using SPSS® for Windows computing program (Version 16.0).

3 RESULTS AND DISCUSSIONS

Although pesticides are highly toxic compounds designed to kill or reduce the effect of target organisms, however, they also become toxic to non-target organisms if they contact them. In spite of great measures taken, there is still risk of contamination with water, food and soil in which the non-target organisms can be badly affected. Although previously reported that the advised and lower than those of advised doses of each pesticide (dimethoate, methyl parathion, alphacypermethrin and dichlorvos) resulted in a dramatic increase in DNA damage [5], it would be more beneficial to find out the genotoxic effects of those pesticides with much lower concentrations in a prolonged period if the human health or environment is of concern. This study revealed that the very low concentrations of pesticides had genotoxic effects on DNA as well as those of advised doses as previously reported [5]. In this work, pesticides such as dimethoate, methyl parathion, alphacypermethrin and dichlorvos were tested with their low concentrations on peripheral lymphocytes. The low concentrations of each pesticide were arranged according to their advised doses, in which one quarter, one eighth and the ultra low concentrations were used. The results showed that the “*lv*” and “**vvlv**” doses of each pesticide except dimethoate, in which only “*lv*” was found toxic, caused significant DNA damages (Tables 2 and 3). L Comet, TM and OTM values of control samples as well as T DNA/H DNA ratio were found significantly lower than those of corresponding treatment groups of pesticides in “*lv*” doses (*p* < 0.05). The significant DNA damage with a lesser extent was also observed in “**vvlv**” doses of the pesticides except dimethoate (*p* < 0.05, Tables 2 and 3). Ultra low doses of the pesticides, “*ulv*”, did not cause any significant DNA damages in lymphocytes.

The comet assay results enabled us to evaluate the damages caused by pesticides with very low concentrations. The important issue here is the genotoxic capacity of those pesticides, especially the ones having with low LD50 values such as methyl parathion, alphacypermethrin and dichlorvos. Many results showed that the pesticides had genotoxic effects on human and animals. For example, in vitro studies with human and Chinese hamster cell lines showed that positive evidence was existed between the genotoxicity and pesticides application [15]. It is important to note that genotoxicity is related with the generation of cancers. For example, Andreotti and Silverman [3] reported that there was a strong correlation between occupationally exposed pesticide workers and pancreatic cancer. Therefore, any doses of pesticides leading to the development of genotoxicity and DNA damage should be of great concern for the environmental safety.

### Table 2 - Names and concentrations of pesticides used for the DNA damage assessment on peripheral lymphocytes.

<table>
<thead>
<tr>
<th>Pesticides</th>
<th>%LD50 mg kg⁻¹</th>
<th>Doses (µg ml⁻¹)**</th>
<th>L Head</th>
<th>L Tail</th>
<th>L Comet</th>
<th>% H DNA</th>
<th>% T DNA</th>
<th>T DNA/H DNA</th>
<th>TM</th>
<th>OTM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td>43</td>
<td>11</td>
<td>54</td>
<td>92.81</td>
<td>7.19</td>
<td>0.077</td>
<td>0.79 ± 1.1</td>
<td>1.00 ± 1.0</td>
</tr>
<tr>
<td>Dimethoate</td>
<td>387</td>
<td>100</td>
<td>59</td>
<td>69</td>
<td>128</td>
<td>66.72</td>
<td>33.28</td>
<td>0.498</td>
<td>22.96 ± 2.0</td>
<td>15.54 ± 2.0</td>
</tr>
<tr>
<td>Methyl parathion</td>
<td>3</td>
<td>90</td>
<td>59</td>
<td>74</td>
<td>133</td>
<td>63.63</td>
<td>36.37</td>
<td>0.571</td>
<td>26.91 ± 3.0</td>
<td>17.93 ± 3.0</td>
</tr>
<tr>
<td>Alphacypermethrin</td>
<td>57</td>
<td>25</td>
<td>47</td>
<td>71</td>
<td>118</td>
<td>60.66</td>
<td>39.34</td>
<td>0.648</td>
<td>27.93 ± 2.0</td>
<td>16.60 ± 2.0</td>
</tr>
<tr>
<td>Dichlorvos</td>
<td>50</td>
<td>100</td>
<td>47</td>
<td>79</td>
<td>126</td>
<td>64.20</td>
<td>35.80</td>
<td>0.557</td>
<td>28.28 ± 3.0</td>
<td>14.52 ± 2.6</td>
</tr>
<tr>
<td>Dimethoate</td>
<td><em>lv</em></td>
<td>50</td>
<td>47</td>
<td>23</td>
<td>70</td>
<td>89.98</td>
<td>10.01</td>
<td>0.113</td>
<td>2.30 ± 1.0</td>
<td>2.38 ± 1.0</td>
</tr>
<tr>
<td>Methyl parathion</td>
<td><em>vvlv</em></td>
<td>45</td>
<td>61</td>
<td>32</td>
<td>93</td>
<td>85.21</td>
<td>14.79</td>
<td>0.173</td>
<td>4.73 ± 2.0</td>
<td>5.28 ± 2.0</td>
</tr>
<tr>
<td>Alphacypermethrin</td>
<td><em>ulv</em></td>
<td>15</td>
<td>47</td>
<td>37</td>
<td>84</td>
<td>78.36</td>
<td>21.64</td>
<td>0.276</td>
<td>8.00 ± 2.0</td>
<td>6.32 ± 2.0</td>
</tr>
<tr>
<td>Dichlorvos</td>
<td><em>ulv</em></td>
<td>50</td>
<td>45</td>
<td>39</td>
<td>84</td>
<td>75.30</td>
<td>24.70</td>
<td>0.328</td>
<td>9.62 ± 2.0</td>
<td>6.79 ± 2.0</td>
</tr>
<tr>
<td>Dimethoate</td>
<td><em>lv</em></td>
<td>10</td>
<td>59</td>
<td>22</td>
<td>81</td>
<td>94.15</td>
<td>5.85</td>
<td>0.062</td>
<td>1.28 ± 1.0</td>
<td>1.94 ± 1.0</td>
</tr>
<tr>
<td>Methyl parathion</td>
<td><em>vvlv</em></td>
<td>5</td>
<td>47</td>
<td>9</td>
<td>56</td>
<td>94.63</td>
<td>5.37</td>
<td>0.056</td>
<td>0.48 ± 0.5</td>
<td>1.03 ± 1.0</td>
</tr>
<tr>
<td>Alphacypermethrin</td>
<td><em>ulv</em></td>
<td>5</td>
<td>71</td>
<td>20</td>
<td>91</td>
<td>91.19</td>
<td>8.81</td>
<td>0.096</td>
<td>1.76 ± 1.0</td>
<td>2.64 ± 1.0</td>
</tr>
<tr>
<td>Dichlorvos</td>
<td><em>ulv</em></td>
<td>10</td>
<td>41</td>
<td>25</td>
<td>66</td>
<td>85.47</td>
<td>14.53</td>
<td>0.170</td>
<td>3.63 ± 2.0</td>
<td>3.00 ± 1.0</td>
</tr>
</tbody>
</table>

* Acute oral effect on rats. ** Concentrations used in this study were selected as the one quarter-, one eighth and ultra low concentrations of the pesticides when compared to their advised doses. *lv* = very low volume", **vvlv** = very very low volume" and *ulv* = ultra low volume".

Parameters with the same letters in the columns are not significantly different from each other at 0.05 levels.
Although there are a few reports of pesticide damages with lessening concentrations on \textit{in vitro} lymphocytes cells [5, 8], the work of Undeger and Basaran [8] showed that the DNA damage was evident with irrespective of the concentrations used in which the lower concentrations of some pesticides somehow resulted in higher DNA damages than those of higher concentrations which caused lower DNA damages on comet tail length. Although their results are quite remarkable for showing DNA damages on lymphocytes cells, however, they arranged the concentrations from high to low without considering the LD$_{50}$ values of each pesticide. Here, in our study, we determined the genotoxic effects of each pesticide with very low concentrations when their advised doses were considered. It is very important to point out that the incubation time of pesticides would play a critically important role for the genotoxicity studies. For example, Ahmed et al. [16] reported that the pesticides endosulfan, malathion, and phosphamidon resulted in in-

<table>
<thead>
<tr>
<th>TABLE 3 - Pesticide damage on peripheral blood lymphocytes.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pesticides</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>Dimethoate</td>
</tr>
<tr>
<td>Methyl Parathion</td>
</tr>
<tr>
<td>Alphacyperme-thrin</td>
</tr>
<tr>
<td>Dichlorvos</td>
</tr>
</tbody>
</table>

*vlv- very low volume", “vvlv- very very low volume” and “ulv- ultra low volume".
increased oxidative and DNA damages with the increase of concentrations on cultured mononuclear cells isolated from peripheral blood samples. These dose-dependent effects on DNA damage and oxidative stress were also time-dependent in that 6, 12 and 24 h incubation of pesticides with the cultured cells resulted in higher damages. In our study, the very low doses which might be considered previously as non-toxic chemicals became highly destructive for DNA structures when the exposure period to the pesticides was increased (1h) compared to the previous works reported [5, 8]. Therefore, the long-term genotoxic effects of pesticides on organisms should not be ignored even the un-toxic doses of pesticides seem to be safe with short incubation period.

4 CONCLUSION

It is clear that the agricultural chemicals are toxic to the environment and to living organisms; therefore, it is important to be of great concern about the environment and human health for our future. For this, every single step towards the control of pests and diseases should be carefully arranged and criticized in detail. In this study, we aimed to determine the toxicity of agricultural pesticides when released with low concentrations to the environment and to report their effects on humans who are exposed to low concentrations of those toxic chemicals. This study showed that the pesticides with low LD₅₀ values were found as toxic in low concentrations as those of other chemicals with high LD₅₀ values when applied in their advised doses. For example, long lasting or chronic effects of pesticides equally disrupted cell metabolism and produced permanent changes in the structures of proteins, lipids and DNA as those of acute effects of the pesticides [17].

The future work with these chemicals should involve the study of reducing their damaging effects by using antioxidant compounds and to evaluate their damaging effects with a further prolonged incubation period with non-toxic doses if the exposure plays an important role.

ACKNOWLEDGEMENTS

This study was conducted at Harran University, School of Medicine, Department of Biochemistry and in the Central Science Laboratories of the University. The authors would like to express their sincere gratitudes to Dr Hasan Biling for his blood donor.

The authors have no conflict of interest with the mention of chemicals and technical facilities here and they have no criticism about similar products which are not mentioned.

REFERENCES

PRACTICABILITY OF 1 mol HCl AS AN EXTRACTANT FOR DETERMINATION OF Cu AND Zn IN CONTAMINATED SOIL

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Institute of Soil Science and Plant Cultivation National Research Institute, Department of Weed Science and Tillage Systems, Orzechowa 61 Str., 50-540 Wroclaw, Poland

ABSTRACT

In this study, a comparison was made of the applicability of various extraction solvents for the determination of available Cu and Zn in contaminated soils. A grey-brown Podzolic Soil (loamy sand) was used, with acid reaction – pH 5.3 and medium level of Cu and Zn.

The soil was contaminated with copper and zinc, in the amounts of 150, 300 and 450 mg Zn kg⁻¹ d.m. of soil. In the soil material, the level of zinc accepted as available for plants was determined in 1 mol HCl. L⁻¹. The total contents of the metals was determined in a mixture of concentrated HCl and HNO₃. To determine the content of the forms of the component in the soil, also sequential chemical extraction of the metal was performed with the BCR (Community Bureau of Reference) method.

As a result of acid extraction in 1 mol HCl L⁻¹, the amounts of Cu released from polluted soils constituted 33-39% of the total content of the element, and in the case of Zn 67-88%. As the level of soil pollution increased, the solution extracted greater and greater amounts of the metals. Correctness of the analyses is evidenced in the comparable amounts of available zinc determined in HCl and in 0.11 mol CH₃COOH L⁻¹ according to the BCR method. Also, similar results concerning the total zinc content were obtained using aqua regia and 6 mol HCl⁻¹ + 14 mol HNO₃ L⁻¹, and summing up the zinc amounts obtained through sequential extraction.

The results of the study demonstrate that 1 mole HCl L⁻¹ solution can be useful for the determination of total forms of studied metals with high levels of soil pollution.

KEYWORDS:
copper, zinc, polluted soils, extraction, bioavailability

1 INTRODUCTION

Copper and zinc are essential nutrients that fulfill important functions in plants physiology. These metals occur universally in nature and are components of chemical compounds. They are also emitted to environment where they cumulate in soil. In light acid soils, copper and zinc easily become mobile and phytoavailable. In such soil these metals are easily transformed to exchangeable – plant available forms. In heavier and more alkaline soils Cu and Zn undergo chemisorption and complex formation with humus. This significantly limits their mobility and phytoavailability. Many studies indicated relationships between the amounts of heavy metals extracted, soil physical-chemical properties and extraction solution used. In general, lesser amounts of examined metals were extracted from light soil than from medium soil. The amounts of heavy metals extracted increased with an increase of soil acidity and a level of soil pollution with heavy metals [1-5].

The accumulation of heavy metals is a growing environmental problem. Chemical extraction procedures are able to predict the changes in the heavy metal mobility or bioavailability in soils. A large number of extracting solutions have been used to assess plant available trace elements [6, 7].

In the World, assays of soil pollution with heavy metals rely on total concentrations of these metals determined on the basis of their from soil with the extraction use of aqua regia (3 HNO₃: 1 H₂SO₄), a mixture of perchloric and nitric acid on in nitric acid. Used to assess the bioavailability of metals extraction is weaker reagents – CaCl₂, DTPA, EDTA, NH₄Cl, 0.1 mol HCl L⁻¹ and 1 mol HCl L⁻¹. In Poland total concentrations of heavy metals extracted with aqua regia are use for identification of contaminated areas. As this is a complicated and rather expensive procedure, a simpler and cheaper method is being looked for.

At present, many researchers base their evaluations of threats to environment, posed by excess of heavy metals, on weaker extractants. A strong positive correlation between the contents of Cu and Zn in plants and the amounts of metals extracted from soil can be observed. The higher values of correlation coefficients are obtained when soft
Available forms of microelements can be determined in a combined extract, using 1 mol HCl L⁻¹. The method has been selected on the basis of high correlations between the extraction results and metal uptake by crop plants from natural soils [8]. However, group extraction of metals gives rise to numerous controversies. This results from the fact that each element occurs in soil in different forms and there is no possibility of finding a solvent that would be equally suitable for the extraction of each of them. In every country, and in almost every laboratory, available zinc is determined in another way. 1 mol HCl L⁻¹, as a strong acid with relatively high level of concentration, extracts greater amounts of microelements relative to their true solubility. A strong positive correlation between the contents of Cu and Zn in plants and the relative to their true solubility. The total contents of the metals was determined in a mixture of concentrated HCl and HNO₃ at the ratio of 3:1. To determine the contents of the forms of the component in the soil, also sequential chemical extraction of the metals was performed with the BCR (Community Bureau of Reference) method, the procedure for which had been developed within the framework on the EC Standards, Measurements and Testing Programme [3, 9]. The analyses were performed for three series of samples, and in the case of the highest concentrations – for one series.

2.1 The 1 mol HCl L⁻¹ extraction procedure

1 mol HCl L⁻¹ was added to air-dry soil at the ratio of 10:1 and shaken for 1 hour using an “over-head” rotary shaker (Rinkis method).

2.2 The optimized BCR sequential extraction procedure

Step 1: Acid extractable fraction

Acetic acid, CH₃COOH (40ml of 0.11mol L⁻¹) was added to 1.0 g of dry soil sample in a 50ml polypropylene tube. The mixture was shaken for 16 hours at 22±5°C at 400 rpm. The extract was separated from the solid phase by centrifugation at 3000 ×g for 20min.

Step 2: Reducible fraction

40 ml of 0.5 mol NH₄OH·HClL⁻¹ from a 1:1 solution containing 25 ml of 2.0 mol HNO₃L⁻¹ (pH 1.5) onto the residue from the first step. The mixture was shaken for 16 hours at 22±5°C, centrifuged for 20minutes at 3000 ×g and then decanted into a beaker.

Step 3: Oxidizable fraction

10 ml of 30% H₂O₂ (pH 2–3) was carefully added in small aliquots to the residue in Step 2 in the centrifuge tube and digested at room temperature for 1 hr with occasional manual shaking. The mixture was heated to 85±2°C for 1 hour and the volume reduced to a few milliliters by further heating in a water bath. A second aliquot of 10 ml H₂O₂ was added to the residue and the digestion procedure was repeated for 1 hour. 50ml of 1.0 mol NH₄OAcL⁻¹ adjusted to pH 2 with HNO₃ was added to the moist residue and shaken for 16 hrs at 22±5.C and centrifuged at 3000 ×g for 20min.

Step 4: Residual fraction

The residual analysis was performed using aqua regia for metals insoluble in the first three steps. To the residue from step 3, 3.0ml of distilled water, 7.5 ml of 6 mol HClL⁻¹ and 2.5ml of 14 mol HNO₃L⁻¹ were added and left overnight at 20°C. The mixture was boiled under reflux for 2 hours, cooled and filtered. After each extraction, the residue was washed by adding 20 ml of deionized water, shaken for 15 min and then centrifuged.

2.3 Total metal analysis


2.4 Analysis of samples

The determination of Cu and Zn in the extracts was performed using a Flame Atomic Absorption Spectropho-
tometer (FAAS), in the air-acetylene flame using the metal hollow cathode lamps as radiation sources. Calibration solutions of the elements were prepared in the corresponding extraction solutions and element concentrations were read from the appropriate calibration curve. Blank samples were also prepared (following the same procedure described above but without the sample) and analyzed.

2.5 Statistical methods

The results were processed statistically (analysis of variance), using the STATGRAPHICS Plus software package. The significance of inter-treatment differences in the analysis of variance for the experiments was assessed using the Tukey test (P < 0.05).

3 RESULTS AND DISCUSSION

The BCR method is currently commonly used for sequential extraction of copper, zinc, lead, iron, manganese, nickel and cadmium [13-15].

Results of sequential analysis showed that with increasing levels of soil contamination with copper and zinc there is also an increase in the content of the first fraction Zn - F1 (Fig.1). That fraction of metals occurs in soil solution in the form of exchangeable ions and carbonates, and is accepted as available to plants [16]. The content of available copper constituted from 60% to 74% of the sum of all four fractions determined and the content of available zinc from 69% to 87.7% (Fig. 1). In the case of soils contaminated with higher doses of copper and zinc, the 0.11 mol CH₃COOH L⁻¹ extracted amounts of the metal close to the total content values. Successive fractions extracted from the soils constituted 19-28% (F2), 4-7% (F3), 3-5% (F4) in the pool of total copper and 6.2-13.6% (F2), 3.2-10.2% (F3) and 2.1-9.3% (F4) in the pool of total zinc.

Under natural conditions, release of heavy metals from strongly contaminated soils under the effect of mineral acids is a very slow process. Factors causing desorption of metals from soil - shaking, excess solution - do not occur under natural conditions. Therefore, metal extraction through shaking in acid solution may provide overestimated data on the susceptibility of metals to desorption.

In the natural soil used in the study, content of available, at 3.7 mg Cu kg⁻¹ and 9.6 mg Zn kg⁻¹, constituted a third of the total form (Table 1,2). The share of available copper constituted from 60 to 74% of the total of all fractions determined, while the content of available zinc – from 69 to 91%.

Studies on the content of copper and zinc in industry-contaminated soils were conducted by Karczewska [2, 3]. In lightly acid soils the amounts of copper extracted by 1 mol HCl L⁻¹ were, depending on the level of soil contamination, 90-99% of the total copper content of (210-1080 mg Cu·kg⁻¹). In the case of zinc the values were lower – within the range of 29 -75% of the total content (36-118 mg Zn·kg⁻¹).

![Figure 1 - Percentage content of particular fractions of copper and zinc obtained with the BCR method](image_url)
TABLE 1 - Available forms of copper and zinc in contaminated soils, in mg·kg⁻¹

<table>
<thead>
<tr>
<th>Contamination [mg·kg⁻¹]</th>
<th>Serial</th>
<th>Cu</th>
<th>Available contents</th>
<th>Zn</th>
<th>0,11 mol CH₃COOH · L⁻¹ (BCR)</th>
<th>1 mol HCl · L⁻¹ (BCR)</th>
<th>1 mol HCl · L⁻¹ (BCR)</th>
<th>0,11 mol CH₃COOH · L⁻¹ (BCR)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>3,7</td>
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<tr>
<td>150</td>
<td>I</td>
<td>68</td>
<td>78</td>
<td></td>
<td>146</td>
<td>120</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>72</td>
<td>70</td>
<td></td>
<td>133</td>
<td>130</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>67</td>
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Correctness of the analyses is evidenced in the comparable amounts of available zinc determined in HCl and in 0.11 mol CH₃COOH · L⁻¹ according to the BCR method. Also, similar results concerning the total zinc content were obtained using aqua regia and 6 mol HCl · L⁻¹ + 14 mol HNO₃ · L⁻¹, and summing up the zinc amounts obtained through sequential extraction.

The concept of estimation of heavy metal threat to the environment, currently promoted in the world, proposes the substitution of determination of total forms with that of available forms of heavy metals in soils [16]. For the purpose of soil monitoring, 1 mol HCl L⁻¹ proves to be especially suitable, as in the light of results of analyses it may extract 70-90% of total zinc forms from heavily contaminated soils [17]. In a strict micro-plot experiment with simulated soil contamination included copper - 25, 50, 100 mg Cu·kg⁻¹ and zinc - 100, 200, 400 mg Zn·kg⁻¹ Stanisławska-Grabia and Korzeniowska [18] obtained very strong correlation between the content of the total forms of these metals and their quantities soluble in 1 mol HCl L⁻¹.

In the study carried out Kashem et al. [19] in Japan was found that in all the extractants (1 M HCl, NH₄Cl, DTPA and CaCl₂) the proportion of extractability of determined metals was higher in contaminated soils than the non-contaminated soils. Among the extractants, 1 mol HCl L⁻¹ extracted the largest proportion of Cu - 61 to 83% and Zn – of 23-52% of total from soils.

Metal extractability by 1 mol HCl L⁻¹ in the sediment samples from Sydney Harbour was high >60% for Cu and >80% for Zn. McCready et al [20] concluded that 1 mol HCl L⁻¹ may have application in evaluating potential bioavailability. The authors believe that the use of 1 M HCl may recommended for first – level screening of soil contamination with heavy metals. The other four weak extractant are believed to provide a better assessment of bioavailable, mobile metals content in soils than 1M HCl extractant [21].

Similar results were obtained for zinc in the study of Chowthury et al. [22]. Among the six extractants (CaCl₂, EDTA, NH₄Cl, DTPA, 0,1 M HCl and 1M HCl) 1 M HCl

TABLE 2 - Content of total forms of copper and zinc in contaminated soils, in mg·kg⁻¹

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<td>LSD α = 0,05</td>
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extracted the highest proportion of Zn (28.2 to 60.3%) from soils of industrial contamination area. The study indicates that a mild extractant like 1 mol HCl L⁻¹ could be used to assess the Zn phytoavailability.

4 CONCLUSIONS

1. In the light of results of the analyses, 1 mol HCl · L⁻¹ may extract 70-85% of total zinc forms from heavily contaminated soil. 1 mol HCl · L⁻¹ may extract less amounts of total copper forms from 44 to 58%.

2. Extraction procedure with 1 mol HCl · L⁻¹ proves to be especially suitable for the purpose of monitoring soils heavily contaminated with zinc.

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SUPRAMOLECULAR CHEMISTRY AND CYCLODEXTRINS:
KEY OF MANY GREEN SOLUTIONS IN FUTURE PROBLEMS

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ABSTRACT

The important properties and the unique chemical architecture of Cyclodextrins (CDs) were investigated because of the continuous increasing applications and usages at food, pharmaceuticals, agriculture and in the different chromatography techniques to yield green solutions as they are produced from a renewable natural material, the starch, with environmental friendly technologies and affordable prices in many industrial usages. Through their encapsulation ability with different chemical substances, constitute the key of many future solutions in chemistry problems. Due to their aforementioned ability, they form inclusion compounds with a great number of organic substances and help at the change of molecule solubility, increase the substance stability in the presence of light and oxidization conditions and at the decrease of volatileness of chemical compounds. Also, they can play a dominant role at the environment protection, contributing to solubilization and departure of organic contaminants and heavy metals from the soil, water and atmosphere.

KEYWORDS: Cyclodextrins; suprachemistry; encapsulation; chemical architecture; inclination compounds.

1 INTRODUCTION

The supra molecular chemistry is the scientific field of chemistry, which includes all inter molecular interactions in which there are no covalent bonds between the kinds that interact with one another, i.e. molecules, atoms or radicals. The majority of these interactions is the host-guest type. The loose compounds, which result are supra molecular and they have new and interesting properties. One of the most important molecular categories of host type, which shows intermolecular interactions are the cyclodextrins for the following reasons:

They are produced in great quantities of thousands of tons per year with friendly environmental technologies.

Their initial high prices dropped at levels that make them financially acceptable in many industrial applications.

They have the ability to form inclusion compounds that have great properties and can be greatly modified.

During their usage, remote toxic results appear which can be contained with the choice of the appropriate cyclodextrin or the appropriate product of them or the appropriate application method of them.

The cyclodextrins can be used as materials for drugs, food or cosmetics preparation.

2 CHEMICAL ARCHITECTURE

The enzymes of some micro organisms have the property to convert the starch in cyclic oligosaccharides products with 6-10 glucose units. These products are called cyclodextrins (CDs). The cyclodextrins are usually composed from six, seven or eight units of $\alpha$-D-glucose (α-, β- and γ-types cyclodextrins correspondingly) and generally have the truncated cone [1]. From these three types the β-cyclodextrin is the one that has the lowest production cost and the most applications. Due to their internal segment, which is relatively hydrophobic and is the appropriate environment for non polar molecules of certain size, form inclusion compounds with a broad spectrum of substrates in aqueous solutions, mimic the action of some enzymes in reactions and are used for the encapsulation of drugs.

3 PHARMACEUTICAL EXCIPIENTS

The cyclodextrins are known for over a hundred years, from 1891, [2] but the chemical properties of cyclodextrins are explored at the first half of the last century [3, 4]. Initially, only small quantities of cyclodextrins could be produced, which were relatively dirty and they had high cost of production [5-7]. With the recent biotechnological progress, important improvements have been achieved in

\* Corresponding author
the cyclodextrins production, the production cost is lowered, and high purity cyclodextrins and their products are produced. In pharmaceutical industry the cyclodextrins have been used mainly as factors of forming inclusion compounds with intention to augment the solubility of some drugs and their stability. They are used in drugs that can decrease or dissuade the gastrointestinal or the eye disturbances, to decrease or repudiate the unpleasant scents or tastes while they can dissuade the interactions between drugs with additional substances or even they can convert the oils and liquid drug into microcrystalline or unformed dust. Every year the cyclodextrins is the subject of almost thousand scientific papers and big part of them is referred on drugs and products that are related with these drugs. In addition, new numerous applications have been described, which include the cyclodextrins (over a thousand patents or applications for patents the last five years). One monograph for the β-cyclodextrin is already available on the American pharmaceutical industry and soon will be shown up on the European pharmaceutical industry. One monograph for the 2-hydroxypropyl-β-cyclodext rin is on the stage of preparation for the American pharmaceutical industry and many others monographs for cyclodextrins are included in medical manuals i.e. the manual of medical excipients [8]. Thereafter, almost a century after their discovery, cyclodextrins are fully acceptable as pharmace ugal excipients. One drug’s substrate must have a certain degree of solubility in order to reach easily at the cell’s membrane but it needs also to be enough hydrophobic for penetrating it. One of the unique properties of cyclodextrins is their ability to corroborate the drugs penetration through the biologic membranes, which is decreased if the cyclodextrin is added in greater concentration than the required one in order to form the inclusion compounds between cyclodextrin and the drugs. Cyclodextrins, in aqueous solutions, form inclusion compounds with many drugs, storing the whole drugs molecule or some non polar segment of its into the hydrophobic concaveness of its structure. These non covalent inclusion compounds show a great variety of advantages which are related with physico-chemical properties of the drugs, such as the increment to their solubility to the water and their stability in the form of solution and the formed inclusion compounds are partitioned easily to drugs molecules and cyclodextrin molecules [9]. Cyclodextrins molecules are relatively big molecules (their molecular weight varies from 1000 to over 1500), have hydrate outer surface and under normal conditions penetrate the biological membranes with great difficulty [10, 11], act as vehicles to the drugs transport at the surface of the biological membrane i.e on the skin, mucilage membrane or at the cornea jacket of the eye, sustaining the hydrophobic drugs molecules in solution form. The relatively lipophilic membrane has a very small relation to the hydrophobic molecules of cyclodextrins and thus due to that, the drugs molecules are separated from the inclusion compounds and penetrate the membrane. This passive drugs penetration through the membranes depends on how much lipophilic are the drugs molecules, but it can be corroborated from the high concentration of drugs at the aqueous outer environment of the membranes, for example the liquid of tears or saliva or with the usage of an aqueous transport vehicle of the drugs. In other words, in order to exist a successful diffusion of the drugs through the membrane, the drugs must have a hydrophilic character, namely to be soluble and simultaneously hydrophobic, to wit to be liposoluble. Cyclodextrins remain at the outer aqueous environment of the membrane. The alcohol and the fatty acids that are the substances compatible penetration corroborators upset the layers of lipids of the biological membranes while the cyclodextrins act increasing the availability of drugs at the surface of lipids. Cyclodextrins have successfully been used in aqueous solution of mouthwash [12], nasal drug suspensions [13], aqueous solution of skin [14], and many eye drops [15-17]. In eye drop, for example, cyclodextrins dissolve the lipophilic and insoluble to water drugs with aim to increase the chemical stability of the drugs or to decrease the local disturbance that leads to the eye. Hydrophilic cyclodextrins such as 2-hydroxypropyl-β-cyclodextrin, proved to be non toxic and tolerated by the eye. Cyclodextrins that are used to drugs, are fully resistant at enzymes that break the starch and only in a low degree they are broke from the α-amylase. The α-cyclodextrin breaks with very slow pace while γ-cyclodextrin breaks quicker from all the other types of cyclodextrins and this is due to the differences in size and pliancy of their molecules. The cyclodextrins break is not fulfilled from the amylases, which are found on saliva or pancreas, but from the amylase that produced from microorganisms of the intestinal flora. Studies that made on the topic of absorption of cyclodextrins, show that only a percentage of 2-4% of them is absorbed from the intestine and the rest breaks and absorbed as glucose, fact that explains their low level of toxicity [18].

**4 MODIFIED CYCLODEXTRINS**

The natural cyclodextrins are effective templates from which, after modification, is produced a wide range of molecules of host type. With the modification, a host molecule, a special guest molecule, are adjusted on a cyclodextrin molecule, in order to cover specific requirements of the host-guest type inclusion compound, opening the way in different new fields of supramolecular chemistry. Metallo-cyclodextrines, rotaxanes, catenanes, as well as surface monolayers from modified cyclodextrins are readily obtained. Simple Cyclodextrins serve as scaffolds on which functional groups and substituents can be concentrated with controlled geometry. With that method, virtually improved recognitions of molecules and procedures of chemical separation, including enantiomer discrimination, through guest type molecules binding can be fulfilled. The access at the choice abilities of the operational teams expands very much the usage of cyclodextrins to the chemical composition and provides catalysts that mimic the entire
range of enzymic activity. Modifications to the cyclodextrins also lead to a wide range of applications of them at photochemistry, such as the construction of molecular device assembly of light and photochemical switches of different frequencies, because it appears the increase on the ability of immediate reaction of molecules type guest. In solutions, modified cyclodextrins have been used to the construction of molecular reactors and molecular temperature and pH sensors. At surfaces, cyclodextrins form semi permeable membranes and sensor electrodes. Generally, natural cyclodextrins through the modifications that can suffer lead to exciting chemistry fields [19].

5 CYCLODEXTRINS IN AGRICULTURAL AND CHEMICAL INDUSTRY

Cyclodextrins form inclusion compounds which show a great range of applications to chemical agricultural products such as agricultural drugs for weeding, insecticides, fungicides, insect repellents, pheromones, growth regulators. Cyclodextrins are used to delay the growth of seeds. When the grains are processed with β-cyclodextrins, the action of some type of amylase is suspended, which break the starch storage at seeds. So initially, the plant grows with slow pace but later this, in a great degree, is offset from a more improved development of the plant, ascribing approximately 20-40% more crop [20]. Cyclodextrins are widely used at chemical industry for the separation of isomers and enantiomers, on catalyze reactions and help on waste removal or to the elimination of their toxicity when they resulting from chemical procedures. Because of their stereochemical structure cyclodextrins play an important role as bio catalysts, increasing the enantioselectivity. Since the inclusion compounds are formed with the use of appropriate chemical molecules of guest type, selective derogation is taking place from only enantiomers of the chemical reactor with result high enantioselectivity to be obtained. It has been referred [21] that during hydrolysis of racemic mixture of arylpropionic esters with protein vehicle of albumin, which is contained in cow serum, results low enantioselectivity (50-81%), while the accession of β-cyclodextrin at the reaction not only increase the enantioselectivity (80-99%) but also accelerates the hydrolysis rhythm. Cyclodextrins can play a dominant role in environment protection contributing on solution and removal of organic waste and heavy metals from the soil, water and atmosphere [22]. They used also at the water process in order to increase the encapsulation and absorption of pollutant substances [23]. With the use of cyclodextrins toxic substances can be removed from industrial liquids through the inclusion compounds. At the main aqueous solution of the insecticide trichlorofonio, the trichlorofonio that cannot be crystallized, is converted to inclusion compound by β-cyclodextrin accession and with one simple process 90% of the toxic substance is removed [20,24]. Aromatics like phenols, p-chlorophenoles and benzol, which are substances that pollute the environment and mainly water, after process with the β-cyclodextrin accession, they are converted to inclusion compounds and their concentration is decreased very much at the aqueous waste [20, 24].

The increase at the solubility of some substances, when they are processed with cyclodextrins, is used at the soil remediation test from different lubricators and their products and also at the better availability at the ground of some fungicides i.e. the fungicides of benzimidazole type [25]. β-cyclodextrins also accelerate the disruption of all the hydrocarbons types, affecting their kinetics of plant development, biomass is produced in higher efficiency and there is better usage of hydrocarbons as energy source. The low production cost and the effective disruption are the two main reasons that make β-cyclodextrins to be a useful tool to the process of biological soil remediation [26].

6 CONCLUSIONS

Cyclodextrins is a compound category, which through the encapsulation ability with different chemical substances, are the key for many future solutions in Chemistry issues. The ability that cyclodextrins have to form inclusion compounds with many molecules of guest type, trapping the whole molecule or part of it at the concaveness that exists at their interior, is a unique encapsulation technique, which affects many of the physicochemical properties of type guest molecules. The ability that cyclodextrins have to form inclusion compounds with a great variety of organic substances help at the change of molecule solubility, at the stability increase of substances at the presence of light, heat and oxidation conditions and at the decrease of chemical compound's volatileness. These properties and their unique chemical architecture resulted at the continuous increasing number of actual applications of cyclodextrins in foods, pharmaceuticals, agriculture and in creative diverse chromatography techniques.

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