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COMPARISON STUDY OF CHLORINATED PESTICIDES IN ANIMAL PRODUCTS WITHIN FOURTEEN YEARS IN JORDAN

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ABSTRACT

Seven studies were conducted in the period between 1995 and 2008 to monitor chlorinated pesticides in animal products of Jordan. Comparison of the results shows that residues of chlorinated pesticides were found to be low in animal products of the successive years from 1995 towards 2008. Percentages in milk product samples containing chlorinated pesticides residues were also low, and less or more than the MRL in progressive years from 1995 towards 2008. Results of the seven studies on red meats and chicken samples showed few or no chlorinated pesticide residues in the last three studies. Chlorinated pesticide residues in egg samples were almost constant in the last three studies. More studies are needed to monitor the residues of pesticides in table eggs.

KEYWORDS: Comparison, seven studies, residues, chlorinated pesticides, animal products, Jordan.

1. INTRODUCTION

Pesticides are toxic chemicals used to control pests attacking crops and livestock. There are numerous pesticides used to control insects in livestock farms.

Industrial livestock farms apply these pesticides to control ectoparasites, particularly insects, mites and ticks on cattle and poultry. The animal feed is also exposed to pesticides to control insects. The pesticide residues accumulate in the fatty tissues of animals, thus reaching human beings through the route of food chains. Many people carry pesticide residues in their bodies, particularly chlorinated compounds [1]. These facts push Ministry of Environment in Jordan to ask several scientific official organizations to monitor the chlorinated pesticide residues in different elements of the environment, particularly animal products.

Despite that chlorinated pesticides have been banned in Jordan since the early eighties of the previous century, the investigations showed their occurrence in various commodities including animal products [2, 3]. Several countries have already done monitoring and researches on the residues of chlorinated pesticides in the environment [4] as well as in animal products [5, 6]. The concern has been adopted on maximum residue levels (MRL) for pesticides in foodstuff of animal origin, depending on standards recommended by developed countries or international organizations. The Ministry of Environment in Jordan, through the Steering National Pesticides Residue Committee, has monitored chlorinated pesticide residues in animal and other product since 1995.

It is the aim of this investigation to compare between residues of chlorinated pesticides found in local and imported animal products in Jordan through seven studies in the period 1995 to 2008.

2. MATERIALS AND METHODS

The international analysis method [7] has been adopted by the laboratories of the Royal Scientific Society in Amman (Jordan) for extracting the residues of chlorinated pesticides from animal products.

2.1. Glassware and Tools

A Soxhlet extractor, separatory funnels with glass or Teflon stopcocks, chromatography columns (id = 20 mm, l = 40-50 cm) with Teflon-stopcocks, round bottom flasks, and volumetric flasks were used. All parts of glassware were washed thoroughly with water, soap water, distilled water, acetone and finally hexane, dried in a drying oven at 120 °C, and kept tightly closed after cooling to room temperature. Each glassware piece was washed several times with acetone prior to use.

2.2. Standards, Solvents, Chemicals and Gases

A certified standard mixture containing all the targeted chlorinated pesticides (purity 99.5-99.9%, 1000 ppm; see...
Table 1) purchased from Ehrenstorfer (Germany) was diluted to 0.1 ppm with n-hexane containing 0.1 ppm isodrin as internal standard.

Petroleum ether (40-60 °C), dichloromethane, acetone, n-hexane, methanol, acetonitrile and diethyl ether of pesticide residue grade were purchased from Riedel-deHaën (Hannover-Germany) and used as solvents without further purification. Double-distilled water was prepared for use.

Anhydrous sodium sulfate (grade “for residue analysis”) was heated at 550 °C for 2 h and kept in a closed container. Florisil of “pesticide residues grade” (60-100 mesh) was also heated at 550 °C for 12 h, kept in a closed container and heated once again at 130 °C for 1 h prior to use. Quartz wool was extracted with petroleum ether. Helium used as carrier gas was of 99.999% purity (grade 5). Argon/methane (95/5% v/v) was used as make-up gas (99.9% purity).

2.3. Gas Chromatography
A HP 5890 gas chromatograph, equipped with two capillary columns and a 63Ni-electron capture detector (ECD), was used. The first column was a DB-1701 (30 m x 0.32 mm, film thickness 0.25 µm, moderately polar). The second column was a HP-5 (30 m x 0.32 mm, film thickness 0.25 µm, non-polar). Carrier gas (He) was used at a flow-rate of 2 ml/min. The make-up gas (Ar-CH₄) was used at a flow-rate of 30 ml/min. Injector temperature was 280 °C, while detector temperature was 300 °C. The column temperature program was as follows: Initial temperature 80 °C (2.2 min), 80-175 °C (30 °C/min), 175-225 °C (10 °C/min), and 225 °C (2 min). HP-3395 integrator operated at a chart speed of 5 mm/min was used. Injection volume was 1 µl, and split ratio was 1:25.

2.4. Extraction Methods
2.4.1. Extraction of Fat from Meat Products
A meat sample of 2-3 kg was cleaned from the bones, and then minced with a meat mixer. A 20-30 g aliquot of the homogenous sample was weighed and mixed with 50 g anhydrous sodium sulfate. The sample was placed in a thimble and extracted for 6 h in a Soxhlet apparatus using 250 ml petroleum ether. The extract was rotary-evaporated (12 mbar) nearly to dryness at 30 °C. The residue was left in desiccators for half an hour, and then the fat residue was weighed to obtain % fat in the sample.

2.4.2. Extraction and Determination of Fat Content from Milk Products
Milk sample with less than 3 g fat was placed in a 250-ml separatory funnel with 15 ml petroleum ether and 30 ml of acetonitrile saturated with petroleum ether, and then mixed thoroughly for 3 min. The organic layer was separated and gathered in a 1-L separatory funnel. Two portions each of 30 ml of acetonitrile saturated with petroleum were added to the aqueous layer in the 250-ml funnel, shaken well and then added to the 1-L funnel. Then, 600 ml distilled water, 40 ml saturated sodium chloride solution and 100 ml petroleum ether were added, and mixed thoroughly. The aqueous layer was separately placed in another separatory funnel with 100 ml petroleum ether, mixed thoroughly and then, the aqueous layer was discarded. The remaining organic layer was added to the 1-L separatory funnel. The pooled organic extracts were passed through anhydrous sodium sulfate, and then evaporated using the rotary evaporator. At this stage, the % fat can be calculated.

The fat residues (meat and milk) were dissolved in petroleum ether and passed through an activated Florisil column for clean-up using petroleum ether/dichloromethane (80/20, v/v) as eluent. The eluate was collected in a 500-ml round bottom flask and evaporated to dryness at 30 °C and 12 mbar. The residues were dissolved in 2 ml n-hexane containing 0.1 µg isodrin per ml (internal standard). This final solution was ready for GC injection.

2.5. Recoveries and Quality Control
For the evaluation of the extraction efficiency, blank samples were spiked with standard solution mixture of the studied pesticides, and the spiked samples were analyzed using the above-mentioned procedure. Table 1 shows the

<table>
<thead>
<tr>
<th>Pesticide</th>
<th>Detection limit (mg/L)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-HCH</td>
<td>0.004</td>
<td>84.4±2.5</td>
</tr>
<tr>
<td>HCB</td>
<td>0.004</td>
<td>85.5±2.7</td>
</tr>
<tr>
<td>B-HCH</td>
<td>0.004</td>
<td>84.3±2.9</td>
</tr>
<tr>
<td>γ-HCH</td>
<td>0.004</td>
<td>83.8±2.2</td>
</tr>
<tr>
<td>Heptachlor</td>
<td>0.004</td>
<td>83.8±2.1</td>
</tr>
<tr>
<td>Aldrin</td>
<td>0.004</td>
<td>82.2±2.8</td>
</tr>
<tr>
<td>a,p-DDDE</td>
<td>0.004</td>
<td>84.9±4.3</td>
</tr>
<tr>
<td>a –Endosulfan</td>
<td>0.004</td>
<td>84.8±3.9</td>
</tr>
<tr>
<td>Dieldrin</td>
<td>0.004</td>
<td>84.0±3.5</td>
</tr>
<tr>
<td>p,p′-DDE</td>
<td>0.005</td>
<td>83.0±3.6</td>
</tr>
<tr>
<td>O,p′-DDD</td>
<td>0.004</td>
<td>81.5±5.4</td>
</tr>
<tr>
<td>Endrin</td>
<td>0.005</td>
<td>71.8±8.8</td>
</tr>
<tr>
<td>β-Endosulfan</td>
<td>0.004</td>
<td>83.6±3.0</td>
</tr>
<tr>
<td>p,p′-DDD</td>
<td>0.005</td>
<td>82.4±5.1</td>
</tr>
<tr>
<td>O,p′-DDT</td>
<td>0.005</td>
<td>82.5±3.1</td>
</tr>
<tr>
<td>p,p′-DDT</td>
<td>0.004</td>
<td>81.2±1.5</td>
</tr>
</tbody>
</table>
TABLE 2 - Number of samples in the different animal products taken from different regions in Jordan in the 7 studies between 1995 and 2008.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk products</td>
<td>248</td>
<td>42</td>
<td>40</td>
<td>46</td>
<td>20</td>
<td>25</td>
<td>60</td>
</tr>
<tr>
<td>Red meat and chicken</td>
<td>252</td>
<td>80</td>
<td>60</td>
<td>54</td>
<td>80</td>
<td>44</td>
<td>75</td>
</tr>
<tr>
<td>Table eggs</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>25</td>
<td>31</td>
<td>55</td>
</tr>
<tr>
<td>Total Animal products</td>
<td>500</td>
<td>122</td>
<td>100</td>
<td>100</td>
<td>125</td>
<td>100</td>
<td>190</td>
</tr>
</tbody>
</table>

calculated percent recoveries, and the detection limits for the used ECD. Quality control program was applied through the food analysis performance assessment scheme (FA-PAS) in Britain. Quality control on samples was done nationally through the cooperation with the pesticides analytical laboratory in the Environment Research Center of the Royal Scientific Society, and with the Ministry of Agriculture, to ensure the accuracy of the analysis.

2.6. Sampling

Sampling was done according to methods adopted by FAO [8], particularly samples collection, storing and transferring from collection regions to the laboratory. The samples were transferred in an ice box. All needed information about samples was recorded, and all collected samples were kept in a special refrigerator for this project.

In these studies, animal products were divided into 4 groups. Group 1 contained fresh milk products (sheep milk, white cheese, yellow cheese, Labaneh and Jameed). Group 2 contained local chicken meat products and imported chicken meats. Group 3 contained red meats and their products, which are Balady (local) and imported meats of lamb and calf. Group 4 contained Balady and farm table eggs.

Table 2 shows the number of animal product samples taken from different regions of Jordan in the course of the 7 studies in the period 1995-2008. The total number of all samples was 1237.

3. RESULTS AND DISCUSSION

The first study conducted in 1995 included 500 samples distributed on all available animal products. The 2000 study included 122 samples of animal products, except eggs, infant’s milk, yoghurt, lamb fat and Jameed. Each of the 2001 and 2003 studies included 100 samples (eggs, infant’s milk, yoghurt, Jameed, local and imported lamb and chicken meats). The 2004/2005 study included 125 samples of eggs, milk products, and lamb meat, local and imported calf meat, as well as local and imported chicken. The 2005/2006 study included 100 samples of eggs, milk, sheep, as well as local and imported lamb meat and chicken. The 2007/2008 study included 190 samples of eggs, milk products, and lamb meat, local and imported calf meat, and local or imported chicken.

Figure 1 shows the results for the 7 studies of all animal products. The highest percentage (43%) of samples containing pesticide residues less than the maximum residue level (MRL, see Table 3) was in the year 2001. This percentage decreased to 35% in the studies of 2003 and 2004/2005. The least percentage of 19% was found in the study of the year 2007/2008. Figure 1 also shows the percentage of the samples containing pesticide residues more than the MRL. The highest percentage (5%) of the analyzed samples was found in 1995, and the least percentages (2-3%) in all other studies. The above-mentioned results in-
TABLE 3 - MRL values of pesticide residues in foodstuff of animal origin (86/363/EEC).

<table>
<thead>
<tr>
<th>Pesticides</th>
<th>Food MRL (mg/kg)</th>
<th>Milk and milk products</th>
<th>Meat</th>
<th>Poultry and eggs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aldrin, Dieldrin</td>
<td>0.006</td>
<td>0.2</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>Total DDTs*</td>
<td>0.04</td>
<td>1</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>Heptachlor**</td>
<td>0.004</td>
<td>0.2</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>(γ)-HCH</td>
<td>0.008</td>
<td>2</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>(α)-HCH</td>
<td>0.004</td>
<td>0.2</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>(β)-HCH</td>
<td>0.003</td>
<td>0.1</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>HCB</td>
<td>0.01</td>
<td>0.2</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>Endrin</td>
<td>0.0008</td>
<td>0.05</td>
<td>0.005</td>
<td></td>
</tr>
</tbody>
</table>

* DDTs (sum of all six DDT isomers); ** Heptachlor (sum of heptachlor and heptachlor epoxide).

FIGURE 2 – Percentages of milk product samples containing chlorinated pesticide residues less and more than the MRL for 7 studies between 1995 and 2008 in Jordan.

Figure 2 shows the results for the 7 studies of milk products. The highest percentage (40%) for samples containing pesticide residues less than the MRL was in the year 2004/2005, and then decreased to 16% in 2005/2006 and 15% in 2007/2008. The least percentage of 5% was found in the study of the year 2000. Figure 2 also shows the percentage of the samples containing pesticide residues more than the MRL, and the highest percentage (10%) was found in the study of the year 2004/2005. This percentage decreased to 5% in 2007/2008, and to 0% in the study of the year 2005/2006. These results lead to the conclusion that percentages of the studied samples containing pesticide residues less or more than the MRL were relatively low in the progressive years of the studies towards the last study in 2007/2008. Nevertheless, milk and milk products present the principle items in the diet of infants and children, and should receive special considerations [1, 10].

Figure 3 shows the results for the 7 studies in red meats and chicken. The highest percentage (47%) for samples containing pesticide residues less than the MRL was found in the study year 2001. This percentage decreased gradually to 27% in the year 2005/2006, but it increased slightly in the study of 2007/2008. Figure 3 also shows the percentage of samples containing pesticide residues more than the MRL. The highest percentage of 3% was in the years 1995, 2000, and 2001 but decreased to 0% in the periods 2004/2005 to 2007/2008. These results lead to the conclusion that few or no pesticide residues occurred in the last three studies. However, there is a need to monitor the residues of chlorinated pesticides in animal feed products through the different steps of animal feeding from storage to feeding [11, 12].

Figure 4 shows the results for 3 studies on table eggs. The highest percentage (24%) of samples containing pesticide residues less than the MRL was in the year 2004/2005, and it decreased to 16% in 2005/2006, but then increased slightly to 18% in 2007/2008. Figure 4 also shows the percentages of samples containing pesticide residues more than the MRL being lowest (4%) in 2004/2005 but slightly increasing to 6% in 2005/2006 and 7% in 2007/2008. Pesti-
cide residues in eggs were approximately similar in the last three studies. More studies are needed to monitor the pesticide residues in table eggs. It is worthy to mention that, in the last joint meeting of FAO/WHO, the total pesticides’ MRL in eggs (shell-free) was recommended not to exceed 0.2 mg/kg [12, 13].

It was observed that 70 and 42% of the pesticides, found in samples of 1995 and 2000 respectively, were from cyclohexane derivatives, particularly lindane, β-HCH and α-HCH. In 1995, 68% of the samples exceeding the MRL contained cyclodienes (heptachlor epoxide and heptachlor), particularly in local liver samples. In 2000, the samples which exceeded the MRL had 33.3% residues, each of heptachlor, aldrin and DDT, particularly in white cheese, imported lamb and calf samples. In 2001, 40 and 44% of the samples containing pesticide residues showed presence of persistent compounds belonging to cyclodiene and DDT family, respectively. There were residues exceeding MRL in one imported “Jameed” sample, local calf meat and “Balady” egg samples. In the study of 2003, 68%, 8%, 24%, 18% and 8% of the samples contained α-HCH, lindane, β-HCH, DDT and heptachlor, respectively. In 2004/2005, 33%, 19%, 19% and 10% of the samples contained α-HCH, β-HCH, p,p’-DDT and p,p’-DDE, respectively (amounts <MRL). However, one sample of farm eggs and
another one from “Balady” eggs contained \( p,p' \)-DDT and heptachlor, respectively, exceeding the MRL. In 2007/2008, 22 and 78% of the analyzed samples contained \( \alpha,\beta,\gamma \)-HCH and \( p,p' \)-DDT, respectively. However, 95% of the samples contained residues <MRL. Seven samples contained residues of \( p,p' \)-DE, \( p,p' \)-DDT, heptachlor, \( \alpha,\gamma \)-HCH, HCB, endrin and aldrin, being >MRL, particularly in one farm egg, one sheep labaneh and one yellow cheese sample.

4. CONCLUSIONS

Relying on the results of these studies, it is recommended to continue monitoring the foods of animal origin in Jordan to be sure that the pesticide residues not exceed the MRL. It is recommended to propose national Jordanian standards for chlorinated pesticides in various animal products.

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INVESTIGATION OF EFFECTS ON PLASMA NITRIC OXIDE, MALONDIALDEHYDE AND TOTAL SIALIC ACID LEVELS OF GLYPHOSATE IN KARS CREEK TRANSCAUCAUSIAN BARB (CAPOETA CAPOETA [GULDENSTAEDT, 1773]) IN TURKEY

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ABSTRACT

In this study, Capoeta capoeta was used to determine plasma total sialic acid (TSA), nitric oxide (NO) and malondialdehyde (MDA) levels in fishes applied glyphosate. The animals in group I in normal water environment, group II and group III sequentially in aquariums added 0.01 and 0.02 mg/L glyphosate were waited for 10 days. Plasma TSA levels in group II animals were higher than group I and group III. There was not significantly difference between groups in terms of plasma NO levels. MDA levels were determined to be higher in group III compared the other two groups.

KEYWORDS: Capoeta capoeta, glyphosate, total sialic acid, peroxidation.

1. INTRODUCTION

Glyphosate [-(phosphonomethyl)-glycine] is a herbicide with wide spectrum inclusive of aminophosphonate, promiscuous, systematic effective used widely in weed control in agricultural regions all over the world [1-3]. It is preferred more than the other herbicide in terms of its half life period, toxicity and mobility [4,5]. It was reported that morphological, physiological, immunological and biochemical changes can appear on aquatic flora and fauna since glyphosate solubility is high in water [6,7].

It is pointed out that tissue and cells of fish are very suitable lipid oxidation because high amount of polyunsaturated fatty acid [8]. As well as one of the most important products of oxidation is malondialdehyde (MDA) which has carcinogenic and mutagen characteristics, levels of that last product are used as an indicator of lipid peroxidation [8,9]. Peroxide occurred in consequence of lipid peroxidation also constitutes peroxynitrite by giving reaction with nitric oxide (NO) affecting organic matters [10,11]. Sialic acids (SA) have the ability of being ionized because of their carboxyl groups in their structures, and are in charge of electrostatic impulse in trombosite, erythrocyte and cancer cells because of negative charge they carry [12].

Capoeta capoeta is declared as the most wide-spread transcaucasian barbs and they are economically and ecologically important in Kars Creek among the fishes [13]. Physiological and biochemical effects of glyphosate on a lot of freshwater organisms especially fishes have been researched [14-17]. However, there were not any studies investigating the effects of glyphosate on Capoeta capoeta from freshwater fishes. Therefore, it was aimed to study the NO, MDA and plasma TSA levels on Capoeta capoeta applied glyphosate.

2. MATERIAL AND METHODS

In this study, 26 Capoeta capoeta whose weights about 200-250 g were used. Fishes caught in Kars Creek were put into tanks each by 500 liters. After adaptions of fishes to laboratory environment during 15 days, 3 groups were constituted of 8 in control group, 9 in other groups. Fishes in group I in normal water environment, group II and group III fishes sequentially were waited in water environments with 22±2 °C, 7.4 pH including 0.01 and 0.02 mg/L glyphosate (N-phosphono-methyl glycine, 480 mg/L, Korfosat 48 SL, alkaline chlorine protection, Izmit, Turkey) for 10 days. At the end of application, blood samples were taken by syringe from fish hearts. Blood samples were taken into the tube with EDTA and centrifuged at 3000 rpm for 10 minutes in +4 °C, and the plasma samples were separated. Specimens were stored at -20 °C until being analyzed.

* Corresponding author
Nitric oxide levels were appointed according to the method described by Miranda et al. [18] as colorimetrically. To this NO levels were fixed with absorbance rates read in 540 nm as spectrophotometrically with total levels of nitrate and nitrite levels in plasma. Plasma TSA analyses were carried out according to method pointed out by Sydow [19] as spectrophotometrically. MDA levels were determined with absorbance levels of pink color occurred through thiobarbituric acid (TBA) and MDA read 532 nm by using spectrophotometric measurement method pointed out by Draper and Hadley [9].

2.1. Statistical Analysis

Descriptive statistic analyses were conducted for statistical evaluations of data. Results were expressed as mean (X) ± standard deviation (SD). Significance of the degree of difference among group averages was determined through analysis of variance (ANOVA) and Duncan multiple comparison test using statistic packet program (SPSS 15.0 for Windows).

3. RESULTS

Biochemical changes indicated in Table 1 and Figure 1 occurred in blood samples taken after 10 days from fishes added 0.01 and 0.02 mg/L glyphosate to their life environments with control group fish not added glyphosate. In plasma TSA and MDA levels than control group in group II and group III statistically considerable increase (p<0.001) were determined in NO levels statistically considerable difference were not been found.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control (n=8)</th>
<th>Glyphosate (n=9) (0.01 mg/L)</th>
<th>Glyphosate (n=9) (0.02 mg/L)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO (µmol/L)</td>
<td>40.03±7.59</td>
<td>41.51±18.05</td>
<td>27.95±11.23</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td>TSA (mg/dl)</td>
<td>67.73±10.29a</td>
<td>108.72±17.61b</td>
<td>78.05±10.91a</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>MDA (µmol/L)</td>
<td>11.66±1.64a</td>
<td>14.74±1.95a</td>
<td>28.14±6.54b</td>
<td>p&lt;0.001</td>
</tr>
</tbody>
</table>

Values with different letters within the same row indicates significant differences (p<0.001)

4. DISCUSSION

Studying of the relationship between oxidative stress and sialic acid levels with glyphosate is important in terms of decreasing of the herbicide pollution and using in appropriate ratio. Fishes may be used as indicators to take earlier measurements against aquatic environmental pollution [20,21]. In studies carried out, was found out that the toxicity was different in a lot of aquatic organisms [22-26]. Ayoola [28] showed that the 96-h LC50 value of glyphosate (herbicide) was 0.04 mg/L. The corresponding glyphosate doses used in the present study are consonance with earlier reports [26-28]. It was pointed out that high glyphosate concentration increased toxicity [28]. Although
MDA is a highly toxic aldehyde, there appears to be no direct data in the literature on its oral LD₅₀ values in humans or experimental animals. However studies have shown that exposure of human erythrocytes to a very low concentration (50 µmolar) of MDA brought about early redox impairment, leading to depletion of reduced glutathione, glucose-6-phosphate dehydrogenase and oxygenated hemoglobin [29]. Mendes et al. [8] reported that MDA level in hake and sardine was about 29 and 700 µmol/kg, respectively [8]. It is declared that when tissue fat ratio in sardine increase, MDA level will also increase [30]. Chaijan et al. [31] claimed that this level could exceed to 8000 µmol/kg. In aquatic environment, because of the increase of toxic matter content, decrease in the amount of dissolved oxygen has been declared [32]. It was suggested that the mechanisms of up-regulation of antioxidant enzymes could involve the response to enhanced ROS level at transitions from normoxia via hypoxia to anoxia [33]. Lushchak et al. [34] found that hypoxia also increased the activities of SOD and catalase in liver of common carp Cyprinus carpio. Also, by exploiting another fish model was demonstrated oxidative stress development in rotan Percottus glenii under hypoxic conditions [35]. This might result from not protecting the balance between ROS production and reduction against variable environmental conditions. In this study, we suggest that one of the main reasons of statistically important increase in Capoeta capoeta MDA levels are related to failure in respiratory function and amount of dissolved oxygen related to glyphosate doses. Fish MDA levels observed in aquarium without glyphosate were lower level than other groups. This demonstrates that the high MDA level seen in samples was due to glyphosate exposure.

Henrick et al. [36] reported that O²⁻ production increased in vascular system when removing SAs in neutrophils. It was claimed that SAs had an antioxidative function which was responsible for removing O²⁻ from vascular system in living organisms [36,37]. Also, it was reported that oxidative stress could start to release of the SAs from the oligosaccharides in cell surface without sialidase activation or induction [38]. In our study, it was determined that TSA levels including both free and bound SA levels significantly changed. Thus, it is confirmed that SAs are important during the stress resulting from certain dose of glyphosate. It was recorded it had harmful effects such as NO’s forming peroxynitrite, DNA damage and disorder in enzyme functions, and it had protective and reduction against variable environmental conditions.

Consequently, with contamination of glyphosate (0.01 and 0.02 mg/L) in aquatic environment, it was observed that plasma TSA and MDA levels related to lipid peroxida-

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SEASONAL DYNAMICS AND PHYLOGENETIC ANALYSIS OF BACTERIOPLANKTON IN LAKE ERHAI, SW-CHINA

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ABSTRACT

The community composition and seasonal variation of bacterioplankton were investigated using 16S rRNA gene sequence analysis in high mount Lake Erhai, China. Restriction fragment length polymorphism (RFLP) analysis revealed remarkable seasonal fluctuations in bacterioplanktonic composition. A total of 1498 clones were obtained from 16 water samples, and most of clones belonged to the clusters α-Proteobacteria, β-Proteobacteria, γ-Proteobacteria, Actinobacteria, Bacteroidetes, Verrucomicrobia, and Cyanobacteria. Among these groups, α-Proteobacteria was the identified as most dominant group in spring and summer. However, the structure shifted toward a β-Proteobacterium-dominated community in autumn and winter. The clone libraries were found having the greatest diversity in winter, and some species only appeared in their specific seasons. The number of operational taxonomic units (OTUs) predicted for the Lake Erhai ranged from 42.7 to 98.0, significantly lower than those detected in other types of environments [6, 7], and their communities structures were sensitive to pH, water temperature, lake water retention time, and other environmental factors [8, 9]. Cyanobacteria, Bacteroidetes, α-, β-, and γ-Proteobacteria, Actinobacteria, and Verrucomicrobia were the most frequent clusters present in freshwater, and many were widely distributed in lakes with different characteristics and different origins [10, 11]. Pearce et al. [12] reported that the bacteria community from an oligotrophic lake contained a cluster of bacteria also known to be important in other freshwater ecosystem. Glücknor et al.[13] did a research in three fresh water lakes with large regional difference and found that although the major groups of bacteria community of the three lakes were all belong to Bacteroidetes, Actinobacteria, α-, β-, and γ-Proteobacteria, between different lakes there existed obvious difference. Lindström’s [14] study on five lakes of Swedish suggests that the region and nutrition status of lakes had obvious influence on the diversity of bacterioplankton.

1. INTRODUCTION

Bacterioplankton play a key role in planktonic ecosystems [1, 2], such as nutrient recycling in aquatic systems [3]. They have a special relationship with other species and the environmental factors. Previous studies showed that composition of bacteria community changed with nutrient levels and other environmental conditions [4, 5]. Recent studies indicated that many bacteria groups found in lakes and rivers were different from those detected in other types of environments [6, 7], and their communities structures were sensitive to pH, water temperature, lake water retention time, and other environmental factors [8, 9]. Cyanobacteria, Bacteroidetes, α-, β-, and γ-Proteobacteria, Actinobacteria, and Verrucomicrobia were the most frequent clusters present in freshwater, and many were widely distributed in lakes with different characteristics and different origins [10, 11]. Pearce et al. [12] reported that the bacteria community from an oligotrophic lake contained a cluster of bacteria also known to be important in other freshwater ecosystem. Glücknor et al.[13] did a research in three fresh water lakes with large regional difference and found that although the major groups of bacteria community of the three lakes were all belong to Bacteroidetes, Actinobacteria, α-, β-, and γ-Proteobacteria, between different lakes there existed obvious difference. Lindström’s [14] study on five lakes of Swedish suggests that the region and nutrition status of lakes had obvious influence on the diversity of bacterioplankton.

Seasonal changes had partial influence on biological communities of lake [15, 16]. Wu et al. [17] collected water samples from the same location of Lake Taihu in March, May, July and September, 2004 and obtained a total of 336 clones and 142 OTUs from four 16S rRNA clone library, which mostly belonged to α-, β-, and γ-Proteobacteria, Bacteroidetes and Actinobacteria. They found that the samples in July were very different from the other three water samples. In addition, phylogenetic analysis showed that sequences of which 53 were divided into six new categories which indicated these may be related to a unique environment. However, most of the results pertaining to bacterioplankton diversity in freshwater were
yielded from analysis on samples of oligotrophic and low altitudes lakes. Studies had shown that the structure of bacterioplankton communities altered by differently environmental conditions in eutrophic lakes [4, 18]. High altitude lakes were a relatively common ecosystem, which remained less intensively studied than lowland lakes. Significantly, the high altitude lake ecosystem is a sensitive reference for global climatic change and other human impacts. Lake Erhai is a shallow eutrophic lake located in Yunnan province of southwestern China. Previous studies performed on phytoplankton, fish, and water quality, but the bacterioplankton community in this plateau lake has not been studied. To this end, the composition of community and seasonal variation of bacterioplankton in Lake Erhai were investigated by constructing clone libraries of 16S rRNA gene and RFLP method.

2. MATERIALS AND METHODS

2.1. Sampling sites

Lake Erhai, located on the Yun-Gui plateau in the southwestern of China (25°25’N to 26°16’N, 99°32’E to 100°27’E, 1972 m above sea level), is the second largest freshwater lake in Yunnan Province, with a surface area of 256.5 km², an average depth of 10.6 m, and a maximum depth of 21.3 m. It is a shallow eutrophic water body with a total volume of about 27.4 million m³.

Surface water samples (top 0.5m) were collected from four different locations (Fig. 1) in July, October, 2009 and in January, April, 2010. Water temperature was measured using YSI550A dissolved oxygen meter. 1L water samples were used to analyze the concentrations of total nitrogen (TN), total phosphorus (TP), ammonia (NH₃-N), and nitrate (NO₃-N) according to the standard methods in the lab [19, 20]. 1L water samples were filtered onto Whatman GF/C filters, air dried and then stored frozen for Chlorophyll a (Chl. a) analysis. The filters were extracted in ethanol and measured spectrophotometrically [21]. For phylogenetic analyses, 1L water samples were prefiltered through 3 μm pore-size filters, and then the filtrates were passed through 0.2 μm pore-size filters and stored at -20 ºC until DNA extraction.

2.2. DNA extraction and PCR analysis

Total DNA was extracted by a standard phenol chloroform method, and the 16S rRNA genes were amplified using the bacterial universal primers 27f (5’-AGAGTTTGTCTCAGGTTACCTTGTTACGACTT-3’) and 1492r (5’-GGTACGAGTACCTTGTTACGACTT-3’). Polymerase chain reactions were performed in 50 μl volumes, containing 1×PCR buffer, 200 μM of each dNTP, 1.5 mM MgCl₂, 0.1 μM of each primer, and 0.5U of Taq DNA polymerase (MBI Fermentas). Thermal cycle conditions included an initial denaturation step at 94°C for 5 min, 34 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1.5 min, followed by
a final extension step at 72°C for 10 min. Reaction products were verified on 1.2% agarose gels and purified using the Axygen PCR clean-up kit.

2.3. Construction of the clone library and 16S rDNA RFLP analysis

The PCR products were ligated into pMD-18T vector (Takara, Japan), and transformed into E. coli DH 5α cells. Positive clones were verified by the means of the amplification reaction described above. Aliquots (10 µl) of PCR product were digested with 10 U each of restriction endonucleases HhaI and XbaI (MBI Fermentas) for 3 h at 37 ºC [22]. Digested products were separated by 2.5% (w/v) agarose gel electrophoresis in 1×Tris-acetate-EDTA buffer. Clones with the same restriction fragment pattern were grouped, and those exhibiting unique restriction fragment length polymorphism (RFLP) patterns were selected for sequencing.

2.4. Sequencing and phylogenetic analysis

According to the RFLP maps, at least one clone from each RFLP pattern was sequenced using M13 forward sequencing primer. Sequences obtained were compared to those in GenBank by BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) and the closest sequences were aligned by CLUSTALX 1.81 [23]. A phylogenetic tree was constructed by MEGA 4 software (http://www.megasoftware.net/).

2.5. Nucleotide sequence accession number

Sequences of the rDNA clones were submitted to GenBank and assigned GenBank accession numbers: HQ852947-HQ853015 and HQ853440-HQ853450.

2.6. Analysis of bacteria diversity

The richness of the bacterial community was estimated by the non-parametric estimator of Chao [24]: S= S_{obs}+a^2/2b, where S_{obs} is the number of OTUs observed in the study; a is the number of OTUs detected only once; and b is the number of OTUs detected only twice. Evenness was estimated by Simpson’ index (D)[25]: D=Σn_i/(N(N-1)).

The coverage of the clone library was computed by a formula: C= [1-(n_i /N)] [26], where n_i is the number of OTUs in the clone library, and N is the total number of clones in the sample.

The relationship between the relative abundance of bacterioplankton and environment factors was analyzed by partial redundancy analysis (RDA) with Canoco for Windows 4.5.

3. RESULTS

3.1. Physical and chemical characteristics of Lake Erhai

As shown in Fig. 2, the physical and chemical characteristics of Lake Erhai varied with seasons. The highest temperature was in July 2009 (22.5 ºC) and the lowest was in January 2010 (15.8 ºC). The concentrations of TN and TP ranged from 0.268-0.981 mg l⁻¹ and 0.004-0.069 mg l⁻¹ respectively, showing similar tendency in seasonal change, as higher in summer and autumn, but lower in winter and spring. The maximal concentration of nitrate (0.302 mg l⁻¹) was detected in April 2010, and the minimum (0.062 mg l⁻¹) in January 2010. The highest concentration (0.0467 mg l⁻¹)
of Chl. \(a\) appeared in October 2009, and then began to decrease and reached the lowest level (0.0027 mg l\(^{-1}\)) in April 2010.

3.2. Clone library construction and RFLP analyses

A total of 1498 positive clones were obtained from the sixteen samples taken from Lake Erhai after removing the negative clones. Eighty unique OTUs were identified by RFLP analysis, and representative clones of each sample were forward sequenced. Phylogenetic analyses showed that 24 OTUs had less than 98% similarity with known sequences, while the others had 98% or greater homology to the known species. In general, 98% of the phylotypes belonged to seven distinct clades: Cyanobacteria, \(\alpha\)-, \(\beta\)-, and \(\gamma\)-Proteobacteria, Actinobacteria, Bacteroidetes, and Verrucomicrobia. Only 2% of the phylotypes were unclassified bacteria. According to the relative abundance of OTUs, Proteobacteria were the most abundant bacteria throughout the year and changed seasonally; among them, \(\alpha\)-Proteobacteria was the most abundant group with relative abundance 44.2% and 55.8% in summer and spring, respectively. \(\beta\)-Proteobacteria dominated the bacterioplanktonic community with a relative abundance of 34.1% in autumn and 51.2% in winter. Actinobacteria was the second abundant group (Fig.3). A phylogenetic tree was constructed using the neighbor-joining (NJ) method based on the sequences and comparisons with their closest sequences (Fig. 4).

3.3. Bacterial community composition and diversity in Erhai Lake

Coverage of the constructed clonal libraries varied from 88.5% to 92.6% in different seasons, which indicated that the clone libraries represented the most ribosomal OTUs in all samples (Table 1). 90% of the OTUs were similar to the uncultured bacteria, while 13 OTUs were present all year. The most abundant clone was A7 (Table 2), which shared 99.9% similarity to the uncultured bacterial clone MYW1 from oligotrophic lakes (YangHe Reservoir and MinYun Reservoir, China) and 99.8% to the uncultured SAR11 cluster \(\alpha\)-Proteobacterium clone ZS-4-50 from temperate lakes. The second abundant clone was B24 (Table 2), which was affiliated to \(\beta\)-Proteobacteria and 99.7% similar to the uncultured clone B07-32-BAC from the Marathonas Reservoir in Greece. The third most abundant clone was B22 (Table 2), which was also \(\beta\)-Proteobacteria and was of 98.7% homologous to the uncultured bacterium clone 3C002712 from marine ecosystem. The percentage of the clone A7, B24 and B22 of the total clones was 34.3%, 10.6% and 6.2% respectively.

The composition and diversity of the planktonic bacteria community varied with seasons. The highest richness and diversity were observed in winter (Table 1), while the second highest richness of bacterial community structure in summer. Some phylotypes were only detected in specific seasons.

\(\alpha\)-Proteobacteria was the second abundant group in autumn and winter (25.7% and 20.2%), but its relative abundance in winter was the lowest for the whole year. Among this group, clone A7 was the most abundant one out of 14 OTUs. Some phylotypes were only detected in specific seasons, for example, A8 clone was only detected in summer, A19 and A21 clones were only present in autumn, A4, A6, A12, A14, and A15 only in winter, and A23-24 only in spring.

\(\beta\)-Proteobacteria was one of the most common clusters in this study and was the second most abundant group in summer and spring (19.8% and 27.8%). Besides B24 and B22, clones B8 and B17 had a higher abundance in autumn and winter. They were closely related to the uncultured bacterium clone DP7.5.26 and clone D15 respectively from Lake Dongping. Clones B6, B16, and B20 appeared only in winter and their closest relatives were all from aquatic environments.
FIGURE 4 - Phylogenetic tree based on 16S rRNA partial sequences obtained from samples of Lake Erhai and its closest sequence from other environmental clones. Bootstrap values of <50% are not shown. Arcobacter nitrofigilis (L14627) was used as outgroup.

TABLE 1 - Comparison of bacterial diversity in different seasons

<table>
<thead>
<tr>
<th>Season</th>
<th>Total no. of sequences analyzed</th>
<th>No. of OTUs detected</th>
<th>Chao1 estimate</th>
<th>Reciprocal Simpson index</th>
<th>Coverage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Summer</td>
<td>398</td>
<td>41</td>
<td>55.0</td>
<td>4.94</td>
<td>89.7%</td>
</tr>
<tr>
<td>Autumn</td>
<td>331</td>
<td>33</td>
<td>43.7</td>
<td>10.70</td>
<td>90.0%</td>
</tr>
<tr>
<td>Winter</td>
<td>416</td>
<td>48</td>
<td>98.0</td>
<td>13.80</td>
<td>88.5%</td>
</tr>
<tr>
<td>Spring</td>
<td>353</td>
<td>26</td>
<td>42.7</td>
<td>2.97</td>
<td>92.6%</td>
</tr>
</tbody>
</table>
The highest relative abundance of *Bacteroidetes* appeared in autumn (8.5%), and the community succession of this group was similar to Cyanoacteria. Twelve OTUs fell into this group. The most frequent clone was E9; its closest sequence was clone K08-144-BAC from the Marathon Reservoir. In sum, these results demonstrated a remarkable seasonal variation in many species of the bacterial community in Lake Erhai.

The result of partial redundancy analysis (RDA) (Fig. 5) between the relative abundance of bacterioplankton and environmental factor showed that the bacteria community

### TABLE 2 - The main species composition of bacterioplankton observed in Lake Erhai

<table>
<thead>
<tr>
<th>Clones</th>
<th>Closest sequence</th>
<th>Similarity (%)</th>
<th>Phylogenetic affiliation</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>A4</td>
<td>Uncultured alpha proteobacterium clone Hv(lakePohsle), 38 (EF667926)</td>
<td>98.0</td>
<td>α-proteobacteria</td>
<td>Only detected in winter</td>
</tr>
<tr>
<td>A7</td>
<td>Uncultured bacterium clone MYW1 (GU305752)</td>
<td>99.9</td>
<td>α-proteobacteria</td>
<td>Dominant in all seasons</td>
</tr>
<tr>
<td>A15</td>
<td>Alpha proteobacterium 9-29 (AM411930)</td>
<td>97.4</td>
<td>α-proteobacteria</td>
<td>Detected in summer and winter</td>
</tr>
<tr>
<td>A21</td>
<td>Uncultured bacterium clone A07-68-BAC (GQ340094)</td>
<td>99.0</td>
<td>α-proteobacteria</td>
<td>Only detected in autumn</td>
</tr>
<tr>
<td>A23</td>
<td>Hoeflea alexandri strain AM1V3011(AJ786600)</td>
<td>96.7</td>
<td>α-proteobacteria</td>
<td>Only detected in spring</td>
</tr>
<tr>
<td>B2</td>
<td>Uncultured beta proteobacterium clone ZS-2-72 (FN668037)</td>
<td>98.7</td>
<td>β-proteobacteria</td>
<td>Dominant in summer</td>
</tr>
<tr>
<td>B5</td>
<td>Uncultured bacterium clone DP10.4.16(FJ612405)</td>
<td>99.7</td>
<td>β-proteobacteria</td>
<td>Only detected in winter</td>
</tr>
<tr>
<td>B6</td>
<td>Uncultured bacterium clone 3C003438(EU802045)</td>
<td>99.7</td>
<td>β-proteobacteria</td>
<td>Dominant in winter</td>
</tr>
<tr>
<td>B7</td>
<td>Uncultured bacterium clone DP10.2.93(FJ612362)</td>
<td>99.7</td>
<td>β-proteobacteria</td>
<td>Only detected in winter</td>
</tr>
<tr>
<td>B8</td>
<td>Uncultured bacterium clone DPT.5.26(FJ612295)</td>
<td>99.7</td>
<td>β-proteobacteria</td>
<td>Dominant in autumn and winter</td>
</tr>
<tr>
<td>B12</td>
<td>Uncultured beta proteobacterium clone WA0.2-0d-16 (HM153608)</td>
<td>99.1</td>
<td>β-proteobacteria</td>
<td>Only detected in winter</td>
</tr>
<tr>
<td>B14</td>
<td>Uncultured bacterium clone R7C71(DQ450172)</td>
<td>99.2</td>
<td>β-proteobacteria</td>
<td>Only detected in winter</td>
</tr>
<tr>
<td>B16</td>
<td>Uncultured bacterium clone Hot Creek 23(AY168937)</td>
<td>99.6</td>
<td>β-proteobacteria</td>
<td>Detected in spring and summer</td>
</tr>
<tr>
<td>B17</td>
<td>Uncultured bacterium clone D15(EU234291)</td>
<td>99.8</td>
<td>β-proteobacteria</td>
<td>Only detected in summer</td>
</tr>
<tr>
<td>B20</td>
<td>Uncultured beta proteobacterium clone WA0.2-0d-16 (HM153608)</td>
<td>99.7</td>
<td>β-proteobacteria</td>
<td>Only detected in winter</td>
</tr>
<tr>
<td>B22</td>
<td>Uncultured bacterium clone 3C002712(EU801425)</td>
<td>98.7</td>
<td>β-proteobacteria</td>
<td>Dominant in all seasons</td>
</tr>
<tr>
<td>B24</td>
<td>Uncultured bacterium clone B07-32-BAC(GQ340121)</td>
<td>99.7</td>
<td>β-proteobacteria</td>
<td>Dominant in all seasons</td>
</tr>
<tr>
<td>B26</td>
<td>Uncultured bacterium clone 3C002581(EU801319)</td>
<td>95.6</td>
<td>β-proteobacteria</td>
<td>Detected in all seasons</td>
</tr>
<tr>
<td>B28</td>
<td>Uncultured bacterium clone YHW35(GU305530)</td>
<td>99.2</td>
<td>β-proteobacteria</td>
<td>Detected in spring and summer</td>
</tr>
<tr>
<td>B30</td>
<td>Uncultured beta proteobacterium clone 60GS4(EF203860)</td>
<td>99.4</td>
<td>β-proteobacteria</td>
<td>Only detected in summer</td>
</tr>
<tr>
<td>B31</td>
<td>Uncultured beta proteobacterium clone WA0.2-0d-38(HM153624)</td>
<td>99.8</td>
<td>β-proteobacteria</td>
<td>Only detected in summer</td>
</tr>
<tr>
<td>B32</td>
<td>Uncultured bacterium clone 3C003483(EU802084)</td>
<td>100</td>
<td>β-proteobacteria</td>
<td>Only detected in summer and autumn</td>
</tr>
<tr>
<td>B34</td>
<td>Uncultured bacterium clone DP7.4.11(FJ612271)</td>
<td>99.2</td>
<td>β-proteobacteria</td>
<td>Detected in summer and autumn</td>
</tr>
<tr>
<td>C1</td>
<td>Pseudomonas sp. YR07(EU373434)</td>
<td>99.7</td>
<td>γ-proteobacteria</td>
<td>Dominant in summer and winter</td>
</tr>
<tr>
<td>C6</td>
<td>Uncultured bacterium clone DP10.3.24(FJ612378)</td>
<td>98.8</td>
<td>γ-proteobacteria</td>
<td>Dominant in autumn</td>
</tr>
<tr>
<td>C8</td>
<td>Hydrocarboniphaga effusa strain AP102(AJ363244)</td>
<td>98.6</td>
<td>γ-proteobacteria</td>
<td>Only not detected in spring</td>
</tr>
<tr>
<td>C9</td>
<td>Uncultured bacterium clone S0032(FJ820396)</td>
<td>99.9</td>
<td>γ-proteobacteria</td>
<td>Detected in summer and autumn</td>
</tr>
<tr>
<td>C10</td>
<td>Pseudomonas sp. BS1(2009)( GQ281048)</td>
<td>99.9</td>
<td>γ-proteobacteria</td>
<td>Only detected in summer and autumn</td>
</tr>
<tr>
<td>D1</td>
<td>Uncultured bacterium clone MY1-21(GU305741)</td>
<td>100</td>
<td>Actinobacterium</td>
<td>Dominant in summer</td>
</tr>
<tr>
<td>D2</td>
<td>Uncultured bacterium clone 3C002525 (EU801876)</td>
<td>99.8</td>
<td>Actinobacterium</td>
<td>Only detected in autumn</td>
</tr>
<tr>
<td>D5</td>
<td>Uncultured Actinobacterium clone LA1C1(EU117788)</td>
<td>99.6</td>
<td>Actinobacterium</td>
<td>Dominant in autumn and winter</td>
</tr>
<tr>
<td>D14</td>
<td>Uncultured bacterium clone MYY16(GU305709)</td>
<td>99.9</td>
<td>Actinobacterium</td>
<td>Detected in summer and autumn</td>
</tr>
<tr>
<td>D15</td>
<td>Uncultured bacterium clone MYW3(GU305754)</td>
<td>99.9</td>
<td>Actinobacterium</td>
<td>Only not detected in winter</td>
</tr>
<tr>
<td>D19</td>
<td>Uncultured Actinobacterium clone TH1-84(AM690876)</td>
<td>97.7</td>
<td>Actinobacterium</td>
<td>Only detected in summer</td>
</tr>
<tr>
<td>E1</td>
<td>Uncultured bacterium clone DP10.4.3(FJ612397)</td>
<td>99.8</td>
<td>Bacteroidetes</td>
<td>Only detected in spring</td>
</tr>
<tr>
<td>E2</td>
<td>Uncultured bacterium clone SING423(HM129081)</td>
<td>99.3</td>
<td>Bacteroidetes</td>
<td>Only not detected in summer</td>
</tr>
<tr>
<td>E4</td>
<td>Uncultured bacterium clone 2C228668(EU800536)</td>
<td>99.9</td>
<td>Bacteroidetes</td>
<td>Detected in all seasons</td>
</tr>
<tr>
<td>E5</td>
<td>Uncultured bacterium clone DPT.5.43(FJ612299)</td>
<td>99.7</td>
<td>Bacteroidetes</td>
<td>Only detected in spring</td>
</tr>
<tr>
<td>E6</td>
<td>Uncultured bacterium clone A23(FJ660548)</td>
<td>99.1</td>
<td>Bacteroidetes</td>
<td>Detected in all seasons</td>
</tr>
<tr>
<td>E7</td>
<td>Uncultured bacterium clone DP10.9.11(FJ612295)</td>
<td>99.6</td>
<td>Bacteroidetes</td>
<td>Detected in autumn and winter</td>
</tr>
<tr>
<td>E9</td>
<td>Uncultured bacterium clone K08-144-BAC(GQ340327)</td>
<td>99.7</td>
<td>Bacteroidetes</td>
<td>Detected in all seasons</td>
</tr>
<tr>
<td>E11</td>
<td>Uncultured Flexibacter sp. clone ZS-2-316</td>
<td>98.8</td>
<td>Bacteroidetes</td>
<td>Only detected in summer</td>
</tr>
<tr>
<td>F1</td>
<td>Uncultured bacterium clone zih-8-113(GU323634)</td>
<td>99.7</td>
<td>Verrucomicrobia</td>
<td>Dominant in autumn</td>
</tr>
<tr>
<td>F5</td>
<td>Uncultured Verrucomicrobia clone TH1-67</td>
<td>99.8</td>
<td>Verrucomicrobia</td>
<td>Only detected in summer</td>
</tr>
<tr>
<td>F6</td>
<td>Uncultured Verrucomicrobia clone B07-03-BAC(GQ340110)</td>
<td>97.9</td>
<td>Verrucomicrobia</td>
<td>Only detected in autumn</td>
</tr>
<tr>
<td>Y2</td>
<td>Uncultured cyanobacterium clone cln_0TU34C148(AM259252)</td>
<td>98.7</td>
<td>Cyanobacteria</td>
<td>The most abundant Cyanobacteria clones</td>
</tr>
</tbody>
</table>
had certain correlations with the physical and chemical parameters. TN was the most important factors influencing the group of β-proteobacterium, the second was water temperature, and DTN significantly influenced the group of α-proteobacterium. Environmental factors associated with nitrogen (TN, DTN, and NH$_3$-N) may be the key factors on driving the two major groups transition in Lake Erhai. Group γ-proteobacterium and Bacteroidetes closely related with TP and NH$_3$-N, and Cyanobacteria was positively correlated with bacteroidetes.

Similar to other freshwater ecosystems, γ-Proteobacteria was present at low frequency in Lake Erhai. In general, γ-Proteobacteria were the least common cluster in the bacterioplankton population community [28], and Lake Erhai is a typical shallow lake of early eutrophication on Yunnan Plateau, the low frequent appearance of this group is similar to those lakes with lower altitudes and high eutrophic levels.

Actinobacteria was also a bacterial group commonly detected in freshwater habitats all over the world [34]. Clone D1 which belonging to this group was well represented in summer, and showed 100% identity with uncultured bacterium clone MY52 from oligotrophic lakes (YangHe Reservoir/MinYun Reservoir, China), as well as 100% to uncultured bacterium clone A07-79-BAC from the Marathonas Reservoir, and 99.6% similarity to clone TH1-24 from large, shallow subtropical Taihu Lake in China. This indicated that this phylotype was widespread in the aquatic ecosystem at different trophic levels. Previous studies demonstrated that Actinobacteria in freshwater were considerably more resistant to flagellate grazing [35, 36], which may explained why they were so widely distributed and abundant in most freshwater ecosystems [33]. The research of Kolmonen et al. [34] in Lake Jouti-kas found that Actionbacteria were relatively more frequent in oligotrophic lakes. The relative abundance of Actinobacteria in Lake Erhai was next to α- and β-Proteobacteria. In most studies of Chinese lakes, however, including Lake Taihu and Lake Xuanwuhu, Actinobacteria were at relatively low levels, possibly due to eutrophication level, the concentration of TN, TP and NH$_3$-N in Lake Taihu was higher than Lake Erhai [37]. Other reports found that Actionbacteria were relatively more frequent in oligotrophic lakes [34].

Bacteroidetes and Verrucomicrobia were also commonly found taxa in Lake Erhai, consistent with previous reports [12, 38]. Although Planctomycetes were also typical phyla which were widely distributed in freshwater and marine ecosystems, but they were not detected in Lake Erhai. The reason behind this probably resulted from to the sensitivity of RFLP methods which underestimated the diversity of bacterioplankton, or from the impact of PCR bias.

The Chao formula was used to estimate the enrichment of OTUs present in the studied lake (Table 1). The number of OTUs predicted from the lake was significantly lower than those from low-altitude lakes studied by Xing et al. [22]. In summer, autumn, and spring, the enrichment detected was close to the estimated richness predicted which was much larger than predicted only in winter. Expanding the size of screening library might be needed for a better estimation for the diversity of community.

As shown in Fig 3, the composition of bacteria changed seasonally. The relative abundance of Bacteroidetes and cyanobacteria varied in a similar way. Jaspers et al. [39] showed that diversity of Bacteroidetes changed rapidly during phytoplankton blooms. In Lake Erhai, shifts

**FIGURE 5** - Results by using RDA of bacterioplankton community data constrained to environmental parameters.

4. DISCUSSION

Lake Erhai is a relatively clean water body in China, on which the understanding of ecological relationships among the food web is of unique importance for the protection of lake. Results presented in our paper first reported the composition and seasonal changes of the bacterioplanktonic community in Lake Erhai by using molecular biology methods.

Previous studies showed that α-, β-, and γ-Proteobacteria were frequently detected in marine and freshwater plankton [28], while 542 and 503 clones both fell into the α- and β-Proteobacteria groups in terms of our research. β-Proteobacteria are the most numerically dominant cells and clone types in freshwater lakes worldwide [7, 28-30]. In Lake Erhai, clone types belonging to β-Proteobacteria were the most numerous, but α-Proteobacteria had the most abundant clones. Among all of the groups, clone A7 belonging to SAR11 cluster, α-Proteobacterium was the most abundant clone throughout the year. This may be related to the weakly alkaline pH and low salinity of the lake water [31], and previous studies revealed that α-Proteobacteria were more common in lakes with neutral or weakly alkaline pH [32]. Despite their ubiquity, no representatives existed in pure culture, the ecology and physiology of α-Proteobacteria were still largely unknown [33].
in diversity of Bacteroidetes associated with the growth of cyanobacteria (Fig.3). Rashidan and Bird [40] isolated two strains of bacteria belonging to Bacteroidetes that could lyse bloom-forming Cyanobacteria. The authors had also isolated stains of algae-lysing bacteria from Lake Dianchi, among them, one strain affiliated to Bacteroidetes. All the above results suggest that the prevalence and biodiversity of Bacteroidetes cells should be linked to phytoplankton blooms.

In the clone library, there were 13 OTUs represented in every season. Among them, A7 was the most abundant clone of which abundance changed with the season. Some clones only presented in specific seasons, revealing that the compositions of bacterial communities in the four seasons were very different. Many sequences showed 93.8% - 100% homology with known sequences found in other freshwater ecosystems, such as Lakes Dongping Wulian-angshuai, the Marathonas Reservoir, and some from marine ecosystems. These similarities may be related to the low salinity of lake water [31].

In our RDA analysis, TN, DTN and NH₄-N emerged as significant environmental factors affecting the bacterioplankton in Lake Erhai, Haukka et al. [41] reported that nitrogen concentration may have a direct or indirect effect on the bacterioplankton composition. Elevated nitrogen concentration could increase the biomass of phytoplankton [42]. In Wetzel’s [43] research, algal secretions are significantly bioavailable to bacteria. Therefore, the nitrogen concentration may have an indirect effect on the composition of bacterioplankton through the changes in biomass and composition of phytoplankton.

In conclusions, the investigations of seasonal dynamics and the phylogenetic analyses of bacterioplankton provide basic information that valuable for understanding the structure of bacterioplankton and the microfood web in Lake Erhai. Further studies will focus on elucidating the relationships between bacterioplankton, phytoplankton, and zooplankton.

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REFERENCES


GLYCINE-β-CYCLODEXTRIN ENHANCED ELECTROKINETIC REMEDIATION OF SOILS CONTAMINATED WITH PHENANTHRENE

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ABSTRACT
Enhanced solubilization and desorption behavior of phenanthrene by glycine-β-cyclodextrin (G-β-CD) was investigated, and the feasibility of using G-β-CD in electrokinetic removal of phenanthrene from contaminated soil was evaluated. Bench-scale electrokinetic tests were conducted under a voltage gradient of 2.0 V cm⁻¹ for 10 d, and deionized water, 0.05 M Na₂CO₃/NaHCO₃ buffer solution, 0.05 M Na₂CO₃/NaHCO₃ buffer solution containing 2 g/L G-β-CD and 0.05 M Na₂CO₃/NaHCO₃ buffer solution containing 10 g/L G-β-CD were used as anodic flushing solutions, respectively. The results from solubilization experiments showed that the solubility of phenanthrene in 30 g/L of G-β-CD was enhanced about 30-fold. The desorption efficiency of phenanthrene in soil increased with increasing G-β-CD concentration. The experimental results from electrokinetic (EK) remediation tests showed that phenanthrene migration and removal from soils were significantly affected by G-β-CD concentration and cumulative electroosmotic flow (EOF). The deionized water test without pH control exhibited minimal phenanthrene migration, however, the maximum phenanthrene migration was obtained when 0.05M Na₂CO₃/NaHCO₃ buffer solution containing 2g/L G-β-CD was used as anodic flushing solution. In a test with deionized water, only about 2.1% of phenanthrene was removed from the soil near the anode, whereas 13% of phenanthrene was removed using the deionized water test with pH control. When G-β-CD was added to anodic flushing solution, the 2 g/L and 10 g/L G-β-CD flushing solution showed approximately 24.8% and 34.3% removal, respectively. Electrokinetic process combined with G-β-CD flushing and pH buffering may be a good remediation alternative for PAHs contaminant removal from contaminated soil.

KEYWORDS: Phenanthrene; Glycine-β-cyclodextrin; Solubilization; Desorption; Electrokinetic Remediation

1. INTRODUCTION
Polycyclic aromatic hydrocarbons (PAHs) with two or more fused benzene rings are toxic organic contaminants produced through incomplete combustion and pyrolysis of organic matter [1]. PAHs are receiving increasing attention because of their carcinogenic nature, environmental persistence and chronic toxicity [2-3]. They are strongly adsorbed onto soils or sediments because of low solubility in pure water [4-6]. The in situ microbial degradation of PAHs are limited by their low bioavailability and low water-solubility [7], which results in their accumulations in soil, these PAHs can have detrimental effects on both the flora and fauna through food chains. In some instances, they may also pose serious health problems to humans and cause genetic alterations [8]. Thus, the remediation of PAHs-contaminated soil is an important environmental issue. Researchers have reported the remediation of PAHs–contaminated soils by chemical oxidation [9-10], photocatalytic degradation [11], phytoremediation [12-14], bioremediation [15-17] and enhanced desorption [18-20]. However, phytoremediation and bioremediation require highly selective for plants and bacteria, and both of them are time consuming. In addition, desorption and physicochemical processes are only applicable to permeable soils. Electrokinetic (EK) remediation is an emerging technology that can effectively remove soluble organic pollutants from low permeability soils [21]. However, the EK removal of hydrophobic organic chemicals (HOCs) from contaminated soils is rather difficult due to the poor dissolution and minimal desorption efficiency. Thus, various extracting agents have been used to enhance the desorption efficiency of HOCs [22]. Recently, cyclodextrins (CDs) have received increasing attention because of their forming inclusion complex with various guest molecules with suitable polarity and dimension. So they have been proposed as an alternative agent to enhance water solubility of hydrophobic compounds [23]. In addition, cyclodextrins present several advantages over solvents and non-ionic surfactants such as their lower toxicity and their higher biodegradability [24-26]. Cyclodextrins (CDs) are cyclic oligosaccharides made up of six to eight α-D-glucopyranose monomers connected...
at 1 and 4 carbon atoms. CDs with 6-8 α-D-glucopyranose units are denoted α-, β-, and γ-CDs, respectively. However, β-cyclodextrin is the most accessible, the lowest priced, and, generally the most useful, but its application was limited because of low solubility (18.5 g/L). Some modified β-cyclodextrins with high solubility have been used to enhance the solubility of organic pollutants [27].

In this work, a novel modified β-cyclodextrin (G-β-CD) was synthesized, and G-β-CD enhanced solubilization and desorption behavior of phenanthrene in soil were investigated. On the base of it, the performance of G-β-CD on the enhancement of EK removal of phenanthrene from contaminated soils was evaluated. Various EK parameters, as well as the distribution of water content, pH and residual phenanthrene in the soils were measured. This research provided valuable information on the feasibility of using solubility-enhanced electrokinetic remediation as effective in situ remediation method for removing PAHs from contaminated soils.

2. MATERIALS AND METHODS

2.1. Materials

β-CD was obtained from Seebio Biotechnology Inc (Shanghai, PR China), and used without further purification. Phenanthrene was selected as a representative polycyclic aromatic hydrocarbon (PAH) to model the hydrophobic organic contaminants, it was purchased from Jingchun Chemical Reagent Co. (Shanghai, PRC), with a purity of 99%. Glycine was analytical pure, also purchased from Jingchun Chemical Reagent Co. (Shanghai, PR China). Epichlorohydrin was obtained from Fuchen Chemical Reagent Co. (Tianjing, PR China). All other reagents and solvents used were of analytical reagent grade unless otherwise stated. The water used throughout the work was deionised by a Milli-Q Water Purification system.

2.2. Soil characteristics and preparation of contaminated soil

An uncontaminated natural soil was collected from Fuzhou City, China, the soil was air-dried and sieved to obtain particles less than 2 mm in all experiments. The soil has a pH of 6.50 and organic carbon content of 1.82%. The contaminated soil was prepared by dissolving an appropriate quantity of phenanthrene in hexane, and a known weight of soil was added slowly, with continuous mixing. This slurry was mixed thoroughly and the solvent was allowed to evaporate slowly, the dry contaminated soil was transferred into a bottle and tumbled for about a week before the experiments.

2.3. Preparation of glycine-β-cyclodextrin

Glycine-β-cyclodextrin was prepared as described by Dai et al. [28]. Epichlorohydrin (10 g) was added dropwise to a solution of glycine (7.5 g) and potassium hydroxide (6.7 g) in 70 mL deionized water at 50°C, and β-cyclodextrin (8.0 g) was subsequently added to the above solution. The mixture was reacted at 60°C for 1 h, and pH was adjusted to about 5.5 using sulphuric acid after the solution was cooled to room temperature. Then, ethanol (280 mL, 95%) was added to the mixture. The mixture then became composed of two phases, the top layer a solution of salt and other small molecule compounds in 80% ethanol, the bottom layer a solution of glycine-β-cyclodextrin in 80% ethanol. Methanol (350 mL) was added to the bottom layer, and a white cubic crystal was obtained, the white crystal was filtered and washed by ethanol, then put in vacuum drying chamber at 60°C.

2.4. Solubilization experiments

For the solubility measurements, 10 mL of solution containing different CD concentrations were poured in 50 mL conical flasks with caps, and the solid phenanthrene was added in quantities in excess of the solubility limit. The conical flasks were equilibrated on a reciprocating shaker for 24 h at 25°C, and then the mixture solution was centrifuged at 4000 rpm for 30 min. The solubility of phenanthrene was determined by measuring the solution absorbance at 254 nm after being diluted with 50:50 methanol-water. The role of methanol is to decompose the CD inclusion complexes, thereby keeping the UV spectrum unchanged [29].

2.5. Batch desorption experiments

The desorption experiments were performed in 50mL conical flasks containing 1 g of phenanthrene contaminated soil and 25 mL of G-β-CD solution with a concentration of 8 g/L. The conical flasks were equilibrated on a reciprocating shaker for 24 h at 25°C. After equilibration, the mixture solution was centrifuged at 4000 rpm for 30 min. Phenanthrene in aqueous solution was determined by HPLC (Shimadzu, Japan). The mobile phase was methanol/water mixture (90/10, v/v) at a flow rate of 1 ml min⁻¹. The UV detector was set at 254 nm. Measurements were made in triplicate in each experiment with errors less than 5%.

2.6. EK remediation

The EK remediation test setup used in this study was shown in Fig 1. The setup consisted of a cell, two electrode compartments, reservoirs and a power supply. A plexiglas cylinder (Φ5.0 cm × 15 cm) was used as the EK cell. Perforated graphite (Φ5.0 cm × 0.7 cm) was used as anode and cathode. Anode compartment and cathode compartment (60 mL capacity) were assembled at each end. Each electrode compartment contained a filter paper, a porous stone, and a perforated graphite electrode. Gases were provided in the electrode compartments to allow gases resulting from the electrolysis reactions to escape. The anodic flushing solution was continuously supplied to the soil system from anode reservoir, maintaining the constant hydraulic gradient in the anode compartment. The electroosmotic flow (EOF) in cathode reservoir was measured with a scaled bottle, whose top was sealed with gummed tape to avoid the evaporation of water. Approxi-
mately 300g of phenanthrene-contaminated soil was mixed with 200 mL of deionized water. The moist soil was then added into the electrokinetic cell in layers, and each layer was compacted thoroughly using a stainless steel rammer so that the amount of void space was minimized. Once the cell was filled with soil, the anode and cathode compartments and the anode reservoir were connected to the cell, and the anode reservoir was filled with the selected flushing solution. A voltage gradient of 2.0 V cm\(^{-1}\) was applied in all tests, and the experiments were carried out at room temperature. Parameters associated with each experiment were listed in Table 1.

At the end of each test, the anode reservoir and the electrode assemblies were disconnected, and the soil specimen was extruded from the cell using a mechanical extruder. The soil specimen was sectioned into five equal parts. Each part was weighed and preserved in a glass bottle and was used to analyze phenanthrene concentrations. In addition, the water content and pH of each soil section were also determined.

### 3. RESULTS AND DISCUSSIONS

#### 3.1. Solubilization of phenanthrene in CD solution

The solubilization effects of CD on phenanthrene are shown in Table 2. The results show the apparent aqueous solubilities of phenanthrene are linearly increased with increasing CD concentration. This phenomenon is attributed to the formation of 1:1 inclusion complex in solution [30]. The linear relationship can be expressed by as follows:

\[
\frac{S_t}{S_0} = 1 + K_f C_0
\]

where \(S_t\) is aqueous-phase concentration of phenanthrene in the presence of CD, \(S_0\) is concentration of phenanthrene in the absence of CD, \(C_0\) represents the initial concentration of CD, and \(K_f\) is the stability constant of inclusion complexes for phenanthrene with CD, and it was used to evaluate solubilization capacity of CD for phenanthrene. As shown in Table 2, the stability constant of inclusion complex for phenanthrene with \(\beta\)-CD, CMCD and G-\(\beta\)-CD is 0.7890, 0.9635 and 0.7443, respectively. Higher

<table>
<thead>
<tr>
<th>Experiment number</th>
<th>Anodic flushing solution</th>
<th>Soil moisture (%)</th>
<th>Voltage gradient (VDC cm(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1</td>
<td>Deionized water</td>
<td>40%</td>
<td>2</td>
</tr>
<tr>
<td>E2</td>
<td>0.05 mol/L Na(_2)CO(_3)/NaHCO(_3) buffer</td>
<td>40%</td>
<td>2</td>
</tr>
<tr>
<td>E3</td>
<td>10 g/1 G-(\beta)-CD in 0.05 mol/L Na(_2)CO(_3)/NaHCO(_3) buffer</td>
<td>40%</td>
<td>2</td>
</tr>
<tr>
<td>E4</td>
<td>2 g/1 G-(\beta)-CD in 0.05 mol/L Na(_2)CO(_3)/NaHCO(_3) buffer</td>
<td>40%</td>
<td>2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CD type</th>
<th>Solubilization equation</th>
<th>Solubilization coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\beta)-CD</td>
<td>(S_t/S_0=0.80693 + 0.7890 C_0) (R=0.9992)</td>
<td>0.7890</td>
</tr>
<tr>
<td>CMCD</td>
<td>(S_t/S_0=0.250 + 0.7443C_0) (R=0.9974)</td>
<td>0.7443</td>
</tr>
<tr>
<td>G-(\beta)-CD</td>
<td>(S_t/S_0=-1.06451 + 0.9635 C_0) (R=0.9909)</td>
<td>0.9635</td>
</tr>
</tbody>
</table>
binding constant was found for phenanthrene with G-β-CD than with β-CD (or CMCD), which indicates that solubilization capacity of G-β-CD for phenanthrene is higher than that of β-CD (or CMCD) for phenanthrene. The G-β-CD has obvious solubilization for phenanthrene, the solubility of phenanthrene in 30 g L⁻¹ of G-β-CD was enhanced about 30-fold.

Prior to the EK tests, batch experiments were conducted to evaluate the effects of CD on the desorption of phenanthrene from contaminated soil. As shown in Fig. 2a, the desorption efficiency of phenanthrene in soil increas ed with increasing G-β-CD concentration. This is because that solubilization-capacity of phenanthrene by G-β-CD increased with G-β-CD concentration, which results in that phenanthrene adsorbed onto soil surface gradually transferred into aqueous phase through the partition interaction between aqueous and solid phase. Although G-β-CD with high concentration showed higher desorption efficiency for phenanthrene in soil, 8g/L G-β-CD was used in the desorption experiments because of cost concerns.

3.3. Electric parameter variation during EK process
3.3.1. Electric current variation

During the EK process, the electric current was affected by the conductivity of soil pore solution, the composition of the solutions in anode and cathode compartments, and soil moisture [33]. The variation of electric current versus elapsed time was shown in Fig. 3a. The electric current values generally reached a peak at the start of testing, and the quantity of ions in the pore solution was greatest due to the dissolution of salts that were associated with the dry soil particles [34]. As the ions electromigrated toward the electrodes, the current gradually declined. After approximately 220 h, a more stable current value was reached. The initial current of all tests were different because of the different initial conditions. Comparing the results of the test with and without pH control; it can be observed that the tests (E2, E3 and E4) employing the 0.05 M Na₂CO₃/NaHCO₃ solution commonly had higher current values, and this is most likely a result of the additional ions introduced by the Na₂CO₃/NaHCO₃ electrolyte. When Na⁺ and CO₃⁻/HCO₃⁻ ions were introduced, the CO₃⁻/HCO₃⁻ neutralized some of the H⁺ ions generated at the anode by the electrolysis reaction, while Na⁺ ions electromigrated towards the cathode and increased the current. In addition, the current values of E3 and E4 were higher than those of E2, this was because that more ions could be released into soil pore solution with the help of coordination interaction of G-β-CD containing amino and carboxyl groups [35].

3.3.2. Cumulative EOF

The cumulative electroosmotic flow (EOF) was depicted in Fig. 3b. The flow behavior was dependent on the flushing solutions and elapsed time. For all tests, the cumulative EOF increased with increasing elapsed time. Within 240 h EK process, for the deionized water test (E1) without pH control, the maximum electroosmotic flow was 220 mL, however, for tests with pH control, the maximum electroosmotic flow of E2, E3 and E4 was 340, 375, and 510 mL, respectively. When compared with deionized water (E1), Na₂CO₃/NaHCO₃ buffer solution (E2) signifi-
cantly increased the cumulative EOF. It is because that the Na₂CO₃/NaHCO₃ buffer solution leads to higher pH than deionized water. Higher pH leads to more negative ζ potential [21], resulting in the increase of the cumulative EOF. Compared with the test (E3) with 10g/L G-β-CD, the higher cumulative EOF in the test (E4) with 2g/L G-β-CD may be due to relatively higher dielectric constant and lower viscosity of 2g/L G-β-CD solution. Similar results were also reported [36]. In addition, the Electric current represents the transport of ions when the conductive medium has the same resistance. Higher electric current leads to faster ion transport, which results in faster transport of water by electroosmosis [34]. Thus, the higher the electric current is, the higher the cumulative EOF is, and the order of the cumulative EOF is as follows: E4>E3>E2>E1.

3.4. Variation of soil characteristics during EK process

3.4.1. Soil pH

When voltage potential was applied to the electrokinetic cell, the electrolysis of water produces H⁺ at the anode and OH⁻ at the cathode. Migration of two ion species toward the opposite electrode results in a high pH at the cathode and a low pH at the anode. For the test without pH control (E1), the H⁺ ions migrated through the soil all the way to the cathode region, which is evident because acidic pH values occurred along the length of the soil profiles. As shown in Fig.4a, the pH value in the soil was 3.54 near the anode and then gradually increased to 5.98 towards the cathode. However, the test (E2) conducted with pH control had somewhat higher pH values that ranged from 7.21 near the anode to 9.23 near the cathode, which indicates that Na₂CO₃/NaHCO₃ had a larger potential to neutralize H⁺ produced on the anode. The pH of the tests with G-β-CD ranged from 7.42-7.92 near the anode region and to 8.88-9.54 near the cathode region. The pH in both tests (E3 and E4) was almost the same in the corresponding soil sections.

![Image of electric current and cumulative EOF](image)

**FIGURE 3** - Variation of (a) electric current and (b) cumulative EOF with elapsed time

3.4.2. Soil water content

The initial water content of soil was 40%, and the variation of water content after EK treatment was shown in Fig. 4b. In all tests, low moisture regions occurred near anode and soil water content clearly increased from anode to cathode. Changes in water content were attributed to the variations of the cumulative EOF. In the test (E4) with 2g/L G-β-CD, it was observed that water content in most of the sections across the column increased after EK treatment. This could be attributable to the enhanced EOF as a result of high pH produced with pH control at anode. However, in the test (E3) with 10 g/L G-β-CD, it was observed that the water content of all the soil specimens was lower than the initial value. Low dielectric constant with 10 g/L G-β-CD solution was responsible for the
decrease of EOF according to H-Stheory [21], thus decreased the water content in soil.

3.5. Electrokinetic removal of phenanthrene in soil

At the end of EK tests, the residual phenanthrene concentration profiles from anode to cathode were shown in Fig.5. The deionized water test without pH control (E1) exhibited minimal phenanthrene migration, it was because that its movement by electroosmosis was rather difficult due to low solubility of phenanthrene in water. However, the deionized water test with pH control (E2) caused some contaminant mobilization. In our opinion, the Na2CO3/NaHCO3 buffer solution has no obvious solubilization for phenanthrene, but the cumulative EOF in the test E2 was higher than that in test E1. Therefore, the strong flushing action through the small pore spaces was obtained in test E2, which resulted in the adsorbed phenanthrene particles being flushed and transferred towards the cathode because of the high cumulative EOF [34]. When G-β-CD (E3, E4) was used, it was found that phenanthrene concentration increased gradually from anode to cathode. The values of C/C0 were below 1 in the region close to anode, which implied that phenanthrene was partly removed from the soil, however, the values of C/C0 were above 1 in the region close to cathode, which implicated that phenanthrene was accumulated there. The maximum phenanthrene migration was observed in the test (E4) with 2g/L G-β-CD solution. The normalized concentration near anode was 0.75 and it increased gradually to 1.24 in the soil near the cathode. This mobility may be due to partial solubilization of phenanthrene and the increased soil-solution-contaminant interaction resulted from increased EOF. In the test with 10 g/L G-β-CD (E3), the normalized concentration of phenanthrene at section near anode was 0.66 and it increased gradually to 1.10 in the soil near the cathode. Compared with 2g/L G-β-CD (E4), the 10 g/L G-β-CD concentration was sufficient for the solubilization to occur, but mobilization remained minimal because of the low soil-solution-contaminant interaction due to low EOF.

4. CONCLUSIONS

G-β-CD has obvious solubilization for phenanthrene, and it could enhance desorption of phenanthrene in soil. During the EK process, phenanthrene migration and removal from soils were significantly affected by G-β-CD concentrations and cumulative electroosmotic flow. The deionized water test without pH control exhibited minimal phenanthrene migration, however, the maximum phenanthrene migration from anode to cathode was obtained when 0.05M Na2CO3/NaHCO3 buffer solution containing 2g/L G-β-CD as anodic flushing solution. In tests with deionized water, only about 2.1% of phenanthrene was removed from the soil near the anode, while 13% of phenanthrene was removed using the deionized water test with pH control. When G-β-CD was added to flushing solution, the 2 g/L G-β-CD and 10 g/L G-β-CD flushing solution showed approximately 24.8% and 34.3% removal, respectively. EK process combined with G-β-CD flushing and pH buffering may be a good remediation alternative for removing PAHs from contaminated soil.

ACKNOWLEDGEMENTS

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REFERENCES


ABSTRACT

In this study, adsorption of Astrazon Red 6B (AR6B) in aqueous solutions by sepiolite was studied using full factorial experimental design. The interactive effects of the three most important operating variables, initial AR6B concentration (75-175 mg/L), initial pH (2-8) and temperature (20-40 °C), on the amount of dye adsorbed at equilibrium were studied in batch adsorption experiments. A total of 63 experimental runs were set, and the experimental data fitted to the empirical second-order polynomial model for maximum adsorption of AR6B from aqueous solutions by sepiolite. An initial concentration of 175 mg/L, pH 8 and 20 °C were found to be optimal for the maximum removal of AR6B from aqueous solutions. Analysis of variance of the quadratic model showed that the model was highly significant ($R^2 = 0.9962$). The result showed that adsorption capacity of dye increased with increasing dye concentration and pH while it was decreasing with increasing temperature. The equilibrium data were also analyzed by the Langmuir and Freundlich adsorption isotherm models. The adsorption isotherm data fitted well to Freundlich isotherm.

KEYWORDS: adsorption; sepiolite; full factorial experimental design; Astrazon Red 6B.

1. INTRODUCTION

Dyes can be classified according to chemical structure and usage. Acidic, basic, reactive, direct, disperse, mordant, solvent, sulfur, and vat are common types of dyes. Cationic dyes also known as basic dyes are widely used in acrylic, nylon, silk, wool, paper, ink, and polyester dying [1-3]. There are more than 100,000 dyes commercially available, most of which are difficult to decolorize due to their complex structure and synthetic origin. These dyes are harmful to fish and other aquatic organisms. The colored wastewater in the receiving streams reduces the light penetration through the water’s surface and, therefore, reduces removal of such colored dyes from aqueous solutions is significant for environmental, technical, and commercial importance [4].

There are several methods for dye removals in literature, such as coagulation and chemical oxidation, adsorption, membrane separation process, electrochemical treatment, filtration, reverse osmosis as well as aerobic and anaerobic microbial degradation [2]. Among all these, adsorption has been found to be an efficient method for the removal of dyes from aqueous solutions because it produces high quality-treated effluents, and also allows kinetic and equilibrium measurements without any highly sophisticated instruments [5]. However, the adsorption process is limited by the high cost of adsorbents, thus limiting its usage. Due to this reason, many researchers searched for local, less expensive, and efficient alternative substitutes/adsorbents to remove dyes from wastewater. Activated carbons [6-8], agricultural wastes [9-12], fly ash [13, 14], and sepiolite [2, 15-35] were some of these.

Organic cations may bind to sepiolite by different modes: (i) a neutral complex may be formed by the binding of a cation to a monovalent negative site on the sepiolite blocks. This reaction is an electrostatic one, and may be considered for both organic and inorganic cations, (ii) a second organic cation may bind to a neutral sepiolite–organic complex by non-Coulombic interactions, forming a single positively charged complex with two organic cations and a charged site; (iii) neutral sites occur at the external surface in sepiolite, a monovalently charged complex may be formed by the binding of an organic cation and a neutral site. Such a binding is responsible for the very large number of monovalent organic cations adsorbed to sepiolite [17, 36].

The objective of the present study is to investigate the feasibility of adsorption using sepiolite for the removal of AR6B from aqueous solutions, and to develop a mathematical model for the estimation of the maximum adsorption capacity of the adsorbent at possible environmental conditions. The main environmental parameters under investigation were initial dye concentration, pH and temperature. A full factorial experimental design for these parameters was used to predict adsorption capacities for the dye solutions at equilibrium.
2. MATERIALS AND METHODS

2.1. Materials

Sepiolite was obtained from Eskisehir, Turkey. It was ground and sieved, and then dried at 105 °C for further analyses and experimentation. Values of surface area and pore diameter (Quantachrome Instruments Autosorb-1) and analysis of particle size distribution (Malvern–Zeta Sizer Nano ZS) of sepiolite are shown in Table 1. Sepiolite is a natural hydrated magnesium silicate clay mineral, \( (\text{Si}_{12})(\text{Mg}_8)\text{O}_{30}(\text{OH})_6(\text{OH}_2)_4 \cdot 8\text{H}_2\text{O} \), structurally formed by blocks and channels extending in the fiber direction. It has peculiar surface properties and important industrial interest due to its high sorbing capacity [37]. The chemical composition of sepiolite determined by chemical analysis (Philips PW2404 X-ray Fluorescence Spectrometer) is given in Table 2.

![TABLE 1 - Some physical properties of sepiolite.](image1)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
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<tr>
<td>Multipoint BET surface area (m²/g)</td>
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<tr>
<td>Average pore diameter (nm)</td>
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</tr>
<tr>
<td>Total pore volume for pores with diameter &lt;0.85 nm (cm³/g)</td>
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<tr>
<td>Particle size range (mm)</td>
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<tr>
<td>Mean particle size (volume weighted) (mm)</td>
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</tbody>
</table>

![TABLE 2 – Chemical composition of sepiolite by XRF.](image2)

<table>
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</thead>
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<tr>
<td>Al₂O₃</td>
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<td>SO₃</td>
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<tr>
<td>Other</td>
<td>1.20</td>
</tr>
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</table>

2.2. Characterization of Sepiolite

The surface image of sepiolite after the adsorption experiments using Astrazon Red 6B basic dye (AR6B) determined by scanning electron microscopy (SEM, Philips XL30S-FEG) is illustrated in Fig. 1. The sample was gold-coated prior to SEM observation. The adsorbent had an irregular and porous surface, indicating relatively high surface areas. As seen in Fig. 1, the pores of sepiolite were almost packed with dye materials.

![FIGURE 1 - SEM micrograph of sepiolite with AR6B adsorbed.](image3)

AR6B was obtained from Dystar and used for the adsorption study. The structure of AR6B, along with its molecular weight, color index, and wavelength of maximum absorbance in aqueous solution, is given in Table 3.

The X-ray diffraction (XRD) of sepiolite was measured with an automated Rigaku X-ray diffractometer (D-Max Rint 2200 Series instrument) using Cu Kα radiation at 40 kV and 40 mA over the range (2θ of 5–45°). The XRD pattern of sepiolite is shown in Fig. 2. The major phases for the sample were sepiolite (S), kaolinite (K) and quartz (Q).

![FIGURE 2 - XRD patterns of the sample (sepiolite (S), kaolinite (K) and quartz (Q)).](image4)

![TABLE 3 - Structure of Astrazon Red 6B.](image5)

<table>
<thead>
<tr>
<th>Name and Synonyms</th>
<th>Molecular structure</th>
<th>Formula</th>
<th>CAS number</th>
<th>Color index</th>
</tr>
</thead>
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<tr>
<td>Astrazon Red 6B</td>
<td></td>
<td>C₂₁H₂₅Cl₂N₂</td>
<td>6441-82-3</td>
<td>48020</td>
</tr>
<tr>
<td>Basic Violet 7</td>
<td></td>
<td></td>
<td>M.W.</td>
<td>417.41</td>
</tr>
<tr>
<td>Brilliant Red 6B</td>
<td></td>
<td></td>
<td>λ&lt;sub&gt;max&lt;/sub&gt; (nm)</td>
<td>543</td>
</tr>
<tr>
<td>Cationic Red 6B</td>
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<td></td>
<td></td>
</tr>
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</table>
Fourier transform infrared (FTIR) spectroscopy is a useful tool in determining whether the adsorptive interaction between sepiolite and AR6B is physical or chemical. The FTIR spectrum of sepiolite is presented in Fig. 3. Sepiolite and dye-loaded sepiolite sample after adsorption were placed in an oven at 105 °C for 2 h. Samples were made as pellets, and then the infrared spectra of AR6B on sepiolite before and after the adsorption process were recorded in the range 4000–650 cm$^{-1}$ on a Bio Rad FTS 175C spectrophotometer (Fig. 3). The bands at 3630 and 3550 cm$^{-1}$ corresponded to stretching vibrations of hydroxyl groups (indicated as H–O–H stretching vibrations of water molecules). The bands at 1650 cm$^{-1}$ and 1580 cm$^{-1}$ may be attributed to the hydroxyl bending vibration which was related to presence of bound water and –CH=CH– vibration, respectively. The band at 1210-750 cm$^{-1}$ was produced by the Si–O bonds [38]. FTIR spectra given in Fig. 3 clearly indicated that dye-sepiolite complexation was physisorption.

2.3. Method

Adsorption runs were executed in a waterbath thermostatic shaker. The initial pH of the solutions were adjusted by using either 0.1 M KOH or 0.1 M HCl, and 100 ml solutions of known dye concentration ($C_0$) and initial pH ($pH_0$) were placed in screw-cap conical flasks with 0.05 g of sepiolite, and agitated at a speed of 200 rpm at 3 different constant temperatures (20, 30, 40 °C) for 72 h (previously determined for the equilibrium). The dye concentrations of solutions were determined by spectrometric analysis (PG Instruments T80+ UV/VIS spectrophotometer). The maximum wavelength of absorbance for AR6B was determined experimentally as $\lambda_{max} = 543$ nm. A calibration equation for this study was constructed between 0 and 50 mg/L of dye concentrations. Samples were filtrated at 10,000 rpm (Beckman Coulter Allegra 64R Centrifuge), and the final concentrations of dye solutions were determined. The dye concentration retained in the adsorbent phase ($q_e$, mg/g) was calculated by using the following equation:

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

where, $C_0$ is the initial dye concentration (mg/L), $C_e$ is the equilibrium dye concentration (mg/L), $V$ is the volume of solution (L), and $W_s$ is the mass of the adsorbent (g).

The effect of conductivity at 125 mg/L was investigated between 0-3.5 mS/cm by using NaCl as the supporting electrolyte. The removal efficiency was changed from 99.64 to 99.76% as conductivity increased in the range of 0-3.5 mS/cm (Fig. 4). Therefore, conductivity of 0.1 mS/cm was used throughout this study, since the removal efficiency was not affected with increase in conductivity.

Desorption experiments were carried out by immersing the sepiolite loaded with dye in 100 ml of desorption solution for 24 h at room temperature. In the batch desorption process, different desorption solutions were tested, and the mixtures of KCl in ethanol/water solutions (e.g. 0.5 N KCl in 50% ethanol or 0.5 N KCl in water). The dye concentration in the desorption solution was analyzed spectrophotometrically.

2.4. The Experimental Design

The 3-level full factorial experimental design for the three factors was used. A total of 27 ($=3^3$) experiments was required for a 3-level/3-factorial experimentation. Eight experiments for the center-point, and one replicate for each of the other points were also added to experimental design (61 experiments were done). Independent factors were initial dye concentration ($C_0$), initial pH ($pH_0$) and temperature ($T$), and the dependent response variable was the amount of dye adsorbed at equilibrium ($q_e$). The independent factors were coded to the (-1, 0, 1) interval where the low and high levels were coded as -1 and +1, respectively. The ranges of the variables are shown in Table 4. The regression analyses and analyses of variance (ANOVA) were carried out using Minitab 16.1.1 [39].

### Table 4 – Experimental range and levels of independent variables.

<table>
<thead>
<tr>
<th>Independent variables</th>
<th>Codes</th>
<th>Range and levels (coded)</th>
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</thead>
<tbody>
<tr>
<td>$C_0$ (mg dm$^{-3}$)</td>
<td>$x_1$</td>
<td>-1  75  0  125  175</td>
</tr>
<tr>
<td>$pH_0$</td>
<td>$x_2$</td>
<td>2  5  8</td>
</tr>
<tr>
<td>$T$ (°C)</td>
<td>$x_3$</td>
<td>20  30  40</td>
</tr>
</tbody>
</table>

FIGURE 3 - FTIR of sepiolite and dye-loaded sepiolite.

FIGURE 4 - Effect of conductivity on adsorption of AR6B onto sepiolite.
3. RESULTS AND DISCUSSION

3.1. Statistical Analysis

In this study, the relationship between 3 independent variables and response was established with the quadratic model. The general form of the empirical quadratic model may be given as follows:

\[ q_e = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 \]

(2)

Herein, coded variables \( x_i \)s are the independent variables (initial dye concentration, initial pH and temperature) and \( q_e \) is the dependent response variable (adsorption capacity). The experimental conditions including results of experimental adsorption capacities (\( q_{exp} \)) and predicted adsorption capacities (\( q_{pred} \)) are shown in Table 5. Adequacy of the empirical model can be justified by analysis of variance (ANOVA). The results of ANOVA are given in Table 6.

At first glance, larger probability of values (>0.05) of individual coefficients of the terms \( x_2 x_3 \), \( x_2^2 \) and \( x_1 x_3 \) indicated that these coefficients may be insignificant. Then, these terms were eliminated one by one by observing the probability values and \( R_{adj}^2 \) values, until a model was obtained for all the individual coefficients being significant. The resulting model had a 7-coefficient regression model as given in equations 3 and 4. The probability of the individual coefficients and analysis of variance of the resulting model are given in Tables 6 and 7. The smaller p-values and larger t-values in Table 7 verified that the remaining coefficients in the model were significant. The regression coefficients (\( R^2=99.62\% \) and \( R_{adj}^2=99.58\% \)) also suggested that the resulting model was a good estimate. In Table 8, F-ratio obtained by dividing the mean square (MS) of model to that of error (2345.2) was greater than that of tabulated \( F_{20,34,0.05} \) value (2.27). This model was adequate with 95% significance level. The adequacy of the model can also be verified via lack-of-fit test. In Table 8, F-ratio obtained by dividing the MS of lack-of-fit to the MS of pure error (1.77) was smaller than that of tabulated \( F_{20,34,0.05} \) value (1.89). The p-value in Table 8 (0.0689 = \( F_{dist}(1.77,20,34) \)) was greater than 0.05 which indicated that lack-of-fit was not significant, and the model was adequate.

<table>
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<tr>
<th>Run</th>
<th>( x_1 )</th>
<th>( x_2 )</th>
<th>( x_3 )</th>
<th>( q_{exp} ) (mg/g)</th>
<th>( q_{pred} ) (mg/g)</th>
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3.2. Effect of Experimental Factors

3D response surface graphs with sepiolite for combined effect of initial temperature-initial pH, initial concentration-temperature and initial concentration-initial pH on adsorbent capacity dependence are shown in Figs. 5a-c. Figure 5 generated using equations 3 and 4 predicted the combined effect of initial temperature-initial pH, initial concentration-temperature and initial concentration-initial pH. For AR6B adsorption, a significant increase in the amount adsorbed with temperature was observed while initial pH of dye solution increased from 2 to 8. This expected observation was a result of the increase in the negative surface charge on the adsorbent as pH increased, leading to a higher degree of cationic species adsorption. Some studies with other cationic dyes and adsorbents reported an increase in adsorption capacity with pH [40, 41]. For higher pH values, a decrease in temperature favored adsorption on sepiolite, suggesting that chemical forces might be responsible for the adsorption at high pH.

3.3. Equilibrium Adsorption Isotherms

The Langmuir adsorption isotherm describes the surface as homogeneous assuming that all the adsorption sites have equal adsorbate affinity, and that adsorption at one site does not affect adsorption at an adjacent site. The Langmuir equation is described by the following equation:

\[
q_e = \frac{Q_b}{1 + b \cdot C_e}
\]

where, \(q_e\) is the amount of dye adsorbed at equilibrium (mg/g), \(C_e\) is the equilibrium concentration (mg/L), \(Q_b\) is the monolayer adsorption capacity (mg/g), and \(b\) is Langmuir constant (L/mg) related to the free adsorption energy.

The Freundlich isotherm describes the equilibrium on heterogeneous surfaces. The Freundlich equation is described by the following equation:

\[
q_e = \frac{Q_a \cdot b \cdot C_e}{1 + b \cdot C_e}
\]

TABLE 6 – ANOVA results for response parameters.

<table>
<thead>
<tr>
<th>Sources of variation</th>
<th>Degrees of freedom</th>
<th>Sum of squares</th>
<th>Mean squares</th>
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<tr>
<td>(x_2^2)</td>
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<td>2</td>
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<td>(x_3^2)</td>
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<tr>
<td>Lack-of-fit</td>
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<td>18</td>
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<td></td>
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</tbody>
</table>

**R**²=0.9964, Adj **R**²=0.9958

\[
q_e = 230.639 + 70.0746 \cdot \left( \frac{C_0 - 125}{50} \right) + 4.0788 \cdot \left( \frac{pH_0 - 5}{3} \right) - 2.2797 \cdot \left( C_0 - 125 \right) + 1.9998 \cdot \left( \frac{pH_0 - 5}{3} \right) - 13.7078 \cdot \left( \frac{C_0 - 125}{50} \right) + 2.3115 \cdot \left( C_0 - 125 \right) + 2.705611 \cdot C_0 - 0.306915 \cdot pH_0 - 1.614869 \cdot T + 0.013332 \cdot C_0 \cdot pH_0 - 0.00548312 \cdot C_0^2 + 0.023115 \cdot T^2
\]

**R**²=0.9962, Adj **R**²=0.9958

**TABLE 7 – Individual coefficients of the final quadratic model.**

| Term          | Coefficient | Standard error | t-ratio | Prob>|F |
|---------------|-------------|----------------|---------|-----|
| Intercept     | 230.639     | 0.8485         | 271.812 | 0.000|
| \(x_1\)      | 70.0746     | 0.5967         | 117.446 | 0.000|
| \(x_2\)      | 4.0788      | 0.5967         | 6.836   | 0.000|
| \(x_3\)      | -2.2797     | 0.5967         | -3.821  | 0.0053|
| \(x_1 \cdot x_2\) | 1.9998 | 0.7307         | 2.737   | 0.0084|
| \(x_1 \cdot x_3\) | -13.7078 | 0.9487         | -14.449 | 0.0000|
| \(x_2 \cdot x_3\) | 2.3115 | 0.9487         | 2.437   | 0.0182|

**TABLE 8 – ANOVA results of the final quadratic model.**

<table>
<thead>
<tr>
<th>Sources of variation</th>
<th>Degrees of freedom</th>
<th>Sum of squares</th>
<th>Mean squares</th>
<th>F-ratio</th>
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<td>0.0689</td>
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<tr>
<td>Pure Error</td>
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<td>339</td>
<td>10</td>
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<tr>
<td>Total</td>
<td>60</td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

**R**²=0.9962, Adj **R**²=0.9958

\[
q_e = \frac{Q_b \cdot b \cdot C_e}{1 + b \cdot C_e}
\]
where, $k_F$ is a constant for adsorption capacity of the adsorbent, and the constant $1/n$ indicates the intensity of the adsorption [42].

Parameters of Langmuir and Freundlich isotherms were obtained from the model (equation 3). For different temperatures and pH0 values, data were fitted to Langmuir and Freundlich isotherm equations by means of nonlinear regression. Adsorption equilibrium isotherms for the dye including sum of squares of error (S) and graphs are depicted in Table 9 and Fig. 6. As seen in Table 9, the data fitted well with Freundlich isotherm because lowest value of S was 0.3489 corresponded to 30 °C and pH 8. Figure 6 supported Freundlich isotherm, since the experimental data correlated well with the model. Although both isotherms were very close to each other, deviations from the model were highest for Langmuir isotherm (Table 9).

A comparative evaluation of the adsorbent capacities of various types of sepiolite for the adsorption of basic and acidic dyes was also carried out and listed in Table 10. The adsorption capacity of the sepiolite used in this study had the highest uptake capacity of the dye that makes sepiolite suitable for color removals in textile industry.
TABLE 9 – Langmuir and Freundlich isotherm parameters derived from nonlinear regression analysis.

<table>
<thead>
<tr>
<th>T (°C)</th>
<th>pH</th>
<th>Langmuir</th>
<th>Freundlich</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Q₀ (mg/g)</td>
</tr>
<tr>
<td>20</td>
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<td>291.373</td>
<td>0.487351</td>
</tr>
<tr>
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<td>5</td>
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<tr>
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<td>5</td>
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<tr>
<td>40</td>
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<tr>
<td>40</td>
<td>5</td>
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<td>0.499546</td>
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**TABLE 10 - Adsorption capacities of sepiolite for various dyes.**

<table>
<thead>
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<th>Dye</th>
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</thead>
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<tr>
<td>Astrazon Red 6B</td>
<td>291.373</td>
<td>This study</td>
</tr>
<tr>
<td>Astrazon Blue FGRL</td>
<td>152-209</td>
<td>[2]</td>
</tr>
<tr>
<td>Black B</td>
<td>170</td>
<td>[23]</td>
</tr>
<tr>
<td>Everzol Yellow 3RS H/C</td>
<td>169</td>
<td>[17]</td>
</tr>
<tr>
<td>Acid Red 57</td>
<td>135</td>
<td>[18]</td>
</tr>
<tr>
<td>Everzol Black B</td>
<td>121</td>
<td>[17]</td>
</tr>
<tr>
<td>Acid Blue 294</td>
<td>112</td>
<td>[18]</td>
</tr>
<tr>
<td>Everzol Red 3BS</td>
<td>109</td>
<td>[17]</td>
</tr>
<tr>
<td>Basic Red 46</td>
<td>94-108</td>
<td>[25]</td>
</tr>
<tr>
<td>Methylene Blue</td>
<td>81-92</td>
<td>[30]</td>
</tr>
<tr>
<td>Methylene Blue</td>
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<td>[31]</td>
</tr>
<tr>
<td>Crystal Violet</td>
<td>77</td>
<td>[26]</td>
</tr>
<tr>
<td>Basic Astrazon Yellow 7GL</td>
<td>63-88</td>
<td>[28]</td>
</tr>
<tr>
<td>Methylene Blue</td>
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<td>[21]</td>
</tr>
<tr>
<td>Crystal Violet</td>
<td>73</td>
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<tr>
<td>Reactive Blue 21</td>
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<td>[22]</td>
</tr>
<tr>
<td>Acid Yellow 49</td>
<td>1.9</td>
<td>[16]</td>
</tr>
</tbody>
</table>

3.4. Desorption Studies

Batch AR6B desorption results are shown in Fig. 7. The use of aqueous 0.5 N KCl and 1.0 N NaOH for AR6B desorption was hardly affected, since some complex formation might have taken place between the active sites of sepiolite and the cationic group of AR6B leading to very low desorption rates. In Fig. 7, the mixtures of aqueous ethanol solutions with 0.5 N KCl and pure ethanol did improve desorption of AR6B up to 37%. These results indicated that AR6B was bound onto sepiolite through an electrostatic interaction binding force.

**FIGURE 6 - Isotherm graphs for a) pH=2 and T=40 °C, b) pH=8 and T=20 °C.**

**FIGURE 7 - Batch desorption of AR6B (conditions: 125 mg/L, 298 K, 24 h, 0.5 g/L).**

4. CONCLUSIONS

The adsorption modeling of AR6B on sepiolite from aqueous solutions was investigated in a batch study. Modeling of AR6B adsorption process was performed by varying three independent parameters (initial dye concentration (75-175 mg/L), initial pH (2-8), temperature (20-40 °C)) using a full factorial experimental design. A total of 63 experimental runs were set, and the experimental data fitted to the empirical second-order polynomial model for the maximum adsorption of AR6B in the aqueous solution. The optimum conditions for removal of AR6B from aqueous solutions were initial concentration of 175 mg/L, pH 8 and 20 °C. The experimental data were analyzed by Langmuir and Freundlich adsorption isotherm models.
The adsorption isotherm data obeyed to Freundlich isotherm model. Predicted adsorption capacities, maximum and minimum by the model, were 297.7 mg/g (175 mg/L, pH 8 and 20 °C) and 144.2 mg/g (75 mg/L, pH 2 and 34.9 °C). The mathematical model gave valuable information on interactions between the factors which led to identification of feasible optimum values of the factors studied.

REFERENCES


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THE EFFECT OF SALINITY ON AMMONIA-OXIDIZING BACTERIAL COMMUNITY IN THE CONVENTIONAL SEQUENCING BATCH REACTOR (CSBR) AND INTERMITTENTLY AERATED MEMBRANE BIOREACTOR (IAMBR)

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ABSTRACT
The effect of salinity on the community compositions and abundance of ammonia-oxidizing bacteria (AOB) were investigated in the conventional sequencing batch reactor (CSBR) and intermittently aerated membrane bioreactor (IAMBR). The salinity loadings were set at 0, 5 and 10 g/L (referring to NaCl) respectively. The removal of total organic carbon (TOC) and conversion of ammonia-nitrogen (NH$_4^+$-N) were stable in the IAMBR, while significant decreases were observed in the CSBR under high salinity. Denaturing gradient gel electrophoresis (DGGE) analysis revealed that the dominant AOB species were *Nitrosospira*-like and *Nitrosomonas*-like species in the two reactors. The cluster analysis showed significant changes of the AOB populations with the increase of salinity in the CSBR. The results of Real-time PCR also showed higher AOB content in the IAMBR than that in the CSBR. These can interpret the better performance of the IAMBR than CSBR under increased salinity stress.

KEYWORDS: conventional sequencing batch reactor; intermittently aerated membrane bioreactor; ammonia-oxidizing bacteria; molecular biotechnology; salinity wastewater

1. INTRODUCTION
In water environments, the existence of ammonia causes problems at the consumption of dissolved oxygen (DO), toxicity to fish at relatively low concentrations, as well as eutrophication of the receiving water bodies [1]. Therefore, elimination of ammonia is one of the key issues in wastewater treatment plants. Conventional biological nitrification is one of the most common processes for ammonia removal. Nitrification is the microbiological process of conversion of ammonium to nitrite by ammonia-oxidizing bacteria (AOB). Nitrite is subsequently oxidized to nitrate by nitrite-oxidizing bacteria (NOB) [2]. Because of the low growth rate and poor cell yield of AOB, aerobic ammonia-oxidation is generally considered as the rate-limiting step in nitrification process.

The growth of AOB is influenced by various environmental factors such as DO, pH, temperature [3-5] and inhibitory compounds such as salinity [6]. Previous studies always have shown poor removal performance of organic compounds and ammonia when salinity increase in conventional biological wastewater treatment systems, including conventional sequencing batch reactors (CSBR) [7, 8]. In general, high salinity results in higher viscosity of mixed liquor and higher osmotic pressure, and subsequently leads to poor settling characteristics of the sludge and low activity of the microorganisms in conventional systems. Previous studies have attributed to a transition of AOB population for the impact of elevated salinity level on ammonia removal in biological treatment systems [9, 10]. In membrane bioreactors (MBRs), high tolerance of salinity shock loading is achieved by the complete retention of sludge. But, the conventional MBRs have some limitations for the wide application, such as membrane fouling and high energy consumption [11]. As an improve, intermittently aerated MBRs (IAMBRs) can effectively reduce energy consumption [12].

To reach a good nitrification performance, it is important to get a better understanding of the ecology and microbiology of AOB in different wastewater treatment systems. The main shortcomings of conventional microbiological techniques which use optical microscopy observation and/or cultivation-dependent techniques have been well documented [13, 14]. Especially for AOB groups, they
are difficult for the isolation of pure cultures. Modern molecular biotechnology such as polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE), Real-time PCR and fluorescence in situ hybridization (FISH) are preferred for the analysis of AOB communities in various environments. To increase the analytical sensitivity and precision of quantification of uncultivable or difficult to culture microbes in environmental samples, Harms et al. [15] used the Real-time PCR to quantify the nitrifying bacteria in a municipal wastewater treatment plant. In another study on the enrichment of anammox cultures, Tsushima et al. [16] reported the newly designed primer sets were successfully used for quantifying anammox bacteria. Xia et al. [17] presented the diversity and quantification of nitrobacteria in bathing wastewater treatment using PCR-DGGE and FISH.

Although current molecular bio-techniques have been widely applied on the identification and enumeration of AOB species in wastewater treatment processes, the effect of salinity on AOB populations from CSBRs and IAMBRs are still not well understood. In present study, the effect of various salinity levels on AOB community composition was investigated using PCR-DGGE, sequences analysis and Real-time PCR in a CSBR and IAMBR fed by synthetic salinity wastewater.

2. MATERIALS AND METHODS

2.1. Descriptions of CSBR and IAMBR

The schematic diagram of the CSBR and IAMBR systems is shown in Fig. 1. Two bench-scale reactors, one for CSBR and another for IAMBR, were operated in parallel, each with 4.8 L working volume. The sludge seed inoculum was fetched from QuYang municipal wastewater treatment plant (Shanghai, China) and adapted for one month in the two reactors for the acclimatization. After the sludge was matured, the reactors were initially operated at the mixed liquor suspended solids (MLSS) concentrations of 2000 ± 500 mg·L⁻¹. Both reactors were operated under an intermittent aeration mode (air flow rate 0.6 m³·h⁻¹) with the air blower switch on/off, and with a solids retention time (SRT) of 20 days. Oxygen was provided by a fine bubble diffuser located at the bottom of each reactor, and an agitator was used for mixing during the anoxic phase. Synthetic wastewater was fed into the two reactors during the experimental period.

The IAMBR was equipped with a hollow fiber polyvinylidene fluoride (PVDF) ultrafiltration (UF) membrane module (Litree Company, Suzhou, China) with a total surface area of 0.072 m² and nominal pore size of 0.02 µm. The outer and inner diameters of the fibers were 1.45 mm and 0.85 mm, respectively. Permeate was extracted by a peristaltic pump at a constant sub-critical permeate flux of 10 L·m⁻²·h⁻¹ with mode of 10 min filtration and 2 min rest. The transmembrane pressure (TMP) was monitored by a vacuum meter (Weiken YN-60, Shanghai). The IAMBR was operated with a 3-hours cycle mode: 1 hour agitation without aeration and filtration (anoxic phase), followed by 2 hours aeration and membrane filtration (aerobic phase). The synthetic wastewater was fed to the reactor at the beginning of the anoxic phase for about 10 minutes until water level reaching 4.8 L. During the filtration period, the water level was dropping until 3.6 L at the end of each cycle. Therefore, the hydraulic retention time (HRT) was 12 hours in the IAMBR.

**FIGURE 1 - Schematic diagram of the bench-scale CSBR and IAMBR.**
The operation of the CSBR was similar to the IAMBR, but with additional 40 minutes for sludge settling and 20 minutes for supernatant discharging. Therefore, the reaction time in the CSBR was the same as the IAMBR, but it was a 4-hours cycle mode with the HRT of 16 hours.

2.2. Synthetic wastewater preparation and chemical analysis

The synthetic wastewater was prepared comparable to domestic wastewater according to Xia et al. [18]. It was composed of 250 mg L\(^{-1}\) of glucose, 250 mg L\(^{-1}\) of corn starch, 28 mg L\(^{-1}\) of peptone, 133.75 mg L\(^{-1}\) of NH\(_4\)Cl, 30.8 mg L\(^{-1}\) of KH\(_2\)PO\(_4\), 9 mg L\(^{-1}\) of MgSO\(_4\)\(\cdot\)7H\(_2\)O, 6 mg L\(^{-1}\) of MnSO\(_4\)\(\cdot\)7H\(_2\)O, 0.3 mg L\(^{-1}\) of FeSO\(_4\), 8 mg L\(^{-1}\) of CaCl\(_2\), and 120 mg L\(^{-1}\) of NaHCO\(_3\). The mineral compounds were diluted from stock solution and the organic compounds were freshly made every day. The salinity levels were expressed as the concentration of sodium chloride (NaCl) and gradually increased from 0 to 5, and to 10 g·L\(^{-1}\) by adding NaCl in the two reactors.

The influent and effluent were collected daily from the two reactors. The total organic carbon (TOC) was measured instead of Chemical oxygen demand (COD), because the limitations of COD when measuring the organic matter in wastewater samples with chlorides higher than 2000 mg·L\(^{-1}\). TOC, ammonia-nitrogen (NH\(_4\)\(^+\)-N), MLVSS (Mixed Liquor Volatile Suspended Solid) and MLSS were measured according to Chinese NEPA standard methods [19].

2.3. DNA extraction and PCR amplification

The mixed-liquor samples were taken at the end of each operation phase. Genomic DNA was extracted with a FastDNA Spin kit (MP Biomedicals, LLC, France) and further purified using a DNA Gel Purification Kit (UNIQ-10, Sangon Co., Ltd., Shanghai, China) according to the manufacturer’s instructions. For minimizing the variations in the DNA extraction, the templates used for the nested PCR and Real-time PCR were prepared from the DNA mixture which was extracted in the triplicate for each sample.

<table>
<thead>
<tr>
<th>TABLE 1 Sequences of the PCR primers used in this study</th>
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<tr>
<td>Name</td>
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<tr>
<td>CTO189AB</td>
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<tr>
<td>CTO189C</td>
</tr>
<tr>
<td>CTO654</td>
</tr>
<tr>
<td>F357</td>
</tr>
<tr>
<td>518R</td>
</tr>
<tr>
<td>GC-clamp</td>
</tr>
</tbody>
</table>

For analyzing the AOB community, a nested PCR was performed. The PCR amplification of a 465 bp fragment of the 16S rRNA gene from Proteobacteria beta-subgroup AOB was done with the primers CTO189AB, CTO189C and CTO654 (Table 1) in the first PCR round. PCR amplification was carried out with a total volume of 50 µL in a 0.2-mL tube using a DNA thermocycler (Thermo, USA). The PCR mixture contained 1.25 U of Taq polymerase (Takara, China), 1×PCR buffer, 2 mM MgCl\(_2\), 0.5 µM of each primer, 200 µM of each deoxynucleoside triphosphate, and around 40 ng of template DNA. The PCR amplification conditions were set at 5 min of initial denaturation at 94 °C and 30 cycles of 45 s at 94 °C, 1 min at 57 °C, and 1 min at 72 °C. Finally a 7 min final extension at 72 °C was attached.

The second round re-amplified the PCR products obtained from the first round with the total bacterial primers F357-GC and 518R. The amplification program used in the second PCR round was similar to the first round except using 32 cycles instead of 30 cycles. The PCR products were then electrophoresed on a 0.7% (w/v) agarose with ethidium bromide staining for confirming the product size.

2.4. DGGE analysis and phylogenetic tree

DGGE analysis was performed with a D-code System (Bio-Rad, USA) according to the standard method [21]. The PCR products from the second round were loaded onto 8% (w/v) polyacrylamide gels (37:5:1 of acrylamide/bisacrylamide) using 30% to 55% denaturant. The 100% denaturant was defined as 7 M urea and 40% formamide in the 1×TAE buffer. The gels were run first at 200V for 5 min and then at 120 V for 8 h at a constant temperature of 60 °C, and stained with SDNA-Nucleic Acids Stain Dye (BIO BASIC INC., Canada), then washed with sterile water and visualized with a UV transilluminator (FR-980A, Furi Tech. Ltd., Shanghai, China). DGGE images were processed with Smartview software (Furi Tech. Ltd., Shanghai, China).

The specific DGGE bands were manually excised from the gel, re-amplified, cloned and sequenced according to Xia et al. [17]. All nucleotide sequences were determined by Generay Co., Ltd. (Shanghai, China). The sequences obtained were then manually proof-read and corrected. These nucleotide sequences were compared with known sequences in Genbank using the BLAST program (http://www.ncbi.nlm.nih.gov). The sequences with more than 94% homology taxonomically were selected for the following analysis. Phylogenetic evolutionary analyses were conducted with MEGA3.1 based on neighbor-joining method. Bootstrap resampling analysis for 1000 replicates was performed to estimate the confidence of tree topologies.

2.5. Statistical analysis of DGGE banding patterns

The Shannon-diversity index \(H^\prime\) [22] was introduced to examine the structural diversity of the AOB community of the two reactors under different salinity levels. The \(H^\prime\) index was calculated by:

\[
H^\prime = - \sum_{i=1}^{s} (P_i \ln(P_i))
\]

where \(H^\prime\) is the Shannon biodiversity index, \(s\) is the number of bands in the sample and \(P_i\) is the intensities proportion of sample \(i\).
Cluster analysis was used to investigate the relationship between DGGE profiles. Similarities between the band patterns generated by DGGE were analyzed using the Pearson correlation coefficient and displayed as a dendrogram which was calculated according to UPGMA (unweighted pair group method with arithmetic averages) of the clustering algorithms. The cluster analysis and dendrogram were carried out by NTSYSpc (2.10, Exeter Software, USA).

2.6. Real-time quantitative PCR assays

Real-time quantitative PCR was carried out using a Rotor-Gene 3000 system (Corbett research, NSW, Australia). Quantification of the 16S rDNA genes of the total bacteria was performed using the primers 1055F and 1392R. The primer pair CTO189f (CTO189AB and CTO189C)/RT1r along with amoA1F and amoA2R were used for the amplification of the AOB 16S rDNA and amoA gene. The Real-time PCR primers were listed in Table 2.

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<th>Name</th>
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<td>1392r</td>
<td>ACCGGCGGTGTGATCC</td>
<td></td>
<td></td>
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<tr>
<td>CTO189AB</td>
<td>GGAGRAAAGCGGAGGATCG</td>
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<td>[23]</td>
</tr>
<tr>
<td>CTO189C</td>
<td>GGAGAAAGTGAGGGAGTCC</td>
<td>(AOB β-Proteobacteria)</td>
<td>[23]</td>
</tr>
<tr>
<td>RT1r</td>
<td>CGTCCCTCAGACCACTCTAGTCTG</td>
<td>amoA gene (AOB β-Proteobacteria)</td>
<td>[24]</td>
</tr>
<tr>
<td>amoA</td>
<td>ATTACCCGGGCTGTGG</td>
<td>amoA gene (AOB β-Proteobacteria)</td>
<td>[24]</td>
</tr>
</tbody>
</table>

Each PCR mix (total volume of 25 µL) for all triplicate Real-time PCR assays consists of 12.5 µL 2×SYBR Premix TaqTM (Takara, China), 0.625 µM of forward and reverse primers, and approximately 5 ng of DNA template. The standard DNA template was prepared according to Zhang et al. [25]. Tenfold serial dilution of the prepared standard vector plasmids ranged from 10^2 to 10^10 were used as the standard DNA template, to determine the calibration curves by triplicate. The standard curves were obtained using a series dilution of Real-time PCR products from the standard DNA (R²>0.99). All PCR runs also included a negative and a positive control reaction using an HPLC-grade H2O without a template and a previously amplified template, respectively.

3. RESULTS AND DISCUSSION

3.1. Performance of the IAMBR and CSBR

The two reactors were operated in parallel for about 110 days with three successive phases under 0, 5, and 10 g·L⁻¹ salinity levels, which are represented as phase 1, 2, and 3 respectively. The removal of TOC and conversion of NH₄⁺-N at different salinity are shown in Fig 2 for IAMBR and Fig 3 for the CSBR. In the IAMBR, 95% of TOC was removed and 96% of NH₄⁺-N was converted stably during the experimental period, indicating no negative impact on the system performance from the variation of salinity. While in the CSBR, removal efficiencies were achieved in the phase 1 and 2 with average 92% of TOC and 96% of NH₄⁺-N conversion. However, when the salinity increased to 10 g·L⁻¹ in phase 3, the TOC and NH₄⁺-N removal efficiencies therefore dropped to 85% and 80% respectively, indicating a significant impact on the performance of the CSBR at high salinity level.

One of the most important reasons could lie on the loss of the sludge. MLSS concentrations play a very important role during wastewater treatment performance [26]. It can be seen from Fig. 4 that the MLSS concentrations in the IAMBR increased slightly during the whole operation, while in the CSBR, it was stable in the phase 1 and 2, but decreased significantly in the phase 3. This result might be caused by the poor settling property and part of the sludge were lost from the discharging in the phase 3.
Another important reason is the change of sludge activity. The $f$ (MLVSS/MLSS) was used to characterize sludge activity. The $f$ in the IAMBR was around 0.635±0.006 during the whole operation, which showed that the IAMBR exhibited a good performance of sludge activity. While in the CSBR, similar to the variation of TOC and NH$_4^+$-N removal, the $f$ was stable and around 0.635±0.07 in the phase 1 and phase 2, but deceased significantly in the phase 3. At the end of the operation, the $f$ in the CSBR was only around 0.51. This result also leads to the poor performance of the pollutants removal and the loss of AOB abundance in the CSBR.

Furthermore, the loss of the sludge concentrations and activity probably lead to the change of the bacterial community. Therefore it is interesting to get an insight into the bacterial community of the IAMBR and CSBR under different salinity levels.

3.2. DGGE fingerprints and statistical analysis

DGGE fingerprints were used for revealing the shift of the AOB populations in the two reactors under different salinity levels (Fig. 5a). DGGE separations were conducted in triplicate to assess the reproducibility, exhibiting highly similar gels. Only one of the replicates was shown in this paper as an example.

Clustering analysis based on the DGGE profiles showed that salinity had a significant effect on the AOB communities (Fig. 5b). It was observed that the similarity of AOB of the two reactors gradually decreased with the increase of salinity. The intensities of the AOB changes were different in the IAMBR and CSBR. In the phase 1, the AOB communities in the IAMBR exhibit a high similarity to that in CSBR. In the phase 2, the similarity to the phase 1 shows 94% in the IAMBR and 88% in the CSBR. While a high dissimilarity is observed in the phase 3. But the similarity in the IAMBR is higher than that in the CSBR between the phase 1 and phase 3. The higher similarity change indicates a bigger shift of AOB communities in the CSBR than that in the IAMBR under salinity stress. In addition, clustering analysis also demonstrates that the AOB communities have no difference between the anoxic and aerobic phases in both the reactors.

AOB diversity was determined by Shannon-Wiener index ($H'$) (Table 3). The diversities of the AOB community showed no significant variation between the phase 1 and 2, while decreased obviously in the phase 3 in both the reactors. The results are similar to Li and Jin [27] which reported a decrease in bacterial diversity with salinity increasing. Similar to the AOB similarity, the decrease of the AOB diversity in the IAMBR was slower than that in the CSBR.

<table>
<thead>
<tr>
<th></th>
<th>CSBR</th>
<th>IAMBR</th>
</tr>
</thead>
<tbody>
<tr>
<td>phase 1</td>
<td>2.47</td>
<td>2.54</td>
</tr>
<tr>
<td>phase 2</td>
<td>2.45</td>
<td>2.55</td>
</tr>
<tr>
<td>phase 3</td>
<td>1.95</td>
<td>2.32</td>
</tr>
</tbody>
</table>

$^4$ S and M represent the CSBR and IAMBR, under 0, 5 and 10 g·L$^{-1}$ salinity levels, respectively.

The identification of the selected DGGE bands (Fig. 5a) was investigated by sequencing. The closest matches of the obtained sequences to known species were then determined by comparison to the NCBI database (Table 4). Thereafter the phylogenetic affiliations were conducted with MEGA.
3.1 based on neighbor-joining method (Fig. 6). Phylogenetic analysis demonstrated that there were three main groups in Betaproteobacteria subdivision. Four of the seven sequenced bands corresponded to the β-AOB group, one (band 1) was similar to *Nitrosomonas*, the other three (band 5, 6 and 7) were closely related to *Nitrosospira*. The rest three (band 2, 3 and 4) were found to belong to other uncultured Betaproteobacteria.

![FIGURE 5 - (a) DGGE fingerprints and (b) cluster analysis of AOB communities at different salinity levels in the CSBR and IAMBR. S and M represent the CSBR and IAMBR, A and O represent the anoxic and aerobic phase, under 0, 5, and 10 g·L⁻¹ salinity levels, respectively. Number 1 to 7 in (a) are the selected DGGE bands.](image)

![TABLE 4 - NCBI Blast similarity analyses of the sequenced excised bands in the denaturing gradient gel. Only the highest similarities are indicated.](table)

<table>
<thead>
<tr>
<th>Band</th>
<th>Closest relatives (Accession No.)</th>
<th>Identity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Uncultured Nitrosomonas sp. (FN429864.1)</td>
<td>98%</td>
</tr>
<tr>
<td>2</td>
<td>Uncultured beta proteobacterium (AF204248.1)</td>
<td>96%</td>
</tr>
<tr>
<td>3</td>
<td>Uncultured beta proteobacterium (DQ110077.1)</td>
<td>99%</td>
</tr>
<tr>
<td>4</td>
<td>Uncultured beta proteobacterium (DQ110045.1)</td>
<td>99%</td>
</tr>
<tr>
<td>5</td>
<td>Uncultured Nitrosospira sp. (GU086577.1)</td>
<td>97%</td>
</tr>
<tr>
<td>6</td>
<td>Uncultured Nitrosospira sp. (GQ325303.1)</td>
<td>100%</td>
</tr>
<tr>
<td>7</td>
<td>Uncultured Nitrosospira sp. (EU661952.1)</td>
<td>99%</td>
</tr>
</tbody>
</table>
FIGURE 6 - Phylogenetic tree of ammonia-oxidizing bacteria in MBR. The scale bar represents 2% estimated sequence divergence.
In both the reactors, the band 5 consistently and strongly presented in all of the samples. The band 3 also consistently presented in all of the samples, but the intensities increased with the increase of the salinity level, indicating the bacterium gradually being prominent under higher salinity conditions. The band 4 clearly presented at the phase 3, but was faint in the phase 1 and 2. The band 1 was very pronounced in the phase 1 and 3, but weak in the phase 2. The synchronism of the AOB species between the two reactors shows some differences. The bands of 6 and 7 appeared in the phase 1 and 2, while in the phase 3 were faintly observed in the IAMBR and disappeared in the CSBR. The band 2 only appeared in the phase 3, but clearly appeared in the CSBR and was weak in the IAMBR.

Phylogenetic analysis shows that three *Nitrosospira* sp. and one *Nitrosomonas* sp. are dominant in the reactors, indicating that *Nitrosospira* sp. and *Nitrosomonas* sp. were the main AOB species under the operation conditions and play an important role for ammonia conversion. The results also have been confirmed by many studies [28, 29].

The results revealed that the decreased performance in the phase 3 of the CSBR was not only because of the loss of the sludge concentrations and activity, but also the rapid change of the AOB community under high salinity level. In the IAMBR, the AOB community was relatively stable, therefore exhibited a high tolerance to salinity change.

### 3.3. Real-time quantitative PCR

Three sets of primers were used for quantifying the total bacteria, AOB 16S rDNA and amoA gene copy numbers. Because the AOB 16S rRNA assay has the potential to produce false positives and the amoA assay has the potential to produce false negatives, the use of the two assays in the samples provides complimentary data for the detection of AOB [25]. Two independent Real-time PCR measurements of the triplicate DNA extraction were performed.

The results are presented in Fig. 7. In the phase 1, corresponding to high NH$_4$-$\text{N}$ conversion in both the two reactors, the numbers of amoA gene and AOB 16S rDNA were $4.58 \times 10^7$ and $2.12 \times 10^9$ copies/g MLSS$^1$ of the mixed liquor in the CSBR and $4.37 \times 10^8$ and $8.14 \times 10^9$ copies/g MLSS$^1$ in the IAMBR. The total bacteria numbers in the CSBR and IAMBR were around $1.27 \times 10^{12}$ and $3.30 \times 10^8$ copies/g MLSS$^1$ respectively. In the phase 2, the total bacteria, amoA gene and 16S rDNA increased slightly in the IAMBR, while no significant change was observed in the CSBR. In the phase 3, numbers of total bacteria, amoA gene and AOB 16S rDNA showed different trends in the two reactors. The numbers of those in the CSBR considerably dropped to $1.02 \times 10^{10}$, $3.59 \times 10^8$ and $3.30 \times 10^8$ copies/g MLSS$^1$ respectively. In contrast, the numbers of those in the IAMBR increased to $6.36 \times 10^7$, $2.15 \times 10^9$ and $1.94 \times 10^9$ copies/g MLSS$^1$ respectively. The decreased performance in the phase 3 of the CSBR may be attributed to the loss of the sludge. But, it also can be noticed that the numbers of amoA gene and AOB 16S rDNA in IAMBR are higher than those in the CSBR during the operation time. These results may be attributed to the advantage of MBRs which provide better retention of slow growing microorganisms like AOB [30].

It is not well established whether a correlation between the abundance of AOB populations and nitrification activity in numerous research. Zeng et al. [31] suggested that the AOB population tended to be stable during steady nitritation. LaPara and Ghosh [32] revealed a weak correlation between the quantity of AOB populations and nitrification efficiency. On the contrary, Zhang et al. [33] indicated that an increase in the concentration of AOB populations is linked to improve effluent quality in wastewater treatment bioreactors. Similar to Zhang et al., the results in this study showed that the more numbers of AOB populations, NH$_4$-$\text{N}$ conversion is higher. On the contrary, is lower.

Additionally, it was very interesting that the percentages of AOB in total bacteria with salinity were lower than those without salinity, indicating higher salinity level had an inhibitory impact on the increasing AOB populations [34, 35].

### 4. CONCLUSIONS

This study compared the performance and the community compositions and abundance of AOB in the CSBR and IAMBR treating the same synthetic wastewater under 0, 5 and 10 g·L$^{-1}$ salinity levels. The results of the removal of TOC and conversion of NH$_4$-$\text{N}$ showed the better performance of the IAMBR than CSBR under increased salinity stress. DGGE sequence analysis showed that *Nitrosospira*-like and *Nitrosomonas*-like species were the dominant AOB species in the two reactors. The results...
of cluster and Real-time PCR analysis further revealed that the IAMBR exhibited a high tolerance to salinity change.

ACKNOWLEDGEMENTS

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SILVER NANOPARTICLE LOADED ON ACTIVATED CARBON AS AN ADSORBENT FOR THE REMOVAL OF SUDAN RED 7B FROM AQUEOUS SOLUTION

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ABSTRACT

In this research adsorption and removal of Sudan red 7B (SR7B) from aqueous solution using silver nanoparticle loaded on activated carbon (Ag-NP-AC) has been investigated. Equilibrium data are mathematically modeled using the Freundlich Langmuir, Tempkin and Dubinin-Radushkevich (D–R) adsorption models. The SR7B adsorption kinetics on to Ag-NP-AC was studied in terms of pseudo-first-order, pseudo-second-order, Intraparticle diffusion and Elovich models. A maximum adsorption capacity of 90.909 mg.g\(^{-1}\) based on Langmuir as most applicable model at equilibrium is achieved. The high capacity and low removal time show the suitability and usefulness of Ag-NP-AC alternative adsorbent for the removal of SR7B in wastewater treatment.

KEYWORDS: Sudan red 7B (SR7B); Silver nanoparticle loaded on activated carbon (Ag-NP-AC); Kinetic.

1. INTRODUCTION

Dyes as multiple varieties generally resist the breakdown of long-term exposure to sunlight, water and other atrocious conditions. Therefore, the dye treatment of wastewater is a difficult task. Azo dyes among overall category of synthetic textile dyestuffs consist of half of global production and during dyeing operation processes emerged to wastewaters [1]. The removal of dye (as widely applicable modern industry) from textile effluents is one of the most significant environmental problems synthetic origin and complex aromatic molecular structures dye are inert and difficulty biodegradable and harmful to aquatic life in rivers. The occupational exposure of workers in the textile industries is linked to a higher bladder cancer risk. The use of hair coloring products and breast cancer has also been linked [2-4].

* Corresponding author

FIGURE 1 - Chemical structure of Sudan Red.

The group of color additives known as Sudan dyes (Fig.1) consists of a number of red colors, e.g., Sudan I through IV, Sudan Orange G, Sudan Red B, Sudan Red G, and Sudan Red 7B. Since their degradation products carcinogens and teratogens, their application as food-additives is forbidden. However, in some countries, these dyes are still occasionally used to intensify the color of bell pepper and chili powders. The EU-Rapid Alert System (RASFF) disseminated a series of notifications concerning the presence of Sudan dyes in chili products and other foods (spices, tomato sauces, pastas and sausages) [5]. Synthetic dyes treatment from wastewater before discharging to environment before their offering to public use is essential for the protection of health and environment is required. Some of the techniques used in treatment of wastewaters containing dyes are flocculation, coagulation, precipitation, adsorption, membrane filtration, electrochemical techniques, ozonation and fungal decolorization [6]. Among these techniques, adsorption is an effective technique in term of efficiency, capacity and large scale applicability to as well as the regeneration recovery and recycling potential of adsorbents [6–10]. Some commercial systems currently use activated carbon as an adsorbent to remove dyes in wastewater [11-14]. However, the high cost of activated carbon and high removal times restricts its comprehensive use. Although the adsorption behaviors of aromatic adsorbate on gold and silver nanoparticle surfaces have been extensively investigated, the enhancement mechanism is not completely understood. In this paper, we describe the adsorption of SR7B from aqueous solutions onto this Ag-NP-AC adsorbent has reported. Equilibrium
adsorption isotherms were measured and the experimental data were analyzed to commonly used models including Langmuir, Freundlich, Tempkin and Dubinin–Radushkevich isotherm equations.

2. MATERIALS AND METHODS

2.1. Instruments and Reagents.

Sudan red 7B (Merck, Darmstadt, Germany) stock solution was prepared by dissolving require amount of its solid material in double distilled water. The test solutions daily were prepared by diluting their stock solution to the desired concentrations. The pH measurements were done using pH/Ion meter model-682 (Metrohm, Switzerland, Swiss) and absorption studies were carried out using Jasco UV-Visible spectrophotometer model V-570. All chemicals include KOH, HNO₃, KCl from Merck (Darmstadt, Germany).

2.2. Measurements of dye uptake.

Concentrations of SR7B in solution were estimated quantitatively using the linear regression equations obtained by plotting its calibration curve over a range of concentrations. The dye adsorption capacities of adsorbent were determined at the time intervals in the range of 0-35 min and at various temperatures (10–60 °C). The equilibrium was established after 15 min for SR7B respectively. The effect of initial pH on both dyes adsorption was studied at initial concentration of 10 mg L⁻¹ in the pH range of 2-9 by the addition of HCl or KOH. Dye adsorption experiments were also accomplished to obtain isotherms at room temperatures in SR7B concentration range of 5–100 mg L⁻¹. The amount of dye adsorbed by adsorbent, qₑ (mg g⁻¹), was calculated by the following mass balance relationship:

\[
qₑ = (C₀ − Cₑ) V/W
\]

Where C₀ and Cₑ are the initial and equilibrium dye concentrations in solution, respectively (mg L⁻¹), V the volume of the solution (L) and W is the mass (g) of the adsorbent used.

2.3. Preparation of SNPC

MWNTs were treated according to the previous procedure [13]. Nano-silver coated multi-walled carbon nanotubes were prepared by chemical plating method [14]. Firstly, 1.0 g purified and functionalized MWNTs was mixed with 50 mL mixture solution of 38% formaldehyde, absolute ethyl alcohol and double distilled water (volumetric ratio 3:10:10). Secondly, 50 mL mixture solution of 35 g L⁻¹ silver nitrate (AgNO₃) solution and 25% ammonia solution (volumetric ratio 1:2) was dropped one by one into the mixture of MWNTs–formaldehyde–alcohol–water solution. Keeping the pH value of reacted solution is 8–9, the reaction is processed under strong stirring. After reaction, the product is centrifugated and washed by double distilled water twice, dried in vacuum oven at 60°C [15, 16].

3. RESULTS AND DISCUSSION

3.1. Structural properties and amount of Ag NP-AC

Figure 2A shows the UV-Vis absorption spectra correspond to surface plasmon resonance (SPR) of Ag nanoparticle obtained at different time intervals after mixing AgNO₃ aqueous solution with soluble formaldehyde aqueous solution at 50 °C. The maximum SPR at 400 nm was achieved after 24 h [17]. The broadband indicates a relatively high polydispersity, both in size and shape of the Ag particles.

X-ray diffraction (XRD) pattern of silver nanoparticles powder is shown in Figure 2B. The pattern exhibits peaks at 20 angles of 38.17, 44.21, 64.32, and 77.12 that correspond to the [111], [200], [220], and [311] crystal planes of a cubic lattice structure of silver nanoparticles, respectively [18]. From the full-width at half-maximum of diffraction peaks, the average size of the silver nanoparticles has been calculated using the Debye-Scherrer equation [19]. The calculated average size of Ag nanoparticles was around 55 nm.

The FESEM image of the Ag nanoparticles (Fig. 2C) show the semi-spherical in shape and quite uniform in size distribution of Ag nanoparticle in the range of 15–80 nm that has good agreement with that determined by the XRD results.

3.2. Effect of contact time

The studies involving different contact time helps in determining the uptake capacities of the dye at varying time intervals at fixed value of adsorbents. Adsorption of SR7B onto Ag-NP-AC was monitored spectrophotometrically by the procedure described above. Absorbance data, obtained in 1-min intervals until equilibrium, were converted into concentration data using the corresponding calibration relations [10]. It was established (0.02 g), 15 min of contact time was found sufficient to acquire equilibrium. Within the first 5 min almost 99 % adsorption occurred for Ag NP-AC (Fig. 3). The adsorption rate was found to decrease with increase in time [20].

3.3. Effect of pH

The pH is one of the most important parameters in controlling the adsorption process. The pH of the solution was controlled by the addition of HCl or NaOH. The effect of pH on the adsorption of SR7B ions on Ag-NP-AC is shown in Figure 4. The uptake of SR7B ions was minimum at pH 3 and maximum at pH 4. However, when the pH of the solution was increased (more than pH 4), its uptake was increased. It appears that a change in pH of the solution results in the formation of different ionic species. It seems that SR7B can be absorbed on Ag-NP-AC via soft-soft interaction with silver atom of adsorbent.
FIGURE 2 - A (Temporal evolution of UV-visible absorption spectra after addition of AgNO₃ solution into soluble formaldehyde solution at 50 °C), B (X-ray diffraction pattern of the starch-stabilized Ag Nanoparticles), C (FESEM image of the Ag nanoparticles loaded onto activated carbon).
3.3. Effect of pH on the removal of SR7B

or via hydrogen bonding with various functional group of AC. At lower pH, the AC functional group and SR7B molecules get positive charge and due to repulsive force removal percentage decrease. At higher pH, functional group of AC and SR7B deprotonated and the strong interaction of SR7B with nano silver particle with high surface area was achieved.

At pH value higher than 4, the existence of Ag-NP-AC surface OH- creates a competition between ionic dye and decreases the aggregation of SR7B [21-25].

3.4. Effect of amount of Ag-NP-AC on SR7B removal percentage

The amount of Ag-NP-AC is determines the capacity of adsorbent for a given initial concentration of dye solution. The effect of amount of Ag-NP-AC on the SR7B removal percentage is shown in Fig. 5. It was observed that initially the removal percentage increased rapidly with the increase in amount of Ag-NP-AC and after 0.02 g for 50 mL SR7B solution the removal percentage almost reached a constant value. The SR7B removal percentage increased from 90% to 99% respectively with the increase of adsorbent dose from 0.02-0.09 g. The increase in dye removal percentage was due to increased available sorption surface such as silver atom and AC functional group and the availability of more adsorption sites [26]. This may be attributed to the increase in the availability of surface active sites resulting from the increased dose and conglomeration of the adsorbent [27, 28]. When the adsorbent dose was increased to 0.02 g, the ratio of SR7B adsorbed to adsorbent showed no significant difference. Therefore, 0.4 g/L of adsorbent was chosen for later studies [29].

3.5. Effect of Initial Dye Concentration.

The effect of the initial SR7B concentration in the range of (5.0 to 100.0) mg L-1 on its adsorption rate and amount of adsorbed SR7B onto Ag NP-AC was studied in the pH of 4.0 and 0.02 gL-1 of Ag NP-AC. It was seen that, increasing the initial SR7B concentration (the con-
FIGURE 5 - Effect of amount of adsorbent on Sudan Red 7B removal at dye at pH and room temperature.

Adsorption from the liquid phase was carried out to verify the nature, porosity and the capacities of the samples. Sudan red (SR7B) were employed as the adsorbates in the adsorption experiments. An aqueous solution with a concentration of 500 mg/l was prepared by mixing an appropriate amount of SR7B with distilled water adsorption experiments were conducted by placing 0.02 g of the Ag-NP-AC samples and 50 ml of the aqueous solution in a 250 ml glass-stoppered flask. The flask was then put in a constant-temperature shaker bath with a shaker speed of 300 rpm. The isothermal adsorption experiments were performed at 25 ±2°C [31].

After reaching the equilibrium, the experimental data was fitted to conventional models such as Langmuir, Freundlich and Tempkin isotherms and their constants were calculated. The Langmuir isotherm is based on the assumptions that the molecules of the adsorbate are adsorbed at well-defined, energetically equal sites without interacting with each other, and each site can hold only one molecule [32].

The Langmuir isotherm is represented as follows:

\[ q_e = \frac{K_L q_m C_e}{(1 + K_L C_e)} \]  

The equation of Langmuir isotherm is represented as follows:

\[ q_e = \frac{K_L q_m C_e}{(1 + K_L C_e)} \]  

where \( q_e \) is the equilibrium concentration (mg/L), \( q_m \) is the amount of adsorbents sorbed per unit mass of adsorbent at equilibrium (mg/g), \( K_L \) is the Langmuir isotherm constant related to the energy of adsorption (L/mg). A well-known linear expression for the Langmuir isotherm is represented as follows:

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

where the values of \( K_L \) and \( q_m \) can be determined from the slope and intercept of the linear plot of \( C_e/q_e \) versus \( C_e \) [33].

The validity of Freundlich adsorption model was established using the following relation: \( \log q_e = \log K_F + \frac{1}{n} \log C_e \) [33].

The Freundlich constants derived from these straight lines are presented in Table 1.

Tempkin isotherm takes into account the effects of indirect adsorbate–adsorbate interactions on adsorption, and suggests that the heat of adsorption of all the molecules in the layer would decrease linearly with coverage due to these interactions [34]. The linear form of Tempkin isotherm is expressed as follows:

\[ q_e = B \ln A + B \ln C_e \]  

Where \( B \) is the Tempkin constant related to heat of adsorption and \( A \) is the equilibrium binding constant (L mg⁻¹).

The constants A and B can be determined by a plot of \( q_e \) versus \( \ln C_e \) (Table 1) [32].
### TABLE 1 - Isotherm constant parameters and correlation coefficients calculated for the adsorption

<table>
<thead>
<tr>
<th>Isotherm</th>
<th>Equation</th>
<th>Parameters</th>
</tr>
</thead>
</table>
| Langmuir-1:      | \[
\frac{1}{q_e} = \frac{1}{(K_R Q_m C_e)} + \frac{1}{Q_m} \]
A plot \( C_e/q_e \) versus \( C_e \) should indicate a straight line of slope \( 1/Q_m \) and an intercept of \( 1/(K_R Q_m) \). |
| Q_m (mg/g)       | 90.91                                                                     |            |
| K_R (L mg^{-1})  | 0.52                                                                      | R^2        |
| R^2              | 0.985                                                                     |            |
| Freundlich:      | \[
\ln q_e = \ln K_F + \left(\frac{1}{n}\right) \ln C_e
\]
The values of \( K_F \) and \( 1/n \) were determined from the intercept and slope of linear plot of \( \ln q_e \) versus \( \ln C_e \), respectively. |
| K_F (L/mg)       | 39.26                                                                     |            |
| R^2              | 0.905                                                                     |            |
| Tempkin:         | \[
q_e = B_1 \ln K_T + B_1 \ln C_e
\]
Values of \( B_1 \) and \( K_T \) were calculated from the plot of \( q_e \) against \( \ln C_e \). |
| K_T (L/mg)       | 18.52                                                                     |            |
| R^2              | 0.889                                                                     |            |
| Dubinin and Radushkevich (D-R): | \[
\ln q_e = \ln Q_s - B \varepsilon^2
\]
The slope of the plot of \( \ln q_e \) versus \( \varepsilon^2 \) gives \( K_T \) (mol^2 (kJ^2)^{-1}) and the intercept yields the adsorption capacity, \( Q_s \) (mg g^{-1}). |
| Q_s (mg/g)       | 59.3                                                                      |            |
| B                | 13.13                                                                     |            |
| E (kj/mol)       | 1581.1                                                                    |            |

### TABLE 2 - Kinetic parameters for the adsorption of SR7B onto adsorbent.

<table>
<thead>
<tr>
<th>Model</th>
<th>Equation</th>
<th>Parameters</th>
</tr>
</thead>
</table>
| First-order kinetic | \[
\log (q_e - q_t) = \log(q_e) - \left(\frac{k_1}{2.303}\right)t
\]
Where \( q_e \) and \( q_t \) (mg·g^{-1}) are the amount adsorbed at equilibrium and time \( t \) (min), respectively, \( k_1 \) (min^{-1}) is the rate constant of Lagergren first-order adsorption (min^{-1}). The values of \( \log(q_e - q_t) \) were calculated from the kinetic data. |
| \( k_1 \)       | 0.44                                                                      |            |
| \( q_e \) (calc) | 2.53                                                                      |            |
| R^2             | 0.962                                                                     |            |
| Second-order kinetic | \[
(t/q_t) = (1/(k_2 q_e^2)) + (t/q_e^2)
\]
Where \( k_2 \) (g mg^{-1}·min^{-1}) is the rate constant of pseudo second-order adsorption. If the second-order kinetics is applicable, the plot of \( t/q_t \) versus \( t \) should give a linear relationship and it is not needed to know any parameter beforehand. |
| \( k_2 \)       | 0.51                                                                      |            |
| \( q_e \) (calc) | 25.6                                                                      |            |
| R^2             | 0.99                                                                      |            |
| Intraparticle diffusion | \[
q_e = K_{id} t^{1/2} + C
\]
The values of \( K_{id} \) and \( C \) were calculated from the slopes of \( qt \) versus \( t^{1/2} \). |
| \( K_{id} \)    | 0.52                                                                      |            |
| \( C \)         | 23.48                                                                     |            |
| R^2             | 0.935                                                                     |            |
| Elovich | \[
q_e = 1/\beta \ln(a\beta) + 1/\beta \ln(t)
\]
Plot the values of \( q_t \) versus \( \ln(t) \) to give a linear relationship from which \( a \) and \( \beta \) can be determined from the slope and intercept, respectively. |
| \( \beta \)     | 2.5                                                                       |            |
| R^2             | 0.85                                                                      |            |
| \( q_e \) (exp) | 24.98                                                                     |            |

### 3.7 Kinetic studies

The behavior of the SR7B adsorption is analyzed using the Lagergren first-order kinetic model, pseudo second-order kinetic model and intraparticle diffusion [35].

A linear form of the Lagergren first-order model expression is:

\[
\log (q_e - q_t) = \log(q_e) - \left(\frac{k_1}{2.303}\right)t
\]

Where \( q_e \) and \( q_t \) (mg·g^{-1}) are the amount adsorbed at equilibrium and time \( t \) (min), respectively, \( k_1 \) (min^{-1}) is the rate constant of Lagergren first-order adsorption (min^{-1}). The values of \( \log(q_e - q_t) \) were calculated from the kinetic data.

The kinetic data are further analyzed using the pseudo second-order kinetics expressed as:

\[
t/q_t = (1/(k_2 q_e^2)) + (t/q_e^2)
\]

Where \( k_2 \) (g mg^{-1}·min^{-1}) is the rate constant of pseudo second-order adsorption. If the second-order kinetics is applicable, the plot of \( t/q_t \) versus \( t \) should give a linear relationship and it is not needed to know any parameter beforehand.

The adsorbate species are most probably transported from the bulk of the solution into the solid phase with an intraparticle diffusion process, which is often the rate-limiting step in many adsorption processes. The possibility of intraparticle diffusion is explored by using the intraparticle diffusion model [36],

\[
q_t = K_{id} t^{1/2} + C
\]

Where \( C \) is the intercept and \( k_{id} \) is the intraparticle diffusion rate constant from the plot of \( q_t \) versus \( t^{1/2} \), the values \( k_{id} \), \( C \) and the corresponding linear regression correlation coefficient \( R^2 \) are given in Table 2 that show the intraparticle rate constants calculated are 0.516 mg·g^{-1}·min^{-1/2} [37].

### 4. CONCLUSIONS

Based on the experimental results, the following conclusions can be made:

1. Ag-NP-AC shows excellent adsorption potential for SR7B removal.
2. The Langmuir and Freundlich isotherm parameters confirmed that the adsorption of SR7B onto Ag-NP-AC was favorable and improved by increase in pH, concentration, temperature, speed of agitation and amount of Ag-NP-AC.
3. The Langmuir isotherm model and Ho’s pseudo-second order model were found to be the best fitting isotherm and kinetic models.

4. The proposed sorbent with high adsorption capacity and short analysis time is a suitable for waste water treatment.

REFERENCES


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CHARACTERISING ASSOCIATIONS BETWEEN GEOCHEMICAL LEAD FRACTIONS AND SELECTED PROPERTIES OF A SEWAGE SLUDGE-AMENDED MONTMORILLONITIC VERTISOL

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ABSTRACT

Multivariate analytical techniques have been used to understand total concentration of heavy metal association with soil properties but not many of these studies have investigated the association between heavy metals concentrations in different geochemical fractions of sludge-amended soils and soil properties. This study investigated the relationship between selected properties of a montmorillonitic vertisol, and the concentrations of lead (Pb) in different geochemical fractions of the vertisol before and after sludge addition. The vertisol was amended with sludge at different ratios of sludge: soil. Lead concentrations in the exchangeable, carbonate bound, reducible, oxidizable and residual fractions of the vertisol were extracted using a five stage sequential extraction procedure. Results of concentrations from laboratory analyses were subjected to correlation and principal component analyses (PCA). Lead concentrations in the different fractions of the montmorillonitic vertisol without sludge followed the order residual fraction > oxidizable fraction > exchangeable fraction > carbonate bound fraction > reducible fraction and correlated with Fe, cation exchange capacity (CEC) Mn, and pH. In the sludge-amended vertisol correlations were observed between CEC/Pb fractions and organic matter (OM)/Pb fractions at an SAR of 5%, and CEC/Pb fractions, OM/Pb fractions, Fe/Pb fractions and Mn/Pb fractions as sludge application rate increased. No associations were observed at higher sludge application rates. Sludge affected the properties of the vertisol and consequently the concentration of Pb in the different fractions and their association with these properties. Correlation and PCA analyses can be useful in understanding effects of sludge addition on the association between heavy metals in different geochemical fractions of soils and soil properties.

KEYWORDS: Multivariate analyses, sequential extraction, sludge application rate, Pb mobility

1. INTRODUCTION

Meeting up with inevitable nutritional demands of an ever growing global human population has resulted in soil over exploitation and soil nutrients depletion. Attempts to replenish depleted soil nutrients and boost agricultural yields include the application of manure, and sewage sludge to soil [1, 2]. The benefits of applying sewage sludge to soils are many [1, 3] but possible elevation in concentrations of heavy metals including cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), lead (Pb), nickel (Ni) and zinc (Zn) in sludge-amended soils has contributed to environmental contamination, and constrained sludge use in agriculture [4-6]. Related studies on sludge in Botswana indicated elevated concentrations of Pb and Zn in the sludge. Ngole and Ekosse [7] reported on the bioavailability of Zn in soils amended with the sludge. This paper focuses on Pb.

High Pb levels are health threats to plants and animals [8]. An understanding of factors affecting its mobility in the environment and bioavailability to plants in sludge-amended soils may aid in controlling plant uptake, and eventually reducing the health risks associated with the use of sewage sludge in agriculture. Selective sequential extractions protocols [9-13] have aided in fractionating heavy metals in soils into operationally defined fractions that include exchangeable (S1), carbonate bound (S2), reducible (S3), oxidizable (S4) and residual (S5) fractions, from which the bioavailable fraction could be determined. Segregation of heavy metals in soils into these fractions is influenced by inherent soil properties and geochemical processes which are in turn influenced by soil properties [14]. Applying sludge to soils introduces organic matter (OM) into the soil which on decomposition may affect soil pH and redox conditions [15]. OM decomposition is influenced by soil minerals including montmorillonite [16-18]. Interactions between montmorillonite and sludge added components may affect associations between heavy metals and soil properties and consequently heavy metal bioavailability in sludge-amended soils. Eliciting on these associations could better be demonstrated through multivariate analyses (MVA) including correlation and principal com-
ponents (PCA) [13, 19]. This study thus aimed at investigating associations between different geochemical fractions of Pb and soil properties in a montmorillonitic vertisol amended with sewage sludge at different rates using correlation and PCA techniques.

2. MATERIALS AND METHODS

Montmorillonitic vertisol samples were collected from Pandamatenga (located between latitudes 18°25′S and 18°40′S and between longitudes 25°05′E and 25°47′E) in North Eastern part of Botswana (Figure 1). Pandamatenga plains are overlain by the Stormberg lava stage basalt of Karoo age and bordered by the Kgalagadi sands [20]. The major soil types in the area are the vertisols (also known as the black cotton soils). The mineral composition of the vertisol had been reported in a previous study [21]. It comprised of quartz and Na montmorillonite; (Na0.3(AlMg)2Si4O10(OH)2.6H2O) in the whole soil, and only Na montmorillonite in the clay fraction. Vegetation around Pandamatenga consists of Colophophermun mopane and Acacia species [21] in addition to extensive grassland savannah vegetation. Samples of vertisol were collected randomly at depths of 0 – 50 cm (rooting zone of most plants) from the study area (Figure 1) and homogenized to form a single sample that was representative of the vertisol. Three-year-old anaerobically-stabilized sewage sludge was collected from a municipal waste water treatment plant in Botswana which was also homogenized to form a single sludge sample that was representative of the pile. The sludge was mixed with the vertisol at weight percent ratios of 0%: 100%, 5%: 95%, 10%: 90%, 20%:80%, and 40%:80% sludge: soil and left to mature for three months. The three months period was chosen for many reasons. The sludge used had been left to cure for three years and the OM decomposition rate had stabilized. Natvig et al. [22] have also indicated that 90 days should be the minimum time allowed between sludge application to soil and harvesting of food crops grown on the sludge amended soil as the pathogen load in the soils is drastically reduced during this period. The three months also represents the minimum time required for sludge to mature in the soil. According to Ngole [23], three months is also the average time between planting and harvesting in Botswana. The 0:100 sludge-vertisol mixture served as the control. The matured vertisol-sludge mixtures were analyzed for their pH, OM content, CEC, P, Mn, Fe and Pb concentrations. The texture of the vertisol was determined using the hydrometer method whereas pH and EC were determined in a 1:2.5 soil: water suspension as described by van Reeuwijk [24].

![FIGURE 1 - Location map of Pandamatenga showing sampling areas.](image)
The Walkley Black modified method was used to determine OM, whereas CEC and P were determined using the ammonium acetate and Olsen ascorbic acid reduction methods respectively. Details of these techniques are described in van Reeuwijk, [24]. Concentrations of Pb in the different geochemical fractions were determined using a five stage sequential extraction procedure described by Tessier et al. [9], Pérez-Cid et al. [10], and Tokaliglu et al. [11]. The reagents used for the different stages were 1 M MgCl₂ (S1), 1 M CH₃COONa adjusted to pH 5 with CH₃COOH (S2), 0.04 M NH₂OH.HCl in 25% v/v CH₃COOH (S3), 3 ml of 0.02 M HNO₃ + 5 ml of 30% H₂O₂ adjusted to pH 2 with HNO₃ + 3.2 M CH₃COONH₄ in 20% v/v HNO₃ (S4), and 3HCl + HNO₃ (S5).

From each soil-sludge mixture, ten samples were analyzed. The mean and standard deviation of each parameter at the different sludge application rates (SAR) were therefore determined. The properties of the control and sludge-amended vertisol were compared after the three months maturing period and the percentage change in each property calculated and reported as the change caused by sludge addition. Lead mobility factor (MF) of the soils at each SAR was calculated using the ratio of Pb in the S1 and S2 fractions (which represent the most mobile fractions), to the total Pb concentration in the different vertisol-sludge mixtures as described by Badaway and El-Motaium [25].

The whole experiment was repeated twice with each analyses carried out in duplicate. Sample and reagent blanks as well as in-house standards were used for quality control. Student t-test was used to determine differences in properties between the control vertisol and the sludge-amended vertisol. Correlation and PCA were then used to identify relationships between sludge-amended vertisol properties and the concentration of Pb in the different geochemical fractions at different SAR at a confidence limit of 95%.

### 3. RESULTS AND DISCUSSION

The vertisol used had 35.2 weight percent (wt %) sand, 27.2 wt % silt, and 37.7 wt % clay and was therefore classified as having a clay loam texture. Organic matter content of the sludge (23.1%) was higher than that of the vertisol (6.3%). Addition of sludge to the vertisol therefore resulted in an increase in OM content of the sludge-amended vertisol with increase in SAR (Figure 2). Values for OM in the sludge-amended vertisol were however below 12% at all SAR (Table 1). Whereas the pH value of the homogenized sludge (5.7) was slightly lower than the pH of the vertisol, the CEC value of the sludge (39.01 Cmolc kg⁻¹) was significantly lower than that of the vertisol (Table 1).

Sludge addition decreased the pH of the vertisol from 6.4 at SAR of 0% to 5.8 at SAR of 40%, indicating a decrease in pH with increase in SAR (Figure 2). Decomposition of added OM could have lowered the vertisol pH as a result of the accumulation of both organic and inorganic acids produced, justifying the decrease in the vertisol pH with increase in SAR (Table 1) (Figure 2). The CEC values of the sludge-amended vertisol ranged from 76.7 cmol kg⁻¹ soil at an SAR of 5% to 85.3 cmol kg⁻¹ soil at an SAR of 40% (Table 1) reflecting an increase in CEC with increase in SAR (Figure 2). This pattern could be explained by the increase in OM with increase in SAR which may have provided additional exchange sites to the vertisol resulting in the observed increase in CEC of the vertisol with SAR (Table 1).

Phosphorus, Fe, Mn, and Pb concentrations in the sludge were 7319 mg/kg, 2.94%, 270.3 mg kg⁻¹, and 295.5 mg kg⁻¹ respectively. Except for Mn and Pb these values were all higher than those of the same elements in

### TABLE 1 – Mean and standard deviation of soil properties at different SAR.

<table>
<thead>
<tr>
<th>Property</th>
<th>Sludge Application Rate (v/v %)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Clay (wt %)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>37.7 ± 2.3</td>
</tr>
<tr>
<td>CEC (cmol, Kg⁻¹ soil)</td>
<td>67.7 ± 7.1</td>
</tr>
<tr>
<td>pH</td>
<td>6.4 ± 0.4</td>
</tr>
<tr>
<td>OM (%)</td>
<td>6.3 ± 1.8</td>
</tr>
<tr>
<td>Mn (mg kg⁻¹)</td>
<td>546.2 ± 18.5</td>
</tr>
<tr>
<td>P (mg kg⁻¹)</td>
<td>62.7 ± 4.6</td>
</tr>
<tr>
<td>Fe (%)</td>
<td>6.7 ± 0.7</td>
</tr>
<tr>
<td>Exchangeable (S1)</td>
<td>10.0 ± 4.8</td>
</tr>
<tr>
<td>CO₃²⁻-bound (S2)</td>
<td>5.4 ± 0.9</td>
</tr>
<tr>
<td>Fe-Mn oxide bound (S3)</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>OM- bound (S4)</td>
<td>20.3 ± 9.6</td>
</tr>
<tr>
<td>Residual (S5)</td>
<td>27.8 ± 16.4</td>
</tr>
<tr>
<td>Total</td>
<td>64.5 ± 6.1</td>
</tr>
</tbody>
</table>

*Indicate significantly different from control
FIGURE 2 – Changes in vertisol properties caused by sludge addition.

FIGURE 3 – Changes in Pb concentrations in different geochemical fractions of the sludge-amended vertisol.

The vertisol. Sludge application increased the concentration of P in the vertisol from 62.7 mg kg⁻¹ to 245.2 mg kg⁻¹ at an SAR of 40% (Table 1). Total concentrations of Mn and Fe in the vertisol decreased with increase in SAR (Figure 2) with total concentration values of Fe ranging from 5% (at SAR of 40%) to 6.4% (at SAR of 0%), and those for Mn concentrations ranging from 498.3 mg kg⁻¹ (at SAR of 40%) to 546.2 mg kg⁻¹ (at SAR of 0%) (Table 2). Differences were observed between the analysed properties of the control vertisol and the sludge-amended vertisol ($p < 0.05$) at an SAR of 5% except for those of clay content, Fe, Mn, and P. However, P was increased at SAR of 10%. There were also differences ($p < 0.05$) in vertisol properties at SAR of 10%, 20% and 40% when compared with the control and each other.

Lead concentration in the sludge was significantly higher than in the vertisol. Addition of sludge at rates of 5% and 10% resulted in a reduction of Pb in the vertisol but further increase in the amount of sludge added increased the concentration of Pb in the vertisol (Table 1). Lead concentration in the different fractions of the control vertisol followed the order S5 > S4 > S1 > S2 > S3 (Table 1). At SAR of 5%, the order of Pb concentration in the vertisol was changed to S4 > S1 > S5 > S2 > S3 (Table 1) indicating a shift in concentration towards the S4 fraction. Increasing the amount of sludge added to the vertisol further altered the concentrations of Pb in the different fractions to S4 > S5 > S1 > S2 > S3 at SAR of 10%, 20% and 40% (Table 1). At all SAR, the lowest Pb concentration values were observed in the S2 and S3 fractions.
whereas the S4 and S5 fractions had the highest Pb concentration values (Table 1). Changes observed in concentrations of Pb in the different fractions at the different SAR (Figure 3) may have been caused by changes in vertisol properties after sludge addition.

Lead has a high affinity for OM [26, 27] and will preferentially sorb on montmorillonite than kaolinite and goethite [28, 29], illite [29], and calcite [30]. Lead sorption on montmorillonite which is the dominant mineral both in the whole soil and clay fraction of the vertisol and its binding on OM could be responsible for the shift in Pb concentration towards the less available S4 and S5 fractions. Changes in Pb concentrations in the different fractions resulted in changes in Pb MF from 0.24 in the control vertisol to 0.34, 0.33, 0.29 and 0.26 at SAR of 5%, 10%, 20% and 40% respectively. Sludge addition therefore resulted in a slight increase in Pb mobility at SAR of 5% and 10%. Higher rates of sludge application however decreased the mobility of Pb in the vertisol.

Soil properties and the concentrations of Pb in the different fractions of the control vertisol showed some significant correlations (Table 2). Correlations ($p < 0.05$) were observed between pH/S, pH/S3, pH/S4, Fe/S1, Fe/S3 and Fe/S4 CEC/S1 CEC/S2, CEC/S3 and CEC/S4 fractions of Pb (Table 2). No relationship was observed between Pb concentration in the different fractions and wt % clay, OM content as well as Mn and P concentrations in the control vertisol (Table 2). Except for the S4/S5 and S2/ S4 fractions of Pb, all other fractions showed significant correlations with each other. Mobility factor (MF) of Pb in the control vertisol correlated ($p < 0.05$) with pH ($r = 0.85$), CEC ($r = 0.69$), Fe concentration ($r = 0.75$), S1 ($r = 0.97$), S2 ($r = 0.65$), S3 ($r = -0.77$), S4 ($r = -0.75$) and S5 Pb fraction ($r = -0.77$). These patterns were all altered with sludge addition.

### TABLE 2 – Correlation coefficients of selected properties in the vertisol at different sludge application rates.

<table>
<thead>
<tr>
<th>Sludge application rate = 0 %</th>
<th>pH</th>
<th>Clay</th>
<th>CEC</th>
<th>OM</th>
<th>Fe</th>
<th>Mn</th>
<th>P</th>
<th>S1</th>
<th>S2</th>
<th>S3</th>
<th>S4</th>
<th>S5</th>
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<tr>
<td>pH</td>
<td>1.00</td>
<td>0.34</td>
<td>0.44</td>
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<td>0.05</td>
<td>1.00</td>
<td>0.02</td>
<td>1.00</td>
<td>0.32</td>
<td>0.72</td>
<td>1.00</td>
</tr>
<tr>
<td>pH</td>
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<td>0.34</td>
<td>0.44</td>
<td>1.00</td>
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<td>1.00</td>
<td>0.32</td>
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<td>1.00</td>
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### Sludge application rate = 5 %

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<th>Fe</th>
<th>Mn</th>
<th>P</th>
<th>S1</th>
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<th>S3</th>
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<tr>
<td>pH</td>
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<tr>
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<td>0.02</td>
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### Sludge application rate = 10 %

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<th>Fe</th>
<th>Mn</th>
<th>P</th>
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<th>S2</th>
<th>S3</th>
<th>S4</th>
<th>S5</th>
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<tbody>
<tr>
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<td>0.72</td>
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<td>pH</td>
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<td>1.00</td>
<td>0.02</td>
<td>1.00</td>
<td>0.32</td>
<td>0.72</td>
</tr>
</tbody>
</table>

* = significant at 0.05 level, ** = significant at 0.01 level
Except at an SAR of 5% where total Pb concentrations indicated an increase with decrease in clay content, no correlation was observed between clay and Pb in the different fractions of the vertisol at all SAR (Table 2). Correlations were observed between CEC/S2 and CEC/S5 fractions of Pb at SARs of 5% and 10% respectively (Table 2), and between CEC and all fractions of Pb at an SAR of 40%. However, at an SAR of 20%, no correlation between Pb in the different fractions and CEC was observed (data not shown). Organic matter correlated with the S2 fraction of Pb at an SAR of 5%, S2 and S5 at an SAR of 10%, S2 and S4 fractions at SAR of 20% and all fractions of Pb at an SAR of 40%, which further highlights the significance of OM in Pb retention in soils.

At SAR of 5%, Fe showed no correlation with Pb but increasing sludge content in the vertisol to 10% resulted in negative correlations between Fe/S3 and Fe/S4 fractions of Pb (Table 2) and Fe/S3 and Fe/S2 fraction of Pb at SAR of 20% and 40% respectively. The S3 fraction contains Pb bound onto Mn and Fe oxides. Considering that Fe and Mn concentrations in the sludge-amended vertisol decreased with increase in SAR, the amount of Fe and Mn oxides in the sludge-amended vertisol may have reduced. The decrease in Pb in the S3 fraction of the vertisol with increase in SAR may therefore be explained by lack of binding sites on the hydrous oxides onto which Pb could be sorbed.

Addition of sludge had no influence on the correlation between Pb in the different fractions and P. At an SAR of 10%, Pb concentration in the S1 fraction increased with Mn concentration and decreased in the S2 fraction (Table 2). Addition of sludge to the vertisol resulted in significant correlations between MF and S1 fraction of Pb ($r = -0.78$) only at SAR of 5%; CEC ($r = 0.65$), Fe ($r = 0.71$) and S1 fraction ($r = 0.73$) at SAR of 10%; S1 fraction ($r = 0.68$) at an SAR of 20%; and, CEC ($r = 0.92$), OM ($r = 0.73$), S1 fraction ($r = 0.95$), S2 fraction ($r = 0.74$), S3 fraction ($r = -0.94$), S4 fraction ($r = -0.95$), S5 fraction ($r = -0.97$) and total Pb ($r = -0.96$) at an SAR of 40%.

Principal Component Analyses of the analysed soil properties and Pb concentrations in the different fractions of the control vertisol revealed three components with Eigen values above 1.0 and explaining 82.84% of the variance observed. In the sludge-amended vertisol, five (5), four (4), five (5) and three (3) components were extracted respectively for the vertisol with 5 wt %, 10 wt %, 20 wt % and 40 wt % of sludge (Table 3). These components explained a total of 90.37%, 87.08%, 91.75% and 93.5% of the variance, respectively.

### Table 3 - Rotated component matrix of the control and sludge-amended vertisol.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sludge application rate = 5%</th>
<th>Sludge application rate = 10%</th>
<th>Sludge application rate = 20%</th>
<th>Sludge application rate = 40%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PC1</td>
<td>PC2</td>
<td>PC3</td>
<td>PC4</td>
</tr>
<tr>
<td>pH</td>
<td>-0.113</td>
<td>0.128</td>
<td>0.04</td>
<td>0.903</td>
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<tr>
<td>Clay</td>
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<tr>
<td>CEC</td>
<td>0.052</td>
<td>0.109</td>
<td>0.974</td>
<td>-0.005</td>
</tr>
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</tr>
<tr>
<td>Mn</td>
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</tr>
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<td>P</td>
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<tr>
<td>Exchangeable</td>
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<td>0.003</td>
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<td>Carbonate</td>
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<td>0.859</td>
<td>0.1</td>
<td>0.113</td>
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<tr>
<td>Reducible</td>
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<td>0.08</td>
<td>0.463</td>
<td>-0.052</td>
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<tr>
<td>Oxidizable</td>
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<td>-0.419</td>
<td>0.174</td>
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<tr>
<td>Residual</td>
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<td>0.111</td>
<td>0.061</td>
</tr>
<tr>
<td>Total</td>
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<td>0.109</td>
<td>0.123</td>
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<tr>
<td>Eigen Values</td>
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<td>% of Variance</td>
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<td>22.37</td>
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<tr>
<td>Cumulative %</td>
<td>32.33</td>
<td>54.61</td>
<td>72.41</td>
<td>82.15</td>
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</table>
82.84% of variances observed in the vertisol with 5 wt %, 10 wt %, 20 wt % and 40 wt % of sludge respectively. The relevance of the components for the concentration of Pb in the different fraction varied with SAR. At an SAR of 5%, components 1 and 3 were relevant, whereas at an SAR of 10 % and 40 %, it was component 1 and component 2 (Table 3) which were relevant for Pb concentration in the different fractions. The first three components extracted for the vertisol with 20 wt % sludge were all relevant for Pb concentrations in the different fractions (Table 3). The first two components extracted in the control vertisol explained 73.65% of the variance observed and are presented in a bi-plot in Figure 4. The bi-plot showed a close association between CEC, pH, and the S1 and S2 fractions of the control vertisol (Figure 4). Studies by Businelli et al. [28], Naeem et al. [29] and Wahba and Zaghloul [30].

FIGURE 4 – Bi-plots of components extracted at the different sludge application rates.
have all highlighted the role of CEC and pH in Pb sorption behavior in soils.

Lead would be precipitated in neutral to alkaline soils [31]. Below a pH of 5.5, ion exchange plays an important role in Pb sorption in soils which is reflected in the association observed between Pb/pH and Pb/CEC in the vertisol without sludge. Though no correlation was observed between the different fractions of Pb and pH in the vertisol at all SAR, bi-plots of the components relevant for Pb concentrations in the different fractions of the vertisol with 40% sludge showed an association with the S3 and S4 fractions (Figure 4).

The S3 and the S4 fractions represent the less available fraction of Pb in soils and retention of Pb in soil is closely related with pH. At an SAR of 40% the pH value of the vertisol was significantly lower than in the control vertisol and in the vertisol with sludge applied at rates of 5%, 10% and 20%. The changes in pH may therefore explain the association between pH and the S3 fraction whereas the link between pH decrease and OM decomposition in the sludge-amended vertisol could explain the association between pH and the S4 fraction of Pb in the vertisol at an SAR of 40%. Cation exchange capacity was not associated with the concentrations of Pb in the different fractions of the sludge-amended vertisol except at an SAR of 40% where some association was observed between CEC and S5 fraction. Changes observed in the properties of the vertisol including pH, P, and OM content due to sludge addition were more pronounced at an SAR of 40% (Figure 2).

Montmorillonitic soils have a small amount of pH-dependent charge which affects CEC of the soil. Reduction in vertisol pH as a result of sludge addition could have affected the CEC of the vertisol significantly at an SAR of 40% with a consequent increase in amount of Pb sorbed onto the S5 fraction of Pb (Table 1). Lead has a very high affinity for OM [32, 33] with which it forms strong complexes [34]. The concentration of Pb in the less available S4 and S5 fractions is therefore expected to correlate with OM content of the vertisol.

An association was observed between the S5 fraction of Pb and OM, highlighting the role of OM in the retention of Pb by the vertisol. In the sludge-amended vertisol, OM was associated with the S2 and S5 fractions of Pb at an SAR of 5%, S2 fraction at SAR of 20% and S1 and S2 fractions at an SAR of 40% (Figure 4). The S3 and S4 fractions were associated with Mn concentration in the vertisol whereas the S5 fraction showed association with the OM content of the soil (Figure 4). Correlation between Pb and humic substances in soil has also been reported by Castaldi et al. [12]. Though P has been shown to affect Pb sorption in soils, no association was observed between P and the different fractions of Pb at all SAR (Figure 4). The associations of the different geochemical fractions of Pb with different properties of the montmorillonitic vertisol were affected by sludge addition. In the sludge-amended vertisol, pH revealed no association with the different geochemical fractions of Pb whereas in the vertisol without sludge, an association was observed. At a pH of 5.5 and above, Pb is adsorbed on soil clay and OM surfaces, [28] which may explain the association observed between OM and the residual fraction of Pb at all SAR (Figure 4).

Clay content of the sludge-amended vertisol became associated with the S3, S4, Pb at SAR of 10%, 20% and 40%. Under acidic pH conditions, Pb adsorption on soil clay mineral surfaces is due to ion exchange but at higher pH, ion exchange and precipitation are important. The clay fraction of the soil was monominerallc with only montmorillonite identified. Given that Pb would preferentially sorb unto montmorillonite than other clay minerals [28, 29, 30, 35, 36] the association between the clay fraction and the available fraction of Pb is thus explained.

4. CONCLUSIONS

This study has characterised the concentration of Pb in the different fractions of a montmorillonitic vertisol amended with sludge at different rates. Sludge significantly affected the OM, P, Fe, Mn, pH and CEC of the soils and consequently the concentrations of Pb in the different fractions. Whereas the control vertisol displayed natural association with properties such as pH, CEC OM and clay content as revealed by PCA, the sludge amended vertisol had no distinct association. Results from cluster analyses separated the control vertisol and the vertisol with sludge applied at rates of 5% and 10% from the vertisol with sludge applied at 20%v/v. This may indicate that there are no significant differences in the properties of the control vertisol and those with 5% and 10% sludge but at higher rates of sludge application, differences may be significant. The research has indicated the possibility of using multivariate analyses to understand changes in the relationship between heavy metals in different geochemical fractions of sludge-amended soils and the properties of the soils.

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DETERMINATION OF PARAQUAT EMITTED IN THE AIR AFTER ITS APPLICATION DURING RICE GROWING SEASONS IN SUNGAI BESAR, SELANGOR, MALAYSIA

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ABSTRACT

Owing to increased use of paraquat through mist blower sprayer during the rice growing seasons in Malaysia, the need to assess the residue amount of paraquat emitted in the air from treated rice field is imperative. In this study, representative samples were collected before, during and after spray application from three sampling sites of Sungai Besar rice growing area that included: Kampung Simpang Lima, Kampung Sungai Besar, and Kampung Pasir Panjang. Both passive (cotton gauze sampler) and active (quartz fiber filter sampler) air sampling methods were employed before and after paraquat application in a 12h day time period with a 4h sampling intervals. Furthermore, the estimation of breathing zone paraquat during spraying was done through active sampling pump attached with quartz fiber filter cartridges. Study results showed that in day-long sampling events virtually no residue was detected in any of the samples exposed in the pre-event sampling, and obviously, the highest airborne residue was measured during spraying at breathing zone sampling of the spray operator, and subsequently the residue levels were drastically dropped in the post-spray sessions. However, in post-spray sampling sessions the residue level was consistently higher during first 0-4 hours relative to that of second 4-8 hrs sampling events. In comparison between two seasons, the detected residue amount showed insignificant seasonal variation, with higher values found during dry season (February-May) followed by a decrease during wet season (August-November). Interestingly, respirable residue in the air around the spray operator’s breathing zone during spraying was quite close between two sampling seasons and was bit higher in wet season than in dry season.

KEYWORDS: Paraquat, Airborne residue, Active sampling, Passive sampling, Rice field

1. INTRODUCTION

Pesticides use is still invariably an important tool in the pest management programs. Although these are applied to specific targets such as soil, water, or plant foliage, pesticide residues can be unintentionally transported from the target site as vapors and/or particulates to atmosphere [1]. The nature, quantity, and biological activity of airborne residues along with local meteorological conditions determine the potential for damage to non-target foliage, animals, and humans within the spray sites [2].

Due to huge government subsidies and lack of labour, rice farmers in Malaysia generally use large amount of pesticides in a season starting from land preparation until maturity [3]. Among the pesticides, paraquat that is considered an acutely toxic herbicide has been extensively used during the rice growing seasons because of its rapid action, relatively low cost and, broad spectrum of its activity [4]. Consequently, this heavy usage of paraquat may result in potential exposure to workers and other individuals near spray sites as because substantial quantities of agriculturally applied pesticides have shown to become airborne during and after application [5]. Moreover, the airborne paraquat exposure to field workers in Malaysia is amplified due to its application techniques by which all pesticides are generally sprayed by mist blower in a low volume spray (increased concentrations) for better pesticides efficacy that makes fine driftable pesticides-containing droplets [6]. Mist-blowers either mounted on a tractor or carried by workers, produce droplets with relatively small sizes (50-100 µm) that increases the potentiality of high levels of paraquat exposure for operators [7]. Typical mists (with a median droplet diameter of 57 µm) contain about 0.1% droplets with a size of 15 µm [8] those can easily enter the bronchi (but not alveoli if greater than 5-7 µm) [9, 10]. Thus, the deposition of paraquat in the ambient air and the potential health effects for field workers due to this exposure situation in treated rice field has been produced a wave of concern in both public and regulatory agencies. However, there is no information of airborne residue
associated with agriculturally applied paraquat during and after spray application in the rice growing area of Malaysia but the need for such data is becoming acute. Therefore, the aims of the present paper were to determine the amount of paraquat residue levels emitted from treated rice field before and after application, and in the breathing zone of the spray operator to measure the possible amount of paraquat breathed in during field spraying.

2. MATERIALS AND METHODS

2.1. Study area and sampling locations

The study was conducted at three sampling locations—Kampung Sungai Panjang (Kg. SP), Kampung Simpang Lima (Kg. SL) and Kampung Pasir Panjang (Kg. PP) in Sungai Besar, one of the major rice bowls of Selangor state in Malaysia. The study area is a coastal town in the district of ‘Sabak Bernam’ on northwestern part of Selangor, Malaysia. This town is located about 120 km north of Kuala Lumpur. The plain lands of the study area are totally occupied by rice cultivation and the sizes of sampling plots were 1 ha. The three sampling locations in the study area were chosen to give good spatial representation of airborne residue sampling. All of the sampling plots were situated adjacent to farmer’s house, and surrounding sites were also predominantly following rice farming practices.

2.2. Sampling seasons

In line with the rice cultivation, air sampling was done during two seasons: wet (August to November’ 2009) and dry (February to May’ 2010). During two sampling seasons, however, some uniform periodic changes in weather conditions were observed due to the Southwest and Northeast monsoons. During the period of August to November, the area experienced wet weather conditions due to southwest monsoon, marking light south-westerly wind with their speeds around 5-8 m/s dominate the area, and received an above-normal amount of rainfall. Thus resulted in slightly below-normal temperature (24 -30°C) and the mean relative humidity varied from a low of 65% to a high of 87 %. On the other hand, during the period of February to May, the area experienced relatively drier weather conditions due to northeast monsoon, resulting in an easterly or north-easterly winds of 7-10 m/s dominate the area and received below-average amount of rainfall. In this period, temperature was relatively warmer (28 -35°C) and the mean relative humidity was as low as 42% that reached as high as 70 % [11].

2.3. Paraquat application

The sampling plots of all three locations were sprayed by the same respective location’s spray operators and equipments (mist blower) during the entire study periods. During the study period, herbicide paraquat was sprayed just 2-3 days before planting. As practiced by local farmers, paraquat (Gramaxone, a.i 25% - Syngenta Corporation Sdn.Bhd.) was sprayed at 2 L/ha with a spray volume of 160 L/ha. Duration of spray application was recorded by stop watch that varied from location to location.

2.4. Air sampling procedure

Active and passive air samplers were deployed before and after spraying in the treated field in 4h sampling intervals. In this study, cotton gauze was used as passive air sampler (PAS) and quartz fiber filter as active air sampler which typically showed their efficient ability to trap ambient particulates in the air [12]. In passive air sampling, cotton gauze sampler (Gasmed Sdn.Bhd., Malaysia) was taped on five surfaces – west (W), East (E), North (N), South (S), and Top (T) of an identical dimensions (15x15x15 cm) foil-covered box. The box was placed 1 m above the ground surface at three randomly selected points nearer to downwind edges of the sampling plot.

Air was aspirated through active air samplers (quartz fiber filter) using field air sampling pump (Model 1067, Supelco, USA) with a calibrated flow rate of 10L/min operated in the field alongside passive air samplers. The sampling pump was connected by tygon tubing to quartz fiber filter (Supelco, USA) cartridge fitted in front of a glass housing.

For sampling respirable pesticides in air around the breathing zone of spray operator during spraying, sampling cartridge (quartz fiber filter) was connected by flexible tygon tubing to a battery-operated personal sampling pump (Model PAS-500, Supelco Inc. USA) with a calibrated flow rate of 0.3 L/min, and the sampling cartridge was fixed to the operator’s shirt collar to cover the breathing zone.

2.5. Sampling frequency and duration

The first sampling period was started 4-hours before spraying (pre-spray sampling). The second sampling was done during the spray period (during-spray sampling). The third sampling period started immediately after the end of spray application (0-4h post-spray sampling) and the fourth sampling period started just after the end of the preceding sampling (4-8h post-spray sampling).

2.6. Residue analysis

Residue extraction from samples was done according to ‘Method 5003’ with some modification as proposed in the NMAM (NIOSH Manual of Analytical Methods)[13]. Air samples (both active and passive air samplers) were carefully transferred to 15 mL plastic centrifuge tubes by clean tweezers. Ten mL acidic aqueous solvent (0.01N HCL) was added to each tubes. The tubes were capped, and allowed to stand for 30 min to soak samples completely. The tubes were then placed on an orbital shaker at 200 rpm for 1-hour followed by ultra-sonication (Cole Parmer, USA) for 2 hours to desorb analyte. One mL of each sample solution was transferred to HPLC vials and subsequently injected into the HPLC for the detection of the residue.
HPLC analysis was done based on the procedure described by Ouyang et al. [14]. The stationary phase (column) was C18 10µ (150mm x 3.9mm i.d.) from Waters (Ireland). The PAD detector (Waters 2996) wavelength was set at λ 257 nm. The HPLC mobile phase (1000 mL) was prepared by dissolving 5.0 g NaCl into 600 mL HPLC water that was previously adjusted to pH 3.0 with 1N HCL, and the solution was then mixed with 400 mL actonitrile. The flow rate (isocratic) of the mobile phase was maintained at 1 mL/min. A 10 µL volume of sample was injected into the column by auto-sampler system (Waters 717 plus) with a total sample run time of 5 minute.

2.7. Standard calibration curve

In HPLC system, response of paraquat was linear for 6 standard solutions of paraquat at concentrations of 0.01, 0.05, 0.5, 1.0, 5.0, 10.0 ppm. Linearity of calibration was assessed from a linear regression of response (area) versus concentration of paraquat, resulting in an $r^2$ of 0.999. The lowest calibration level (LCL), i.e. the lowest level of calibration standard which run on an instrument with acceptable response is 0.01 ppm. The average retention time of paraquat was 1.30 minute.

2.8. Fortification and recovery percentage

Fortification was done in three replications by spiking 100 µL of three fortification concentrations (0.5, 1.0 and 5.0 ppm) over the surface of sampling media (air samplers). Then the spiked samples were allowed to keep at 4°C overnight. Next day the spiked samples were extracted and analyzed to HPLC-UV. It was evident that paraquat showed good recoveries that ranged from 93% to 115% with a relative standard deviation (RSD) value of 4 - 8%.

2.9. Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The LOD and LOQ were determined via linear regression method using linear calibration curve of paraquat established at 5 standard concentration levels (ranges from 0.01 to 1.0 ppm) with three replicates [15]. The estimated LOD and LOQ of the analytical method for paraquat detection were 0.008 ppm and 0.03 ppm, respectively.

2.9. Quality control (QC) samples

Laboratory and solvent blanks were prepared and extracted as same as the field samples which showed no contamination in solvent and blank samples (fresh unused air samplers). One field blank sample for every 15 samples was submitted for analysis along with the field samples. All blank samples were below the analytical limit of detection (LOD) for paraquat tested.

2.10. Statistical analysis

Data on passive air samplers were analyzed following 2-way ANOVA (analysis of variance) between sampling location and sampler’s orientation. Data on active air samplers were analyzed with one-way ANOVA. Mean separation was done by Tukey’s Honestly Significant Difference (HSD) test using statistical analysis system [16]. Differences were considered significant at p<0.05.

3. RESULTS

3.1. Passive air samplers

Airborne paraquat residue data from field samples collected during wet (August to November) and dry (February to May) seasons were presented in Figure 1. Data indicated that samples collected during pre-spray period did not produce any paraquat residue in any of the samples of three sampling locations. However, exceptionally at Kg. Simpang Lima during wet season where one of the sampling events showed some residue detection due to the spray drift contamination from the nearby field. In post-spray sampling events, the residue amounts measured were consistently higher in the first post-spray events and subsequently observed a sharp decline in the second post-spray events. During first post-spray sampling event (0-4h), statistical analysis of data obtained over wet (F= 3.34; d.f. =2, 5; P = 0.172) and dry (F = 5.72; d.f.= 2, 5; P = 0.094) seasons showed no significant differences among the three locations. However, in the second post-spray events data obtained during wet season showed no significant difference (F= 0.98; d.f. = 2, 5; P = 0.471) but showed significant differences during dry season (F = 15.18; d.f.= 2, 5; P = 0.027).

During wet season sampling at first 0-4h post-spray periods, the highest residue was detected at Kg. Sungai Panjang (16.01 ng/cm²) which was followed by Kg. Simpang Lima (14.37 ng/cm²), and the lowest airborne residue was obtained at Kg. Pasir Panjang (10.66 ng/cm²). On the other hand, during dry season sampling the same highest amounts (21.84 ng/cm²) were observed at Kg. Simpang Lima and Kg. Pasir Panjang followed by Kg. Sungai Panjang (12.82 ng/cm²) that showed significantly 42 % lower amount than the former two locations. In the second post-spray (4-8h) periods during wet season, the highest amount of residue was deposited at Kg. Sungai Panjang (5.56 ng/cm²) and almost same amount was observed at Kg. Pasir Panjang (5.00 ng/cm²) which was followed by Kg. Simpang Lima (3.96 ng/cm²). However, during dry season sampling the highest amount of residue was reported at Kg. Simpang Lima (10.84 ng/cm²) that was significantly followed by Kg. Pasir Panjang (9.68 ng/cm²) and Kg. Sungai Panjang (5.26 ng/cm²).

3.2. Residue variations on orientation approaches of passive air samplers

During wet season at 0-4h post-spray period (Figure 2), among the samplers that positioned at south side recorded the highest amount (20.97 ng/cm²) followed by west (14.57 ng/cm²), east (14.49 ng/cm²), north (13.78 ng/cm²) and the lowest was on top side (4.59 ng/cm²). Considering the 4-8h
FIGURE 1 – Mean ±SD airborne paraquat residue measured by passive air sampler at pre- and post-spray periods in treated rice field during wet season (August – November) and dry season (February – May)

Values followed by the same letter(s) under same sampling seasons, are not significantly different at (P< 0.05)
Values followed by the same letter(s) under same post-spray sampling period, are not significantly different at (P< 0.05)

FIGURE 2 - Mean ± SD airborne paraquat residue measured in terms of passive sampler’s orientation approaches at post-spray periods in treated rice field during wet season.

Values followed by the same letter(s) under same post-spray sampling period, are not significantly different at (P< 0.05)

FIGURE 3 - Mean ± SD airborne paraquat residue measured in terms of passive sampler’s orientation approaches at post-spray periods in treated rice field during dry season.

post-spray period among five orientation approaches, the highest amount was found at east (6.57 ng/cm²) followed by south (6.15 ng/cm²), west (5.97 ng/cm²), north (3.34 ng/cm²) and top (2.18 ng/cm²).

In view of residue amount found at five sampler’s orientation approaches during dry season (Figure 3) at first (0-4h) post-spray measurements, the samples oriented at east produced the highest amount (23.33 ng/cm²) that was followed by significant reduction at south (20.68 ng/cm²), west (18.85 ng/cm²), north (16.82 ng/cm²) and top (14.49 ng/cm²). On the other hand, in 4-8h post-spray measurements the highest amount was recorded in samples oriented at west (11.87 ng/cm²) followed by significant reduction at south (10.01 ng/cm²), east (7.98 ng/cm²), north (7.52 ng/cm²) and top (5.59 ng/cm²).

3.3. Active air samplers

The results of active sampling done at pre- and post-spray periods during wet and dry seasons were summarized in Figure 4. In the pre-spray period of sampling during both seasons, there was no residue effect in any of the samples of all the three locations, but at Kg Simpang Lima one of the sampling events showed some residue detection during wet season due to the contamination from nearby field.

In the post-spray periods, residue amount measured immediately after spraying (0-4h) showed higher amount and then gradually reduced at second (4-8h) sampling event. In the first post-spraying measurements, no significant variations were observed during wet season ($F= 4.04$; d.f. $= 2.5$; $P = 0.14$) as well as during the dry season.
Values followed by the same letter (s) under same sampling seasons, are not significantly different at (P< 0.05)

FIGURE 4 - Mean ± SD airborne paraquat residue measured by active air sampler at pre-and post-spray periods in treated rice field during wet season (August – November) and dry season (February – May)
during spraying (F = 1.51; d.f.= 2, 5; P = 0.352) among the locations. Similarly, in the second post-spray measurement residue amounts were not significant among the locations during wet season (F= 2.11; d.f. = 2.5; P = 0.267) and also dry season (F = 1.29; d.f. = 2, 5; P = 0.394).

During wet season at first post-spray sampling, the highest residue was detected at Kg. Sungai Panjang (0.82 µg/m³) followed by Kg. Simpang Lima (0.51 µg/m³) and the lowest was at Kg. Pasir Panjang (0.47 µg/m³). On the other hand, during dry season the highest amount was found at Kg. Pasir Panjang (0.91 µg/m³) that was closely followed by Kg. Simpang Lima (0.84 µg/m³) and the lowest amount was recorded at Kg. Sungai Panjang (0.52 µg/m³). However, during second post-spray in wet season Kg. Sungai Panjang location recorded the highest (0.26 µg/m³) followed by Kg. Simpang Lima (0.18 µg/m³) and Kg. Pasir Panjang (0.13 µg/m³). However, in dry season the highest amount was at kg. Simpang Lima (0.35 µg/m³) followed by Kg. Pasir Panjang (0.26 µg/m³) and Kg. Sungai Panjang (0.23 µg/m³).

### 3.4. Residue amount at operator’s breathing zone during spraying

Results shown in Figure 5 indicate that the residue amount measured at operator’s breathing zone was statistically significant during wet (F =16.97; df = 2, 11; P = 0.0009) and dry (F = 6.07; d.f. = 2, 11; P = 0.021) seasons among three locations. In wet season, the amounts of airborne paraquat found at operator’s breathing zone at the time of spraying was highest at Kg. Sungai Panjang (148.61µg/m³) followed by Kg. Pasir Panjang (94.22 µg/m³) and Kg. Simpang Lima (86.38 µg/m³). However, the highest amount found at Kg. Sungai Panjang showed 72% increased over the lowest amount of Kg. Simpang Lima, and the amounts found at Kg. Pasir Panjang and Kg. Simpang Lima were statistically insignificant.

On the other hand, the estimation of breathing zone concentration of operators during dry season showed that the peak paraquat concentration was measured at Kg. Sungai Panjang (123.04 µg/m³), whereas the lowest was measured at Kg. Simpang Lima (84.07 µg/m³) that was around 32% significantly lower than the former location. However, the amount found at Kg. Pasir Panjang (118.86 µg/m³) showed no significant differences with the highest amount found at Kg. Sungai Panjang location.

### 3.5. Seasonal variations

Statistical comparison using a paired t-test between the airborne paraquat mean data of wet and dry seasons following active and passive sampling methods were summarized in Table 1.

**TABLE 1 Seasonal variation of airborne paraquat residue between wet and dry seasons**

<table>
<thead>
<tr>
<th>Sampling method</th>
<th>Sampling period</th>
<th>Airborne residue amount (ng/cm²) Mean ± S.E.</th>
<th>Wet season (Aug – Nov)</th>
<th>Dry season (Feb – May)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Passive</td>
<td>Pre-spray</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Passive</td>
<td>Post-spray (0-4h)</td>
<td>13.68 ± 1.58 a</td>
<td>18.83 ± 3.00 a</td>
<td>18.83 ± 3.00 a</td>
</tr>
<tr>
<td>Passive</td>
<td>Post-spray (4-8h)</td>
<td>4.84 ± 0.47 a</td>
<td>8.59 ± 1.70 a</td>
<td>8.59 ± 1.70 a</td>
</tr>
<tr>
<td>Active</td>
<td>Pre-spray</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Active</td>
<td>During spray</td>
<td>109.74 ± 19.56 a</td>
<td>108.66 ± 12.35 a</td>
<td>108.66 ± 12.35 a</td>
</tr>
<tr>
<td>Active</td>
<td>Post-spray (0-4h)</td>
<td>0.59 ± 0.10 a</td>
<td>0.76 ± 0.12 a</td>
<td>0.76 ± 0.12 a</td>
</tr>
<tr>
<td>Active</td>
<td>Post-spray (4-8h)</td>
<td>0.19 ± 0.03 a</td>
<td>0.28 ± 0.03 a</td>
<td>0.28 ± 0.03 a</td>
</tr>
</tbody>
</table>

Values followed by the same letter(s) row-wise, are not significantly different at (P <0.05)

ND = not detected
Moreover, the differences in residue levels in terms of passive sampler’s orientation approaches entirely due to local wind pattern and velocity since the several studies found the correlation of wind direction and speed with subsequent airborne residue deposition [23, 24]. The mechanisms of airborne particulate matter deposition on passive samplers were mainly by the physical mass transfer processes such as particulate diffusion, impaction or gravitation settling which depend on the characteristics of the depositing particles. It was noteworthy that in all cases the residue deposition was significantly lower on the samplers oriented horizontally on the top side due to the less air movement across the samplers that ultimately reduced the overall mass transfer coefficient of particulate matters via wind [25].

On the other hand, the considerable variation of residue concentration of paraquat found in the air surrounding the operator’s breathing zone area was mainly due to two factors, one was pesticides application techniques and other was prevailing weather conditions during spraying. Although paraquat was applied in same dosage rates with similar spraying equipment (mist blower) at all the three locations, but the droplets size distribution of the spray was different due to the differences in spray equipment parameters (such as restrictor size and engine speed). Furthermore, the length of spray lance influences the residue concentration in the surrounding air at operator’s breathing zone as the shorter the spray lance more residue will be around breathing zone. In this study, the highest residue was obtained at Kg Sungai Panjang due to reduced particle size of the droplet along with higher spray discharge height (as the spray lance was shorter) and, whereas at Kg Pasir Panjang spray operator used to spray on increased droplet sizes with longer spray lance. In this context, it is worthy to mention that large droplets having an appreciable fall velocity had less residence time in the air, in contrast, smaller particles with negligible fall velocity increased their residence time in the air [26]. On the other hand, the prevailing weather conditions mainly the air currents were also greatly influenced the spray droplets released by mist blower during spraying to be run-off on operator’s breathing zone. More importantly, the results also conformed to the study done by Makovskii [27] where paraquat residue measured in air during spraying were between 0.13 and 0.55 mg/m$^3$ depending on the mode of application, environmental conditions and rate of application.

4. DISCUSSION

From the result obtained by active and passive air samplers, it was quite evident that pre-spray air sampling in the early morning in the rice field did not show any airborne paraquat residue. This might possibly be explained by the role of wet deposition mainly by night fog/dew that helps complete removal of atmospheric paraquat residue, as the physicochemical properties of paraquat indicated it’s high water solubility [17]. On the other hand, varying levels of airborne paraquat residues among the three locations (although statistically insignificant) by both active and passive sampling in the post-spray measurement could be due to variation in local meteorological conditions as well as treated surfaces (plant and/or soil) characteristics.

Since it was seen during the study in the rice growing area that farmers used to apply paraquat just 3-4 days before transplanting/sowing when the treated surface was mostly bare soil surface rather than weedy surface, indicating the importance of nature and type of the soil surface characteristics (i.e. soil texture, soil water content and soil organic matter) for atmospheric deposition levels of paraquat [18]. In this context, it was worth stressing that paraquat exists primarily in the air as particulate forms [19] and following spraying the airborne paraquat tends to bound strongly with soil/dust particles [20]. It was justifiable to assume considering the paraquat’s insignificant vapour pressure that paraquat’s potential for becoming airborne after application entirely through wind erosion process of soil and/or dust particles on treated surfaces instead of volatilization/vaporization flux from treated surfaces. Factors that influence the erodibility of soil and dust particles of treated surfaces included horizontal wind speed, precipitation, temperature, relative humidity and cultivation practices [21]. Among the local meteorological factors, however, precipitation played the important role in the nature and concentrations of dust particles present in the treated surfaces as well as in the air. The wind erosion process generally affected from wet and moist soil surfaces that ultimately reduced the amount of dust particles in the air [22].

From the table, it was clearly shown that the residue levels of paraquat collected by both active and passive air samplers were higher in the dry season than that of wet season. The statistical differences in pre-spray periods was out of question, since there was no paraquat detection in active and passive samples under both wet and dry conditions. Statistical results showed that post-spray residue levels using both active and passive sampling methods in wet and dry season conditions were statistically insignificant. Similar result was also showed up in case of residue levels collected in the air at operator’s breathing zone area during spraying. However, the levels were quite close between the two seasons and exceptionally little-bit higher in wet season than dry season.

4.2. Seasonal Variations

While studying the variations of residue amount between wet and dry sampling seasons, it was found from the results that a upward trend of residue deposition was seen in the dry season in comparison with wet season. This would be due to the drier conditions in the dry seasons resulting in more emission processes (wind blown particles) into the atmosphere.

Although Malaysian weather is characterized by almost uniform temperature, high humidity and rainfall, but
some uniform periodic changes in weather conditions are observed year round due to the southwest and northeast monsoons. The weather conditions in dry seasons experienced warmer, drier and below-average rainfall in the study area that greatly enhanced the concentration of soil and dust particles loaded with pesticides in the ambient air leading to an increased entry into the atmosphere. These findings were in accordance with the investigation found by Todoir et al. [28] that the highest concentrations of pesticides in air usually occur in the spring and summer months coinciding with application times and warmer temperatures. Similar findings were reported by Stanley et al. [29] who found the highest pesticides air concentrations during summer and drier months at all the study locations.

5. CONCLUSIONS

Once the pesticide has been used for its intended purpose, the fate of the pesticide in the atmosphere becomes an important issue from the national point of view. The study is one of very few to measure the airborne paraquat levels by both active and passive sampling methods before, during and after its application by mist blower sprayer in the field conditions. The study clearly demonstrated airborne paraquat levels emitted from the treated rice fields at three different locations in the rice growing area of Malaysia that provides marked variations within day-long sampling periods and locations as well as between the two sampling seasons. However, residue amount showed spatial (location-wise) as well as seasonal variations during which environmental conditions, spray application techniques and the treated surfaces (plant and/or soil) characteristics played very important role on the atmospheric entry processes (wind erosion) of paraquat. The study recommends that effective spray application techniques should be implemented to assure continuous reduction of paraquat pollution during and after spraying during the rice growing seasons in Malaysia. The findings of this study needs to be considered in future for formulating guidelines in paraquat safety programs strategies and research.

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QUANTIFICATION OF THIAMINE AND COBALAMINE IN BIOLOGICAL FLUIDS BY SOLIDIFICATION OF A FLOATING DROP MICROEXTRACTION AND HPLC-UV

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ABSTRACT

A simple and sensitive liquid phase microextraction procedure based on solidification of a floated organic drop microextraction (SFDME) has been developed for the determination of thiamine and cobalamine in serum and urine samples. The extracts were analyzed by isocratic reverse phase high performance liquid chromatography (RP-HPLC) and UV detection. The procedure has been optimized with respect to type and volume of extraction solvent, salting out, pH, stirring rate, and time of extraction. The calibration graphs were linear for the both analytes over the concentration range of 5-400 µg/L, and coefficients of determination ranged from 0.994 to 0.998. The limits of detection were in the ranges of 3.1-9.2 µg/L. Intra-day and inter-day precisions for peak areas were in the range of 2.2-4.1% and 1.7-4.3%, respectively. The proposed procedure was successfully applied to the determination of analytes in spiked urine and serum samples with satisfactory results. The relative recoveries of spiked urine and serum samples ranged from 90.5 to 106.8%, with relative standard deviations varying from 2.5 to 4.3%. This method is simple, fast, precise, reproducible, linear over a wide range, sensitive, and especially suitable for the determination of trace amount of thiamine and cobalamin in biological samples.

KEYWORDS: Solidification; Floated organic drop microextraction; Thiamine; Cobalamin; Serum; Urine.

1. INTRODUCTION

Water soluble vitamins, thiamine (B₁) and cobalamin (B₁₂), are essential for metabolic process such as synthesis of red blood cell, normal growth, and functioning of the body cell [1-3]. Cobalamin plays several vital roles in the metabolism of certain amino acids and synthesis of DNA. Many diseases including fatigue, weakness, nausea, constipation, and weight loss may be due to deficiency of cobalamin in the animal bodies [4,5]. Thiamine has well-defined process in the metabolism of saccharine [1]. Both a lack and an excess of thiamine may cause significant serious diseases [6]. These vitamins are lost from the body in urine and faeces [4].

Several procedures have been reported for measurement of thiamine and cobalamin including fluorimetry [7, 8], chemiluminiscence [3,9], UV spectrophotometry [10, 11], and high-performance liquid chromatography (HPLC) [1,2,4,12,13].

Liquid-liquid extraction (LLE) is a traditional technique for extraction and preconcentration of many analyte from aqueous samples. However, in addition to time-consuming, tedious, and low sensitivity, LLE often requires large amounts of toxic organic solvents and can be relatively expensive [14,15]. Recently; solidified floating organic drop (SFDME), which is a modified solvent extraction method, was proposed for extraction and determination of organic analytes [14]. SFDME developed based on liquid-liquid microextraction, is a new microextraction technique in which small volume of an organic solvent with a melting point near room temperature (in the range of 10-30°C) is floated on the surface of aqueous solution. Even since, the proposed method has been successfully performed for the extraction and preconcentration of polycyclic aromatic hydrocarbons in water samples [14], fat-soluble vitamin [16], volatile aromatic hydrocarbons [17], pesticides in water samples [18,19], and estrogenic hormones in water samples [20]. The main advantages of this method were high enrichment factor, high extraction efficiency, and minimum organic solvent consumption, sensitive and effective for the removal of interfering matrices, low cost and simple in operation. The main drawback of the proposed method is the limitation on the selection of extraction solvent because of overlapping of solvent peak with some analytes peaks. However, the use of many solvents that have suitable melting points can decrease this limitation [14,19].

The objective of this study is to exploit the potential SFDME for the extraction of thiamine and cobalamin in
human serum and urine samples. Several important parameters affecting the extraction efficiency including the type and volume of extraction solvent, salting out, pH, stirring rate, and time of extraction on the performance of the analytical procedure was investigated.

2. MATERIALS AND METHODS

2.1. Chemicals and solvents

Thiamine hydrochloride (B₁), cyanocobalamine chloride (B₁₂), phosphoric acid, hydrochloric acid, sodium hydroxide, 1-undecanol, 1-dodecanol, 2-dodecanol, n-hexadecane, HPLC grade water, and methanol were supplied by Merck (Darmstadt, Germany).

2.2. Apparatus

The HPLC system (model SCL-10Avp, Shimadzu, Japan) consisted of a SPD-10Avp UV detector operating at wavelength of 290 nm, LC-10Avp dual solvent pumps and an EIG 001 injection valve. The analytical isocratic RP-HPLC separation was performed on a Shim-pack CLC-ODS column (4.6 mm×150 mm, particle size, 5 µm) with a CLC G-ODS guard column. The mobile phase was made up of phosphoric acid buffer (pH 4) and methanol (90:10, v/v); the flow rate was 1 mL/min. The pH measurements were made with a 780 pH meter (Metrohm, Switzerland) equipped with a combine Ag/AgCl glass electrode. The centurion scientific centrifuge (K280R, UK) was used for centrifuging.

2.3. Solidification of a floated organic drop microextraction

20 mL of aqueous sample solution, containing analytes and sodium chloride, was placed into a 25 mL sample vial, 10 µL of organic solvent was added on the surface of the aqueous sample solution using a 100 µL Eppendorf micropipette sampler. The sample vial was sealed and the solution was stirred for 50 min at 800 rpm using a magnetic stirrer bar. After that, the sample vial was transferred into an ice beaker and organic phase was solidified after 5 min. The solidified organic phase was completely transferred to another test tube using a spatula and melted immediately at room temperature. Then 5 µL of extractant was injected into the HPLC-UV for analysis.

2.4. Standard solution and biological sample preparation

Individual standard stock solutions of each analyte were prepared at a concentration of 1000 mg/L in double distilled water. Working solutions were prepared daily by appropriate dilution of the stock solution. Blank urine and serum samples (2 mL) were spiked with aliquots of working solutions to give an eight-point calibration curve 10-400 µg/L for thiamine and 5-400 µg/L for cobalamine. Each one was prepared in three replicates. Quantitative data obtained using based on the peak area of the standard solutions. All solutions were stored in refrigerator and protected from light, and submitted to SFDME method.

3. RESULT AND DISCUSSION

3.1. Effect of extraction solvent and its volume

One of the most important parameters affecting the extraction efficiency is the extraction solvent. The selection of extraction solvent was based on immiscibility with aqueous phase, low volatility, room temperature melting point, good extraction properties, and good chromatographic analysis. Based on these considerations, 15 µL of 1-undecanol (m.p.: 13-15°C), 1-dodecanol (m.p.: 22-24°C), 2-dodecanol (m.p.: 17-18°C), and/or n-hexadecane (m.p.: 18°C) were tested as extraction solvents to analyze the effect of the solvent on the extraction efficiency. Average peak areas as a function of extraction organic solvent were shown in Figure 1. The results demonstrated that 1-undecanol provided the higher extraction efficiency than other solvents. Therefore, 1-undecanol was selected as the most appropriate organic solvent for subsequent experiments.

![FIGURE 1 - Effect of organic solvent on the extraction efficiency. Experimental conditions: volume of aqueous solution=20 mL; volume of organic solvent=15 µL; extraction time=30 min; stirring rate=600 rpm; pH=7; [NaCl]=0%.](image-url)

The effect of volume of organic solvent was examined in the range 5-20 µL. The results demonstrated in Figure 2 show that the analytical signals initially increases with an increase of the organic phase volume up to 10 µL, followed by a decrease in peak areas with further increase in the organic phase volume. These increase and decrease in extraction efficiencies as a function of organic phase volume can be explained as, in the organic phase volume
of 5-10 and 10-20 µL interfacial area and volume of organic phase are predominated, respectively [21]. Therefore, all further experiments were carried out at the optimum volume of 10 µL.

3.2. Effect of salting out

The effect of ionic strength was extensively evaluated in the traditional liquid-liquid extraction; because the addition of a salt is often used to decrease the solubility of hydrophilic compounds in the aqueous phase through a salting-out effect and consequently increase the partition of analytes to the organic phase. In order to investigate the effect of salinity on the extraction efficiency of analytes, varied amounts of sodium chloride (NaCl) were added to 20 mL of 100 µg/L working solutions (pH 7) in the stirring rate of 600 rpm for 30 min. Figure 3 shows that for both analytes, the analytical signals increased with the NaCl concentration up to 3% and followed by decreasing with further increasing NaCl concentration. Hence, a salt concentration of 3% was chosen for further experiments.

3.3. Effect of pH of sample solution

The extraction efficiency of a weak organic base or acid depends on pH value of sample solution. The pH value of sample solution was investigated at 3, 5, 7, 9, and 11 from 20 mL of 100 µg/L working solutions in the stirring rate of 600 rpm for 30 min. The results showed the analytical signals improved with increasing of pH from 3 to 7 and followed by a decrease from 7 to 11 (Figure 4). Based on the above results, a pH value of 7 was recommended for sample solution for subsequent experiments.

3.4. Effect of stirring rate

The effect of stirring rate on the extraction efficiency of above mention vitamins was studied in the range 200-800 rpm using from 20 mL of 100 µg/L working solutions (pH 7) for 30 min. As demonstrated in Figure 5, the extraction efficiencies increased with the increase of stirring rate and highest peak areas were reached at a stirring rate of 800 rpm. Due to instability of organic drops, stirring rate above 800 rpm was not evaluated. Therefore, all further experiments were performed with stirring rate of 800 rpm.

3.5. Effect of extraction time

The proposed method is an equilibrium method and the better repeatability and the extraction efficiency are obtained in the equilibrium conditions. Effect of extraction time was examined over the range from 10 to 60 min under the above optimized experimental conditions. As shown in Figure 6, the extraction efficiencies increased with increasing extraction time from 10 to 50 min and reached equilibrium at 50 min. After 50 min, the curves reached a plateau and no increase in the extraction effi-
ciencies was observed with additional time. Therefore, an
extraction time of 50 min was selected for subsequent
experiments.

3.6. Method validation

Under the above optimum experimental conditions,
the proposed method was validated by linearity, limit
of detection (LOD), precision and accuracy. The linearity
of compounds was calculated using blank urine and serum
samples fortified at different concentration levels. The
 calibration plots were found to be linear in the range of
10-400 µg/L for thiamine and 5-400 µg/L for cobalamine,
with a correlation coefficients more than 0.994 (n=8). For
each concentration level, three replicate extractions were
performed. The limits of detection (LOD, S/N=3) were
found as 4.3 µg/L and 9.2 µg/L for serum and 3.1 µg/L
and 6.0 µg/L for urine samples, respectively, for both
vitamins. The linear ranges, coefficient of determinations,
and LODs are presented in Table 1. As can be seen, the
proposed method has low LODs and can be used for trace
analysis of analytes in urine and serum samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Comounds</th>
<th>Linear range (µg/L)</th>
<th>$R^2$</th>
<th>LOD (µg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>Thiamine</td>
<td>10-400</td>
<td>0.998</td>
<td>9.2</td>
</tr>
<tr>
<td></td>
<td>Cobalamine</td>
<td>5-400</td>
<td>0.994</td>
<td>4.3</td>
</tr>
<tr>
<td>Urine</td>
<td>Thiamine</td>
<td>10-400</td>
<td>0.997</td>
<td>6.0</td>
</tr>
<tr>
<td></td>
<td>Cobalamine</td>
<td>5-400</td>
<td>0.995</td>
<td>3.1</td>
</tr>
</tbody>
</table>

The intra-day and inter-day precisions were calcu-
lated by analyzing replicate (n=5) urine and serum sam-
ple with three different concentration levels (25, 100,
and 300 µg/L) of analytes on the same day and five
consecutive days (Table 2). As can be seen the relative
standard deviations (RSDs) calculated for intra- and in-
day precision are in the range of 2.2-4.1 and 1.7-4.3%,
respectively.

In order to investigate the recovery of the proposed
method, serum and urine samples were collected, spiked
with standards at three concentration levels (25, 100,
and 300 µg/L) and analyzed. The results are listed in Table 3.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Added (µg/L)</th>
<th>Thiamine</th>
<th>Cobalamine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intra-day</td>
<td>Inter-day</td>
<td>Intra-day</td>
</tr>
<tr>
<td></td>
<td>Found (µg/L)</td>
<td>RSD (%)</td>
<td>Found (µg/L)</td>
</tr>
<tr>
<td>Serum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25.0</td>
<td>23.0±0.5</td>
<td>2.2</td>
<td>24.0±0.6</td>
</tr>
<tr>
<td>100.0</td>
<td>98.3±2.5</td>
<td>2.5</td>
<td>99.2±4.3</td>
</tr>
<tr>
<td>300.0</td>
<td>285.0±8.6</td>
<td>3.0</td>
<td>290.0±9.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25.0</td>
<td>25.0±0.7</td>
<td>2.8</td>
<td>22.8±0.4</td>
</tr>
<tr>
<td>100.0</td>
<td>99.1±3.1</td>
<td>3.1</td>
<td>98.0±2.8</td>
</tr>
<tr>
<td>300.0</td>
<td>301.0±7.4</td>
<td>2.4</td>
<td>296.0±8.1</td>
</tr>
</tbody>
</table>
TABLE 3 - Results from determination of recovery of thiamine and cobalamin in spiked serum and urine samples by standard addition method (n=3).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Added (µg/L)</th>
<th>Thiamine Found (µg/L)</th>
<th>Recovery* (RSD) %</th>
<th>Cobalamin Found (µg/L)</th>
<th>Recovery (RSD) %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>0.0</td>
<td>60.5 -</td>
<td>-</td>
<td>112.4 -</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>25.0</td>
<td>87.2 106.8 (4.3)</td>
<td>137.4 100.0 (3.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>100.0</td>
<td>159.4 98.9 (3.9)</td>
<td>208.7 96.3 (3.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>300.0</td>
<td>346.9 95.5 (3.5)</td>
<td>401.8 90.5 (3.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>100.4 (3.9)</td>
<td>97.6 (3.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urine</td>
<td>0.0</td>
<td>65.2 -</td>
<td>143.6 -</td>
<td>95.0 (3.6)</td>
<td>94.1 (3.1)</td>
</tr>
<tr>
<td></td>
<td>25.0</td>
<td>88.6 93.6 (3.6)</td>
<td>168.4 99.2 (3.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>100.0</td>
<td>166.1 100.9 (3.4)</td>
<td>235.6 92.0 (2.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>300.0</td>
<td>336.8 90.5 (4.0)</td>
<td>417.0 91.1 (3.6)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Recovery = (the amount of found in the spiked sample – the amount of found in the sample) × 100 / the amount added.

Table 4 indicates the limit of detection (LOD), linear range (LR), relative standard deviation (RSD), extraction time, recovery, and matrix, using reversed-phase liquid chromatography [1], flow injection with chemiluminescence detection [3], liquid chromatography and UV detection [5], flow injection [7], liquid chromatography-UV detection [22], micellar liquid chromatography [23], and floated organic drop microextraction-high performance liquid chromatography-UV detection (SFDME-HPLC-UV) methods for the determination of B1 and B12 vitamins in different matrices. The proposed method provides similar quantification extraction efficiency, with advantages of being faster and using smaller volume of organic solvents.

4. CONCLUSIONS

A liquid phase microextraction procedure based on solidification of a floated organic drop microextraction (SFDME) coupled to HPLC-UV was developed for the determination of two kinds of water-soluble vitamins in biological samples. The method is simple in operation, sensitive, accurate, high extraction efficiency, and high enrichment factor. The results from validation indicate SFDME is an efficient method and can be applied for routine the determination of above vitamins in serum and urine samples.

ACKNOWLEDGMENT

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REFERENCES


REMOVAL OF Cr(VI) BY MODIFIED EXPANDED GRAPHITE USING RESPONSE SURFACE METHODOLOGY

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ABSTRACT

In this study, modified expanded graphite (MEG) was prepared by sequence processes of mixed sucrose/phosphoric acid solution impregnation, carbonization and in-situ H3PO4 activation. MEG was characterized by scanning electron microscopy (SEM), infrared spectroscopy (IR) and X-ray diffraction (XRD). Adsorptive property of MEG as a porous adsorbent for the removal of toxic Cr(VI) from aqueous solution was investigated. Three process parameters namely initial pH value (2.0-6.0), temperature (20-60 °C) and initial Cr(VI) concentration (50-150 mg·L-1) was optimized to obtained the best response of Cr(VI) removal using the statistical Box-Behnken design in response surface methodology (RSM). The response surface data indicated maximum Cr(VI) adsorption (99%) with initial 50 mg·L -1 of Cr(VI) concentration at pH 2.0, temperature 25°C. The MEG adsorbent could remove >80% Cr(VI) from aqueous solution with initial metal concentration of 50-80 mg·L -1. Adsorption data for Cr(VI) removal were analyzed according to Langmuir and Freundlich adsorption models and it was found that the Langmuir isotherm model best fitted the adsorption data. Kinetic studies were done and can be described by a pseudo-second order rate equation very well.

KEYWORDS: Modified expanded graphite (MEG), adsorption, Cr(VI), response surface methodology (RSM), Box-Behnken design.

1. INTRODUCTION

Heavy metals, such as copper, cadmium, lead, nickel and specially chromium, are a sanitary and environmental threat. Heavy metals in the environment can be accumulated in living tissues throughout the food chain, causing a serious health problem. Even a very small dose can cause severe physiological or neurological damage [1]. Due to the irrational actions of human being, chromium pollution released in quantity from leather tanning, electroplating, manufacturing of dye, paint, paper, and battery, etc [2-4]. Chromium also arises from volcanic activity and weathering of rocks by natural processes. Chromium is a highly reactive element and exists in six oxidation states. Hexavalent and trivalent are two most stable states. And, hexavalent chromium is considered powerful carcinogenic, mutagenic agent that modifies DNA transcription process causing important chromosomal aberration and more toxic than the trivalent form by the International Agency for Research on Cancer [5-7]. Thus, the maximum permission level of hexavalent chromium in potable waters is 0.05 mg·L-1 [8]. Therefore, the removal and recovery of Cr(VI) from contaminated water and wastewater is important to protect the environment and human health.

The commonly used treatment methods for dealing with the chromium metal pollution are ion exchange [9], reduction [10], adsorption [11-13], membrane separation [14] and bio-sorption [15, 16], etc. Among them, adsorption has proved to be a highly effective and economical method to remove heavy metal ions form aqueous solutions. Activated carbons, with well-developed pore structure and high surface area, are widely used as potential adsorbents for the treatment of chromium from aqueous solution [12, 17-21]. Thus, expanded graphite (EG) is an excellent inorganic carbon material, which has many advantages such as low density, non-toxicity, non-pollution and easy disposal. Recently, EG was reported to exhibit excellent adsorption for spilled oil floating on water [22] and gas adsorbent [23] due to the worm-like pore structure, weak polarity, hydrophobic and lipophilic nature. Compressed expanded graphite (CEG)-based carbon/carbon composites as absorbent in environmental applications were prepared by using phenolic resin [24], sucrose [25], etc., impregnated CEG.

In our previous study [26], the experimental results on the use of EG in removing Cr(VI) indicated that under the optimum adsorption conditions (initial pH 3.0, adsorbent dosage 4.0 g/L, and initial Cr(VI) concentration 50 mg/L) the maximum removal efficiency and uptake capacity were only 39.94% and 2.184 mg·g -1, respectively. The objec-
itive of this study was to enhanced the Cr(VI) removal efficiency by modifying the EG. The modified expanded graphite (MEG) was prepared by using mixed sucrose/H₃PO₄ solution impregnated non-extruded bulk EG. The precursor then was carbonized and in-situ activated by H₃PO₄. The SEM, XRD and IR have been used to evaluate precursor then was carbonized and in-situ activated by H₃PO₄ mixture and distilled water is 1.0 g for 4 h. Then the EG uniformly-coated with sucrose/H₃PO₄ solution was subjected to a heating process from room temperature to 180°C at a heating rate of 10°C·min⁻¹. The solidifying process at 180°C was kept for 2 h, which made the precursor consolidate in the mass.

After cooling to the ambient temperature in nitrogen atmosphere, the carbonized products that have been in-situ activated with H₃PO₄ was washed with 1.0 M NaOH to remove excess of acid followed by filtration. The samples were then washed with distilled water until neutral. After that, the final product abbreviated as MEG was obtained after drying overnight in an oven at 105°C, and then they were stored in desiccators for further studies.

### 2. MATERIALS AND METHODS

#### 2.1. Materials and Reagents

Commercially available expandable graphite as the raw material was purchased from Qingdao Nanshu Hongda Graphite Co., Ltd. (China). Sucrose (C₁₂H₂₂O₁₁), potassium dichromate (K₂Cr₂O₇), 1,5-diphenylcarbazide (C₁₃H₁₄N₄O, DPC), acetone (C₃H₆O), phosphoric acid were used without any further purification.

#### 2.2. Preparation of MEG

EG was prepared in our laboratory by microwave irradiation treatment of the expandable graphite in an EM-3011EB1 microwave oven (Sanyo Inc., China) according to the method reported by O.Y. Kwon and B. Tryba et al [27, 28]. The as-prepared non-extruded bulk EG (1.0 g) was immersed into 200 mL of mixed sucrose/H₃PO₄ solution with the fixed concentration (mass ratio of sucrose/H₃PO₄ mixture and distilled water is 1.0) for 4 h. Then the EG uniformly-coated with sucrose/H₃PO₄ solution was subjected to a heating process from room temperature to 180°C at a heating rate of 10°C·min⁻¹. The solidifying process at 180°C was kept for 2 h, which made the precursor consolidate in the mass.

Subsequently, the precursor composite of EG uniformly-coated with consolidated sucrose/H₃PO₄ were placed into a quartz tube reactor (with a length of 120 mm and an inner diameter of 50 mm). Then the composite was heated to 350°C with the rate of 17.5°C·min⁻¹ and kept at this temperature for 1 h under nitrogen protection.

After cooling to the ambient temperature in nitrogen atmosphere, the carbonized products that have been in-situ activated with H₃PO₄ was washed with 1.0 M NaOH to remove excess of acid followed by filtration. The samples were then washed with distilled water until neutral. After that, the final product abbreviated as MEG was obtained after drying overnight in an oven at 105°C, and then they were stored in desiccators for further studies.

#### 2.3. Characterization of MEG

Microstructure of EG and MEG were observed by using a JSM-5600LV scanning electron microscope (JEOL Inc., Japan) at 5.0 kV accelerated voltage. Infrared spectroscopy technique was used to examine the surface functional groups before and after modification of expanded graphite. The spectra were collected by Tensor 27 spectrometer (Bruker Inc., Germany) within the range of wave-number of 400-4000 cm⁻¹. Samples were prepared as KBr pellet. The IR spectrum was then recorded. The background obtained from scan of pure KBr was automatically subtracted from the sample spectra. To determine the crystalline structure of samples, X-ray powder diffraction (XRD) analysis was carried out at room temperature using a D/max-2550PC diffractometer (Rigaku Inc., Japan) with Cu Kα radiation (λ=0.15406 nm), over the 20 collection range of 0-80°.

#### 2.4. Optimization of Process Parameters Using Box-Behnken Model

Three operational parameters (pH, temperature and initial Cr(VI) concentration) important in the adsorption process were studied using Box-Behnken model of RSM with two levels (the minimum and the maximum). To analyze a process or system mutually with a response, $\hat{y}$ which depends on the input factors $X_1, X_2, X_3, ..., X_n$, the correlation between the response and the input process parameters are described in the following quantitative form:

$$\hat{y} = f(X_1, X_2, X_3, ..., X_n) + \epsilon \tag{1}$$

where $f$ is the real response function its format being unknown, and $\epsilon$ is the residual error which describes the differentiation that can be incorporated by the function.

Response surface is obtained by plotting the expected response but the value of $f$ is unknown and may be very complicated. So RSM approximates its value by using a suitable lower order polynomial. If the response varies in a linear manner, it can be represented by his linear function equation:

$$\hat{y} = b_0 + b_1 X_1 + b_2 X_2 + b_3 X_3 + \cdots + b_n X_n \tag{2}$$

But if curvature is there in the system, a higher order polynomial like quadratic model is used which can be stated in the form of the following equation:

$$\hat{y} = b_0 + \sum b_{ij} x_i x_j + \sum b_{ik} x_i x_0 + \sum b_{lj} x_j x_0 + \epsilon \tag{3}$$

where $\hat{y}$ is the response (dependant variables), $b_{ij}$ is the constant coefficient, $b_i$ is the slope or linear effect of the input factor $x_i$, and $b_{ij}$ the linear by linear interaction effect between the input factor $x_i$ and $x_j$, and $b_{ik}$ is the quadratic effect of input factor $x_i$ [29, 30]. Thus, RSM is a sequential procedure of performing experiments rapidly in various combinations to get optimal set of conditions for improved response.
In the experimental design model, pH (2-6), temperature (20-60°C) and Cr(VI) concentration (50-150 mg·L⁻¹), were taken as input variables, while Cr(VI) removal efficiency by the MEG absorbent was taken as the response of the system. The range and levels of three independent variables, viz. pH, temperature and initial Cr(VI) concentration are presented in Table 1.

<table>
<thead>
<tr>
<th>Variables Z</th>
<th>Coded variables</th>
<th>Variable levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>X₁</td>
<td>-1  0  1</td>
</tr>
<tr>
<td>Temp.(°C)</td>
<td>X₂</td>
<td>20  40  60</td>
</tr>
<tr>
<td>Conc. (mg·L⁻¹)</td>
<td>X₃</td>
<td>50  100 150</td>
</tr>
</tbody>
</table>

The regression analysis, statistical significance and response surfaces were obtained to find the most suitable combination of independent variables resulting in the maximum removal efficiency of Cr(VI) by MEG for predicting the responses. The statistical significance of variables was evaluated using the analysis of variance (ANOVA) and Student’s t-test. Adequacy of the constructed models was investigated via lack of fit, coefficient of determination (R²) and F-values. The results of the experimental design were studied and interpreted by Design-expert (StatEase, 7.1.6 trial version) statistical software to estimate the response of the dependent variable.

### 2.5. Batch Cr(VI) Adsorption and Kinetic Experiments

The stock solution of 1000 mg·L⁻¹ Cr(VI) was prepared by dissolving 2.8290 ± 0.0001 g K₂Cr₂O₇ in distilled water. Experimental solutions of desired concentration were obtained by further dilution of the stock standard solution. The required pH of the solutions was adjusted by 0.1 M HCl or 0.1 M NaOH by using METTLER TOLEDO pH meter (Model FE20).

Batch adsorption studies were performed in 17 sets of 100 mL Erlenmeyer flasks containing MEG (2.0 g·L⁻¹) and 50 mL of simulated Cr(VI) solution in varying concentrations (50-150 mg·L⁻¹) with different pH values (2.0-6.0) and the suspensions were shaken at different temperature (20-60°C) at 150 rpm for 12 h using water bath with orbital shaker. The initial and equilibrium concentration of Cr(VI) were analyzed by Xin-Mao spectrophotometer (Model UV-7504) using 1,5-diphenylcarbazide (DPC) as the complexing agent at the wavelength of 540 nm (GB7467-87) [31, 32]. The adsorbed amount of Cr(VI) ion per unit weight of MEG at time t, q_t (mg·g⁻¹), was calculated by the following equation:

\[
q_t = \frac{(C_0 - C_t) \cdot V}{W}
\]  (6)

where \( C_t \) is the Cr(VI) concentrations (mg·L⁻¹) at time \( t \).

### 3. RESULTS AND DISCUSSION

#### 3.1. Characterization of MEG

Fig. 1 reports the SEM micrographs of the produced graphite materials: (a) EG \( \times 300 \) and (b) MEG \( \times 300 \). From Fig. 1a, it is clearly observed that EG is a loose and porous worm-like material. As shown in Fig. 1b, the pyrolyzed carbon derived from sucrose located both at the surface and the interior of graphite flakes. The quantity of pyrolyzed carbon was less in the interior than that uniformly-coated on the surface [24, 33]. The inner pores between the flakes were partly filled with activated carbon, therefore the total amount of the open pores and the total pore volume of the EMG composites were reduced (Fig. 1b).

Fig. 2 shows the IR spectra of produced graphite materials: EG and MEG. The absorbance peaks at about 3444.7, 1635.5, and 1384.8 cm⁻¹ which correspond to the -OH stretching vibration mode, C=O vibration mode of the keto form and -C=O vibration mode of the enol form were still present in the FTIR spectrum of the EG after the modification [34]. After the carbonization and in-situ activation with H₃PO₄, more peaks, such as 1164.9, 1001.0 and 505.3 cm⁻¹, increased in the IR spectrum of the MEG (Fig. 2b). It showed that the anticipant polar groups had been introduced on the surfaces of the MEG with the carbonation and activation.

The EG modified by uniformly-coated with activated carbon after carbonation and in situ H₃PO₄ activation was evidenced by X-ray diffractions (Fig. 3). A significant difference was observed in the intensity of the peaks in the XRD spectrum before and after modification of expanded graphite. All the patterns illustrated in Fig. 3a can be easily indexed as graphite materials, which are corre-
FIGURE 1 - Scanning electron micrograph of produced graphite materials: (a) EG and (b) MEG.

FIGURE 2 - IR spectrum of produced graphite materials: (a) EG and (b) MEG.

FIGURE 3 - XRD patterns of produced graphite materials: (a) EG and (b) MEG.

3.2. Optimization Studies by Statistical Experimental Design

In the present work, experiments were planned following the Box-Behnken design of RSM to obtain a quadratic model consisting of 12 trials plus 5-centre points. The design matrix of the variables in coded units is given in Table 2 along with the predicted and experimental values of response. Each run was performed in duplicate and mean values for % removal efficiency of Cr(VI) are presented in Table 3, while the predicted values of responses were obtained from quadratic model fitting techniques using the software mentioned above.

TABLE 2 - Box-Behnken design matrix for three variables along with observed response.

By applying multiple regression analysis methods, the predicted response, \( \hat{y} \), for can be obtained and given as Eq. (7):

the spectrum of the MEG was widened and weaker compared with that of the EG, which implied that the MEG consisted of graphite particles with more disordered flakes. Intensity of diffraction peaks decreased in MEG [24].
where $\hat{Y}$ is the predicted response and $A$, $B$ and $C$ are the coded values of the test variables, pH, temperature (°C) and initial Cr(VI) concentration (mg·L$^{-1}$), respectively. The statistical significance of Eq. (7) was checked by $F$-test, and the analysis of variance (ANOVA) for response surface quadratic model is summarized in Table 3.

The analysis of variance (ANOVA) of regression model was carried out to find the significance of the main effects and interacting effects of parameters on the adsorption process. Values of “(Prob. > $F$) = 0.0001” less than 0.05 indicate model are highly significant. The goodness of the model was checked by the determination coefficient $R^2$ and multiple correlation coefficients $R$. The value of adjusted $R^2$ (0.9877) suggests that the total variation of 98% for the Cr(VI) removal to the independent variables and only about 2% of the total variation cannot be explained by the model. The value of $R^2$ (0.9946) obtained in the present case indicates good correlation between the experimental and predicted values of the response. Lack-of-fit test was also carried out, which measures the failure of a model to represent the data in the experimental domain at points which are not included in the regression [30]. The non-significant value (0.19) of lack of fit test revealed that the quadratic model is statistically significant for the response and therefore it can be used for further analysis.

The main goal of response surface is to track efficiently for the optimum values of the variables such that the response is maximized. The three-dimensional response surface plots are the graphical representations of the regression equation. By analyzing the plots, the best response range can be calculated. These plots are presented in Fig. 4(a-c). From Fig. 4a it is clearly evident, as the temperature increased from 20 to 60°C, an increase in removal efficiency of Cr(VI) by MEG was observed, which indicates that the adsorption process was endothermic nature. There was maximum removal of Cr(VI) on the adsorption process at pH 2.0-4.0 but above this metal removal declined. Removal of Cr(VI) by different carbon sorbents has been found to vary as a function of pH of the solution. Metal adsorption depends on protonation or unprotonation of various functional groups on the carbon surface. The higher Cr(VI) adsorption capacity at lower pH values could be explained with the electrostatic attraction between positively charged groups of carbon surface and HCrO$_4^-$ ions [36]. The removal efficiency decreases with increase in the initial dye concentrations (Fig. 4b), which might be due to inhibitory effects caused due to repulsive forces between the Cr(VI) ions on the MEG surface. The interaction of initial Cr(VI) concentration with temperature was not significant as indicated by the shape of the contour lines in most of the interacting region (Fig. 4c). The optimum values of the variables can be analyzed by saddle point or by checking the maxima formed by the $X$ and $Y$ coordinates. The conditions obtained at the saddle point for best response. Responses were pH range of 2.0, temperature 25°C and initial Cr(VI) concentration 50-80 mg·L$^{-1}$, up to 80% removal efficiency was achieved under optimum process conditions. These points were located within the experimental ranges, implying that the analytical techniques could be used to identify the optimal conditions.

**Table 3 - ANOVA results of quadratic model for the removal of Cr(VI) by MEG.**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Sum of squares</th>
<th>DF</th>
<th>Mean square</th>
<th>$F$ Value</th>
<th>Prob. &gt; $F$</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>6731.31</td>
<td>9</td>
<td>747.92</td>
<td>144.04</td>
<td>&lt;0.0001</td>
<td>Significant</td>
</tr>
<tr>
<td>A-pH</td>
<td>215.02</td>
<td>1</td>
<td>215.02</td>
<td>41.41</td>
<td>0.0004</td>
<td></td>
</tr>
<tr>
<td>B-Temp.</td>
<td>188.37</td>
<td>1</td>
<td>188.37</td>
<td>36.28</td>
<td>0.0005</td>
<td></td>
</tr>
<tr>
<td>C-Conc.</td>
<td>6093.27</td>
<td>1</td>
<td>6093.27</td>
<td>1173.48</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>AB</td>
<td>11.06</td>
<td>1</td>
<td>11.06</td>
<td>2.13</td>
<td>0.1878</td>
<td></td>
</tr>
<tr>
<td>AC</td>
<td>0.54</td>
<td>1</td>
<td>0.54</td>
<td>0.10</td>
<td>0.7570</td>
<td></td>
</tr>
<tr>
<td>BC</td>
<td>0.03</td>
<td>1</td>
<td>0.03</td>
<td>0.01</td>
<td>0.9409</td>
<td></td>
</tr>
<tr>
<td>A$^2$</td>
<td>91.34</td>
<td>1</td>
<td>91.34</td>
<td>17.59</td>
<td>0.0041</td>
<td></td>
</tr>
<tr>
<td>B$^2$</td>
<td>102.77</td>
<td>1</td>
<td>102.77</td>
<td>19.79</td>
<td>0.0030</td>
<td></td>
</tr>
<tr>
<td>C$^2$</td>
<td>9.93</td>
<td>1</td>
<td>9.93</td>
<td>1.91</td>
<td>0.2091</td>
<td></td>
</tr>
<tr>
<td>Residual</td>
<td>36.35</td>
<td>7</td>
<td>5.19</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lack of fit</td>
<td>4.59</td>
<td>3</td>
<td>1.53</td>
<td>0.19</td>
<td>0.8964</td>
<td>Not significant</td>
</tr>
<tr>
<td>Pure error</td>
<td>31.76</td>
<td>4</td>
<td>7.94</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Correlation total</td>
<td>6767.66</td>
<td>16</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.3. Adsorption Kinetics

The influence of contact time on the adsorption of Cr(VI) using MEG at different Cr(VI) concentration is presented in Fig. 5. According to the results of kinetic study, the Cr(VI) adsorption rates exponentially increased in the first hour for various initial concentrations, and reached equilibrium gradually at 90, 300 and 450 min with 99%, 93% and 92%, corresponding to Cr(VI) initial concentrations of 50, 100 and 150 mg·L⁻¹, respectively (Fig. 5). The fast adsorption rate during the initial period may be due to the higher driving force making fast transfer of metal ions to the surface of adsorbent particles and the availability of the uncovered surface area and the active sites on the adsorbent [37, 38]. As these sites became progressively covered, the rate of adsorption decreased and made it take long time to reach equilibrium. Thus, the increase of time to reach equilibrium with an increase of initial Cr(VI) concentration was because the competition for the active adsorption sites increased. Similar result in Cr(VI) ion removal by activated carbon has been reported in Ref. [36].

It is very important to evaluate the adsorption dynamics, as well as adsorption equilibrium, by using theoretical models in order to design and control the adsorption process units. For that reason, the adsorption kinetics of Cr(VI), three kinetic models, namely pseudo-first order and pseudo-second order models, were used in this study.

The pseudo-first order model was presented by Lagergren [39, 40]. The pseudo-first-order model can be expressed as follows:

\[
\log\left( q_e - q_t \right) = \log q_e - \frac{k_1}{2} \cdot \frac{t}{303} 
\]

where \( q_t \) and \( q_e \) (mg·g⁻¹) are the amounts of Cr(VI) (mg·g⁻¹) adsorbed onto MEG at time \( t \) (min) and equilibrium, respectively and \( k_1 \) is the rate constant (min⁻¹). The rate constant, \( k_1 \) was obtained from slope of the linear plots of \( \log(q_e - q_t) \) against \( t \). Fig. 6 shows a plot of \( \log(q_e - q_t) \) versus \( t \) for the adsorption of Cr(VI) for pseudo-first-order equation. The values of pseudo-first order equation parameters together with correlation coefficients are given in Table 4.
The sorption data was also analyzed in terms of pseudo-second order mechanism. The pseudo-second order kinetic model suggests that adsorption process involves chemisorption mechanism [41] and described by Ho and McKay [42]:

\[
\frac{t}{q_t} = \frac{1}{k_2q_e^2} + \frac{1}{q_e}t
\]  

(9)

where \(k_2\) (mg g\(^{-1}\) min\(^{-1}\)) are the rate constant of pseudo-second order adsorption reaction. If pseudo-second order kinetics is applicable, the plot of \(t/q_t\) against \(t\) of Eq. (9) should give a linear relationship from which the constants \(q_e\) and \(k_2\) can be determined. Fig. 7 shows the application of pseudo-second order equation by plotting \(t/q_t\) versus \(t\). The values of pseudo-second order equation parameters together with correlation coefficients are listed in Table 4.

As given in Table 4, the coefficient of determination, \(R^2\) for the pseudo-first order adsorption model at all the studied concentrations have high value (>0.995), and their calculated equilibrium adsorption capacity \(q_{ec}\) are consistent with the experimental data. These facts suggest that the pseudo-second order adsorption mechanism is predominant. When the initial Cr(VI) concentration increases from 50 to 150 mg L\(^{-1}\), the rate constant, \(k_2\), decreases from \(4.17\times10^{-3}\) to \(0.63\times10^{-3}\) mg g\(^{-1}\)min\(^{-1}\). At lower concentrations, Cr(VI) ions present in the adsorption medium could interact with the binding sites, hence it has a higher rate constant results. At higher concentrations, because of the saturation of the adsorption sites, the rate constant of dye adsorption onto MEG absorbent shows a decreasing trend. After the initial stage of adsorption, the remaining vacant surface sites are difficult to be occupied due to repulsive forces between the Cr(VI) ions on the MEG surface. Moreover, several literatures have been used pseudo-second order adsorption model to express the adsorption of Cr(VI) by different carbon materials [17-21, 36].

### 3.4. Adsorption isotherms

Adsorption isotherms describe how pollutants interact with adsorbent materials and are very important for optimization of adsorption system. The adsorption isotherms for Cr(VI) were studied at 293, 313 and 333K. The analysis of the isotherm data by fitting them to different isotherm models is an important step to find the suitable model that can be used for design purpose. There are several isotherm equations available for analyzing experimental adsorption equilibrium data. In this study, the equilibrium experimental data for adsorbed Cr(VI) onto MEG were analyzed using the Langmuir and Freundlich models [43, 44]. The linear isotherm equations are expressed as the following:

**Langmuir:**

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

(10)

**Freundlich:**

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

(11)

where \(C_e\) is the equilibrium concentration of Cr(VI) (mg L\(^{-1}\)) and \(q_e\) is the amount of the Cr(VI) adsorbed (mg) by per unit of MEG (g), \(q_m\) and \(K_L\) are the Langmuir constants related to the adsorption capacity (mg g\(^{-1}\)) and the equilibrium constant (L g\(^{-1}\)), respectively. \(K_F\) as well as \(1/n\) are Freundlich constants. The values of \(K_F\) and \(1/n\),

![FIGURE 6 - Pseudo-first order kinetics plot for the adsorption of Cr (VI) onto MEG at 313K.](image)

![FIGURE 7 - Pseudo-second order kinetic plot for the adsorption of Cr (VI) onto MEG at 313K.](image)

### Table 4 - Kinetics parameters for the adsorption of Cr(VI) onto MEG at different initial Cr(VI) concentrations. (Absorbent dosage= 2.0 g·L\(^{-1}\), initial pH=2.0, T=313 K)

<table>
<thead>
<tr>
<th>C (mg·L(^{-1}))</th>
<th>Experimental</th>
<th>Pseudo-First Order</th>
<th>Pseudo-Second Order</th>
</tr>
</thead>
<tbody>
<tr>
<td>q_e (mg·g(^{-1}))</td>
<td>K_L</td>
<td>q_e</td>
<td>R²</td>
</tr>
<tr>
<td>50</td>
<td>24.8895</td>
<td>0.01377</td>
<td>11.9501</td>
</tr>
<tr>
<td>100</td>
<td>48.2757</td>
<td>0.00497</td>
<td>26.5993</td>
</tr>
<tr>
<td>150</td>
<td>70.8163</td>
<td>0.00537</td>
<td>34.8498</td>
</tr>
</tbody>
</table>
which roughly correspond to the adsorption capacity and the heterogeneity factor representing the deviation from linearity of adsorption, respectively.

Experimental and theoretical Cr(VI) adsorption isotherms of MEG are shown in Fig. 8. The shape of the curves was of L-type, indicating high affinity of adsorbate towards adsorbent. Isotherm parameters for the Langmuir and Freundlich models for the MEG, evaluated from the linear plots, are reported in Table 5.

![FIGURE 8 - Adsorption isotherms of the removal of Cr(VI) by MEG at 293, 313 and 333K (Initial Cr(VI) concentration=50-300 mg·L⁻¹, absorbent dosage=2.0 g·L⁻¹, pH=2.0).](image)

A further analysis of the Langmuir equation can be made on the basis of a dimensionless equilibrium parameter, \( R_L \) [45] also known as the separation factor, given by Eq. (9):

\[
R_L = \frac{1}{1 + \frac{q_m K_L}{C_0}}
\]

where \( C_0 \) is the initial concentration of Cr(VI) (mg·L⁻¹). The magnitude of \( R_L \) determines the feasibility of adsorption process. The values of \( R_L \) for the adsorption of Cr(VI) onto MEG at different temperature are presented in Fig. 9.

![FIGURE 9 - Langmuir separation factor \( R_L \) for the adsorption of Cr(VI) onto MEG at different temperature.](image)

**TABLE 5 - Isotherm constants and values \( R^2 \) for the adsorption of Cr(VI) onto MEG at different temperature.**

<table>
<thead>
<tr>
<th>Temperature (℃)</th>
<th>Langmuir constants</th>
<th>Freundlich constants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( q_m ) (mg·g⁻¹)</td>
<td>( K_L ) (L·mg⁻¹)</td>
</tr>
<tr>
<td>27</td>
<td>69.2042</td>
<td>0.1561</td>
</tr>
<tr>
<td>36</td>
<td>71.4796</td>
<td>0.1725</td>
</tr>
<tr>
<td>45</td>
<td>75.7576</td>
<td>0.2365</td>
</tr>
</tbody>
</table>

The linear calculations reveal that the correlation coefficients (\( R^2 \)) values of the linearized form of the two isotherm models were higher for Langmuir isotherm than that of the Freundlich isotherm for MEG adsorbent. This reinforces the fact that Langmuir isotherm is useful to explain the adsorption of Cr(VI) from aqueous solution onto MEG when it follows the monolayer mode, rather than the multilayer mode. The separation factor (\( R_L \)), an important parameter of the Langmuir isotherm, can be used to verify if the adsorption in the system studied is unfavorable (\( R_L > 1 \)), linear (\( R_L = 1 \)), favorable (0<\( R_L < 1 \)), or irreversible (\( R_L = 0 \)). As shown in Fig. 9, the value of \( R_L \) in the present investigation has been found 0<\( R_L < 1 \). Therefore, the adsorption process was very favorable. They also indicate that the adsorption of Cr(VI) onto MEG is more favorable at higher initial metal ion concentrations than at lower ones. The decrease in \( R_L \) with an increase in the solution temperature indicates that the adsorption is more favorable at high temperature, too (Fig. 9).

**4. CONCLUSIONS**

Applications of modified expanded graphite (MEG) for removal of Cr(VI) seems to be a practical approach. The present study showed that RSM was a suitable approach to optimize the process parameters for achieving maximum removal of Cr(VI). By adopting Box-Behnken design for the optimization experiments, it was possible to investigate the process variables completely and a high adsorption of Cr(VI) (>80%) was achieved from relatively low initial concentrations of the metal solution which otherwise are difficult to remove using conventional techniques. The adsorption isotherm data were well fitted to Langmuir isotherm while the kinetic data were represented by pseudo-second-order kinetic model. Cr(VI) adsorption by MEG was also evaluated with the aspect of thermodynamics. The results of the present study showed that the MEG prepared from mixed sucrose/H₃PO₄ solution impregnated and in-situ H₃PO₄ activation has been a high-efficiency adsorbent for Cr(VI) removal from aqueous solutions.

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REFERENCES


WATER-SAVING BY IRRIGATING TWO VARIETIES OF SORGHUM (ENERGY PLANT) WITH TREATED MUNICIPAL WASTEWATER: A 3-YEARS STUDY IN CENTRAL GREECE.

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ABSTRACT

In the last few years, the problem of limited precipitations and, consequently, the lack of water along with the rapid climatic changes have been one of the main topics of discussion in environmental forums all over the world. On the other hand, as the world population increases, so does water consumption for civil and agricultural use and urban wastewater. Under these circumstances, the national scientific communities focus their research on the possibility of reusing treated urban wastewater in agriculture as a source of irrigation water.

The effects of treated urban wastewater, by using subsurface drip irrigation (S.D.I.), on yield and growth of two varieties of sorghum, sweet and fiber, used as an energy plant, were studied, as well as the freshwater saving. For this purpose, experiments were made at the Experimental Farm Station of the University of Thessaly in the Velestino area during the years 2005 and 2006 (sweet sorghum) and 2008 (fiber sorghum) that consisted of a fully randomized complete block design with two treatments in four replications. The first treatment was irrigated with freshwater (FW 100ET), whereas the second one was irrigated once with wastewater and twice with freshwater (WW 100ET), in sequence.

The results showed that the rate of growth (height of plants) and the final yield of dry biomass differed but not significantly (P<0.05) in both treatments during the three years of study. However, by using processed urban wastewater, a significant saving in fresh irrigation water was achieved. Irrigating sorghum with treated wastewater seems to be a quite promising method to produce energy from biomass.

KEYWORDS: wastewater, subsurface drip irrigation, sorghum, biomass production, water saving

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1. INTRODUCTION

The increasing population and the continuous degradation of the surface and subsurface water, the unfair allocation of water resources to various uses (agriculture, industry, domestic etc.) and the limited precipitations have necessitated the discovery of new sources of water. In the developed industrial countries, one of the problems is managing the urban and industrial wastewater and finding ways to reduce it.

In the developing countries, especially in those with arid or semi-arid climates, there is a need for available technologies at reasonable costs, in order to increase the available amount of water, conserve the water resources and preserve the environment, in general.

The water quality of treated wastewater depends, to a great extent, on the quality of the municipal water supply, nature of the wastes added during use, and the degree of treatment the wastewater has received. Wastewater quality data routinely measured and reported at the wastewater treatment plant are mostly for treated effluent disposal or discharge requirements, in terms of gross pollution parameters [e.g., biochemical oxygen demand (BOD), chemical oxygen demand (COD), suspended solids (SS)] that are of interest in water pollution control. In contrast, the water characteristics of importance in agricultural and landscape irrigation are specific chemical elements and compounds that affect plant growth or soil permeability. Not all these characteristics are measured or reported by wastewater treatment agencies as part of their routine water quality monitoring program [1].

The recovery and reuse of treated wastewater discharges is considered to be contributing to: a) the development of new water resources, b) the conservation of the existing water resources mainly in coastal areas, where salt water has been found to infiltrate subsurface water-carriers, c) the reduction in the cost of water, and d) the development of a water resources policy with a special

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emphasis on the conservation of sources and the preservation of the natural environment [2].

The reuse of municipal wastewater is a useful practice in Mediterranean countries. Countries such as Cyprus, Israel, Italy, Spain, France, Egypt, Tunisia, Morocco and, lately, Greece, reuse treated wastewater effluents in agriculture. Such reuse practices of wastewater have been previously reported in most countries of the Middle East [3-9].

In Greece, the potential reuse of wastewater in abandoned areas has been studied, and a total of 35 plans of action in the 13 regions of the country have been developed [10, 11].

The efficient reuse of wastewater will contribute towards relieving the problem of water shortages and the protection of the environment in areas with climatic conditions similar to those of Greece, provided that the wastewaters are subjected to additional disinfection treatment so that their microbial load remains within the limits of safety set by international organizations. Treated municipal wastewater effluents have high concentrations of plant nutrients (N, P) and, therefore, their reuse will lessen the need of applying inorganic fertilizers [12].

Based on the above-mentioned aspects, changes in energy production policy and wastewater reuse strategies must be effected. Defending that point of view, the European Union and the Global Community have proposed a research focused on environmentally friendly methods and materials which could be used for maximizing the production of energy from renewable sources (such as biomass). On the other hand, they have also proposed the reuse of conventional fossil fuels so as to reduce their environmental impact. During the last decade, special emphasis has been placed on the farming of energy plants, such as rape, sunflower, sorghum etc, for energy production [13].

The term biomass is used to describe every material derived from living or recently living organisms (plants and animals) [14]. The remnants of the biomass burning for the production of energy are bio-disintegrative and, therefore, less polluting than the respective remnants of fossil fuels. The requirements for the building development of the countryside are laid down, leading to opportunities for restructuring the crops, resulting eventually in the creation of new jobs and a simultaneous dwindling of unemployment [15]. The biomass originating from plants can be used for the production of biogas, biodiesel, ethanol, methanol, oil, petrol and hydrogen [16]. It can also be burnt directly for heating buildings and electrical power generation.

Various plant species can be used for the production of biomass. Of special interest to Greece is sorghum (sweet and fiber). Sorghum (Sorghum bicolor L. Moench) is a tropical C₄ plant that belongs to the family of Agrostis (Poaceae). Among the types of sorghum that can be cropped, is the fruit-yielding sorghum, the sweet and fodder-yielding sorghum [17-19]. Sorghum does not need great amounts of irrigation water, and it is more productive compared to maize in conditions of insufficient irrigation [20]. For a maximum yield, 450-560 mm of water is required depending on the conditions of the cultivation environment. Its maximum requirements for water can reach 7.6 mm/per day during the stage of propagation [21]. Sorghum has also been called “a camel among plants” because of its wide adaptability, its resistance to saline-alkaline soils and water-logging. It is for this reason that sweet sorghum has become a very popular energy plant throughout the world [22]. More specifically, the fiber or cellulosic sorghum constitutes the main type of sorghum used primarily for the production of dry biomass for direct burning and, secondarily, for the production of liquid fuel, in contrast to the sweet sorghum that is mainly used for the production of liquid fuel.

The aim of this 3-year research is to study the effects of treated urban wastewater on the plant growth and biomass production of two varieties of sorghum (sweet and fiber sorghum), as well as on freshwater saving, when using subsurface drip irrigation.

2. MATERIALS AND METHODS

A field study concerning two varieties of sorghum (Sorghum bicolor L. Moench ssp. (Variety Keller and H132)) was conducted during the cultivation periods in the years 2005, 2006 and 2008, at the Experimental Farm Station of the University of Thessaly in Velestino, Magnesia, Greece (latitude 39°23'N, longitude 22°45'E). The crop was sown into a typic xerorthent (calcareous) soil, characterized by a particle-size distribution of 27.5% sand, 33.5% silt and 39% clay. The pH value was 7.8 and the organic matter was 0.97% [23]. The cultivation practices as well as the procedures used were all the same during the 3-years study. Two fully randomized treatments in four replications were organized [24]. Each experimental plot had a length of 5 m and a width of 4 m, namely, an area of 20 m², and consisted of 4 rows of plants. Each replicate was separated by a 1-m long strip. Two treatments were installed: 1) Subsurface drip irrigation (FW 100ET) with freshwater and 2) subsurface drip irrigation (WW 100ET) with a mixture of freshwater and wastewater.

The wastewater was obtained from the Municipal Enterprise for Water and Sewage of Volos. Nitrogen and phosphorus were removed from wastewater during the secondary treatment. There was also close monitoring of the waste quality by measuring its physical-chemical parameters. Treated urban wastewater contained a large amount of salts and chlorine ions in such a concentration that is forbidden for plant irrigation. For this reason, each wastewater irrigation was followed by two applications of freshwater, according to the following equation provided in the bibliography [25-27] regarding the ratio of water capacities:
The following taken as initial values (by analysis):
\[ C_a = 22 \text{ mg/L}, \quad C_b = 1400 \text{ mg/L} \]
Also it is true that:
\[ Q_a + Q_b = 1 \quad \text{and} \quad C_{\text{fin}} = 500 \text{ mg/L} \]
So equation 1 becomes:
\[ 22 \text{ mg/L} \times Q_a + 1400 \text{ mg/L} \times (1-Q_a) = 500 \text{ mg/L} \]
\[ 1378 \times Q_a = 900 \text{ mg/L}; \quad Q_a = 0.651 \]
Therefore:
\[ Q_b = 0.351 \]
So, \[ Q_a = 2Q_b \]

So, it can be seen that two freshwater irrigations and one wastewater irrigation are required. With this technique, salts were leached and the disfavored sequences of their concentration were avoided as much in the soil as in the plants. The amount of supplied water in the two treatments was equal to 100% of the daily evapotranspiration (ETc), which is the crop evapotranspiration calculated by multiplying a crop coefficient (Kc) and the reference crop evapotranspiration (ETo) as measured in an A class Evaporation Pan (Kpan = 0.77) [28]. Polyethylene laterals of 20-mm inside diameter with self-regulated and self-cleaning emitters were used, spaced 0.6 m apart and delivering water at a rate of 3.6 L h\(^{-1}\). The distance between the pipelines was 0.8 m. Subsurface laterals were installed at a depth of 0.45 m, and the subsurface system was additionally equipped with a vacuum breaker valve to prevent any water suction and emitter clogging when irrigation is paused. A disk filter, enriched with the herbicide trifluralin injected during irrigation to prevent root intrusion, was also installed.

The cultivation of the experimental field followed the common practice of the region regarding corn cultivation. The sowing took place in the middle of May in each of the three years of study using a 6-row sowing machine. The variety of sorghum that was used in the years 2005 and 2006 was “Keller”, while “H132” was used in 2008. The seed quantity per hectare was 6.5 Kg. During all cultivation periods, there were no fertilizer or chemical treatments upon sorghum biomass. The SPSS 14 statistical package was used and t-test applied to evaluate statistical differences among treatment means [24].

During the three cultivation periods, the sorghum height, the leaf area index, and the production of fresh and dry biomass were measured.

### 3. RESULTS AND DISCUSSION

#### 3.1. Meteorological data

The area of the experimental field is characterized by a typical Mediterranean climate with hot and dry summers and cool-humid winters. The air temperature and precipitation (10-day average values) prevailing in the experimental site during the growing periods of the years 2005, 2006, 2008 and during the last 25 years, is schematically presented in Fig. 1. It can be seen that the air temperature during the 3-year study period did not fluctuate much in relation to its values of an average year. Apart from May and June 2008, in which generally the temperature was higher than the average and the autumn was colder, in June and July, the temperature reached its highest level in the area and the plants needed more water. In 2008, during these two months, we noticed a rate of convergence to the previous years of the study. In 2005 and 2006, which were similar to each other, it can be seen that the temperature was slightly lower than the average (up to 1 or 2 °C) in early summer, ranging from about 19 °C in mid-May to 25 °C in late June. It remained constant at about 25–26 °C during July and early August, and dropped between 19 and 23 °C from mid- August to early September, and remained below 17 °C until the first fortnight of October. Except for May, as well as the autumn months, with sufficient rainfall in all three years of the study, during the summer months, the conditions were hot and dry, especially in the summer of 2005, when the phenomenon was intense (total rainfall 30 mm). Under such conditions, and more generally, under the conditions prevailing in Central Greece, most summer crops, including sorghum, need irrigation in order to reach acceptable yields (Fig. 1).

#### 3.2. Growth analysis (plant height)

Measurements of plant height were taken from early June until late September in each of the three years of the study. Our aim was to determine the plant growth for both varieties (sweet and fiber sorghum) and define the period of maximum biomass production. As it is obvious from Fig. 2, in the year 2005 (var. Keller), the plants in the treatment FW 100ET reached an average height of 3.15 m at 127 days after sowing (D.A.S.), whereas in the treatment WW 100ET, they reached an average height of 3.05 m (127 D.A.S.).
During the whole cultivation period of 2005, the plant height in WW 100ET treatment was higher than in the FW 100ET treatment, apart from the last two measurements at 127 D.A.S. (maximum growth) and 134th D.A.S. The possible reason behind this slight surpassing in height is the fact that the plants irrigated with wastewater took up the nutrients N and P that the processed wastewater contains, even in small quantities. It must be noted that there were not any statistical significant differences, at the level of 0.05 between the two treatments. Statistically significant differences were found only in the measurement at 85 D.A.S. (Fig. 2).

As presented in Fig. 3, in the year 2006 (var. Keller), the plants in the treatment FW 100ET reached an average height of 3.18 m (125 D.A.S.), whereas the average height in the treatment WW 100ET was 3.03 m (125 D.A.S.). It can be observed that the growth rate of the plants was similar to that of 2005 cultivation period.

However, up to day 54, the plant height of both treatments was similar. During the period between 66 and 91 D.A.S., height in WW 100ET treatment was greater than in FW 100ET. The possible reason for this little surpass is the same as in 2005. From 105 D.A.S. until 125 D.A.S., the FW 100ET treatment surpassed the WW 100ET treatment (Fig. 3). No statistically significant differences at a 0.05 level were observed between treatments in both measurements, and at a level of mean values.
In Fig. 4, it is shown that in 2008 (var. H132), the average plant height in the treatment FW 100ET was 3.34 m (122 D.A.S.), whereas in the treatment WW 100ET, the plants reached an average height of 3.25 m (126 D.A.S.). The average maximum height of the plants surpassed the corresponding plants’ height of previous years but was recorded during the same time period (end of September).

However, between 66 and 94 D.A.S., the height of the plants in the WW 100ET treatment surpassed the height of the plants in the FW 100ET treatment. The possible reason of this slight surpassing in height is the same like in the previous years of study.

Between 94 and 136 D.A.S., the FW 100ET treatment surpassed slightly the WW 100ET one, in terms of the plants’ height (Fig. 4). It should also be added, that no statistically significant differences at a 0.05 level were noted between the two treatments in both measurements, and at a level of mean values.

From the above study, it is observed that the average maximum height of the plants of both sorghum varieties was reached in the same period (end of September), as well as that there are not any statistically significant differences between them (P < 0.05).

### 3.3. Production analysis (dry biomass production)

Figures 5, 6 and 7 present the averages of the production of dry biomass from both treatments (FW 100ET, WW 100ET), in the years 2005, 2006 and 2008, respectively.

Figure 5 shows (var. Keller, 2005) that there is an increase in both treatments, in dry biomass, observed between the 1st measurement (52 D.A.S.) and the 4th measurement (127 D.A.S.) whereas, in the 5th measurement, a decrease in the production of dry biomass can be noted due to the aging of the plants (Fig. 5).

The maximum yields were recorded at 127 D.A.S. (33.70 Mg/ha of dry biomass in wastewater treatment, and 34.20 Mg/ha of dry biomass in freshwater treatment; Fig. 5).

Figure 6 shows (var. Keller, 2006) that there is an increase in both treatments, in dry biomass, recorded be-
between the 1st measurement (45 D.A.S.) and the 5th measurement (133 D.A.S.) whereas, in the 6th measurement, a decrease in the production of dry biomass can be noted for the same reason as above (age of plants).

The maximum yield was recorded at 133 D.A.S. (31.89 Mg/ha of dry biomass in wastewater treatment; 32.58 Mg/ha of dry biomass in freshwater treatment; Fig. 6).

Figure 7 shows (var. H132, 2008) that there is an increase in both treatments, in dry biomass, recorded between the 1st measurement (42 D.A.S.) and the 4th measurement (125 D.A.S.) whereas, in the 5th measurement, a decrease in the production of dry biomass can be noted for the same reason stated above.

The maximum yield was recorded at 125 D.A.S. (38.95 Mg/ha of dry biomass in wastewater treatment, and 38.78 Mg/ha of dry biomass in freshwater treatment; Fig. 7).

Generally, the maximum yields in dry biomass, for treatments of both sorghum varieties during the 3-years study were reported around mid and late September (125 – 133 D.A.S.), following the corresponding maximum height development in plants. It should be mentioned that, in both treatments, the maximum production of dry biomass ranged at quite high levels. It is worth-mentioning that in the conclusions of the experiment made in south Romania [29], the maximum production in dry biomass of sweet sorghum (water needs covered by 100% of evapotranspiration) reached 20.80 Mg/ha.

According to the statistical analysis, as far as the biomass is concerned, for both varieties of sorghum, in all three years of the study, there were no statistically significant differences between the two treatments at the 0.05 level, in both separate measurements and at a level of mean values.
3.4. Water saving

Table 1 shows the amount of water applied in both treatments, which was equal to 100% of daily ETc, as well as the amounts of fresh and wastewater used by subsurface drip irrigation (S.D.I.). The total amount of water is that applied by sprinkler irrigation (SP.I.) for germination and the first stages of cultivation as well as the amount of water applied by S.D.I.

Figure 8 illustrates schematically Table 1, and it is obvious that the largest total amount of water in both treatments, i.e. 683.93 mm, was recorded in 2008, as compared to 2005 during which the total amount of water used with irrigation was 540.40 mm. This was justified mainly by the higher temperatures that prevailed locally in 2008 (Fig. 1), since there were not any differences between the two varieties regarding their needs for water.
2006 was the year in which the largest saving in fresh irrigation water (25.76%) was observed, compared to the other two years of the study in which the fresh irrigation water saving was 20.73 (2005) and 24.56% (2008), respectively (Fig. 8).

4. CONCLUSIONS

The effects of treated urban wastewater, by using subsurface drip irrigation, on the yield and growth of two varieties (Keller and H132) of the energy plant sorghum were studied, as well as the freshwater saving during three cultivation periods (2005, 2006 and 2008). From this study, it can be concluded that during all cultivation periods, no statistically significant differences at the 0.05 level between the FW 100ET treatment and the WW 100ET treatment, with regard to the plants’ height as well as the dry biomass, were recorded. The water saving reached a level of 25.76% in 2006.

The positive effects of using treated urban wastewater encourage their use for the irrigation of energy plants, such as sorghum, with excellent results in biomass production based on the presumption that one application with wastewater is followed by two fresh water applications, and under conditions that the wastewater is being controlled in terms of its physical and chemical parameters.

The significant yield of the crop in biomass and the saving of water, following the use of subsurface drip irrigation and treated urban wastewater, prove the possibility of using fiber sorghum as an alternative crop for the production of biomass and energy.

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METABOLIC RESPONSES OF PLEUSTONIC AND BURROWING FRESHWATER CRABS EXPOSED TO ENDOSULFAN

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ABSTRACT

This work focused on the effects on metabolism of environmental concentrations of endosulfan in the freshwater crabs Dilocarcinus pagei and Zilchiopsis collastinensis. The concentrations used were 0, 6 and 62 µg of endosulfan l-1. Oxygen consumption, oxyregulation capacity and critical oxygen concentration were modified by the presence of endosulfan in D. pagei. In contrast, Z. collastinensis crabs showed a decrease in the ammonia-N excretion rate, but the oxygen consumption was similar between exposed and control crabs. A significant increase in the O:N ratio was evidenced in Z. collastinensis exposed to this biocide, demonstrating a shift toward lipid and carbohydrate primary metabolism. Dilocarcinus pagei controlled its oxygen consumption through isolation as a defense mechanism, while Zilchiopsis collastinensis modified the ammonia excretion. These different defense mechanisms might be related to the dissimilar habitats that each species occupies.

KEYWORDS: Pesticide pollution; metabolism shifts; freshwater crabs; defense mechanisms.

1. INTRODUCTION

Decapod crustaceans are present in freshwater environments associated with agricultural activities. Dilocarcinus pagei and Zilchiopsis collastinensis (Decapoda, Trichodactyliidae) are two common crab species occurring in ponds and rivers. As other crabs, they have a key role in matter and energy transport between water and terrestrial phases. The straight relationship with the sediment that crabs have promotes a constant exposition to the lipophilic pesticides adsorbed there, increased by the bioturbation caused with walking, feeding and burrowing activities [6-8].

When exposed to stressful situations, as constant biocide exposure, there is a shift in metabolism and in energy substrate. Some pesticides, polycyclic aromatic hydrocarbons and metals not only modify the energy substrate but also produce physiological changes that alter the oxygen consumption [9-11]. The exposure to biocides may also modify the energy expenditure [12]. Modifications in metabolism and energy expenditure caused by biocides may change the normal activities of decapods, e.g., growth, trophic activity and reproduction, which, in turn, could alter their density and affect aquatic food webs [13, 14].

Despite of the importance of crabs, there is little information regarding the effects of endosulfan on metabolic physiology of freshwater crabs, especially in the species of the Trichodactyliidae family. The aim of this study was to determine the effects of sublethal concentrations, which regularly occur in the environment, of a widely used insecticide, endosulfan, on the metabolism of D. pagei and Z. collastinensis juveniles to gain insights into their metabolism and their response to insecticides.

2. MATERIALS AND METHODS

Six-month-old juveniles were obtained from the crab hatchery of the Instituto Nacional de Limnología (CONICET-UNL). They were grown in captivity conditions at 25 ± 1° C. Mean (± SD) carapace width was 10.49 (± 1.46) mm in Z. collastinensis and 16.46 (± 3.23) mm in D. pagei crabs. Mean (± SD) weight was 0.48 (± 0.19) g for Z. collastinensis and 2.78 (± 1.21) g for D. pagei crabs. One

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2. MATERIALS AND METHODS

Six-month-old juveniles were obtained from the crab hatchery of the Instituto Nacional de Limnología (CONICET-UNL). They were grown in captivity conditions at 25 ± 1° C. Mean (± SD) carapace width was 10.49 (± 1.46) mm in Z. collastinensis and 16.46 (± 3.23) mm in D. pagei crabs. Mean (± SD) weight was 0.48 (± 0.19) g for Z. collastinensis and 2.78 (± 1.21) g for D. pagei crabs. One
hundred and five individuals of each species were selected. They were isolated without being feed for 4 days. The aquaria were cleaned every day. Molted crabs, if any, were removed and were not included in the experiments.

Commercial grade pesticides were employed, which contained 35% of active ingredient, i.e., endosulfan (Zebra Ciagro®; Red Surcos S. A., Argentina). The commercial product, with all the components used in farmland applications, was diluted with distilled water to obtain solutions with different concentrations. The nominal concentrations were 0, 7 and 70 µg of endosulfan l⁻¹ (C₀, C₁ and C₂ respectively). Endosulfan concentrations were measured by gas chromatography following the ASTM D 6520-06 method [15]. Oxygen consumption and ammonia-N excretion were measured for each crab. Thirty replicates were used for each treatment and control. At the beginning of each test, crabs were transferred to individual, sealed, respiratory, 500 ml-capacity glass chambers equipped with an oxygen sensor connected to an oxygen meter (HANNA model HI9143) [16, 17]. Temperature was kept at 25 ± 1°C and a photoperiod was set at 12:12 h Light – Dark.

Dissolved oxygen (DO) was measured at each hour. Data of the first four hours were used for detecting rapid responses to toxicant exposure. Data collected up until 22nd hour were used because oxygen consumption brought the DO to concentrations lower than 2 ppm in several replicates, and the hypoxia and the accumulation of metabolic waste products may introduce measurement errors [18]. In 10 animals per concentration, dissolved oxygen was measured until crabs become lethargic as a result of hypoxic stress or dual stresses of hypoxia and endosulfan. Oxygen consumption was converted to energy equivalent using an oxycalorific value of 20.11 J ml O₂⁻¹. Oxygen consumption and ammonia-N excretion rates as well as O:N ratio and energy expenditure rate were analyzed. Differences were compared with Kruskal-Wallis and Mann-Whitney U tests (p < 0.05). Regression lines, slopes and Y-interceptions were compared with the Student t-test [22].

3. RESULTS

Endosulfan concentrations were similar to nominal concentrations. These environmentally relevant concentrations had lethal effects on some crabs after 22 hours of exposure. Yet, survival was high in both species (Table 1). The oxygen consumption rate increased as the dissolved oxygen decreased in both species. The presence of endosulfan changed this behavior in *D. pagei* crabs. Regression lines between OCR and DO of control and exposed to C₁ crabs had similar slopes (p > 0.05) but different Y-interception (p < 0.05). Regressions line of control and exposed to C₂ crabs had different slopes and Y-interception (p < 0.05). Slopes and Y-interceptions were similar for both ex-

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**TABLE 1 - Endosulfan concentrations and crab survival**

<table>
<thead>
<tr>
<th></th>
<th>Endosulfan concentrations (µg l⁻¹)</th>
<th>Survival (%)</th>
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<tbody>
<tr>
<td></td>
<td>Nominal</td>
<td>Real</td>
</tr>
<tr>
<td>Control</td>
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<td>0</td>
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<tr>
<td>Concentration 1</td>
<td>7,00</td>
<td>6,00</td>
</tr>
<tr>
<td>Concentration 2</td>
<td>70,00</td>
<td>62,00</td>
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**TABLE 2 - Slopes values from regression analysis among dissolved oxygen and oxygen consumption rate of crabs exposed to endosulfan**

<table>
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<tr>
<th>Groups</th>
<th>Slope</th>
<th>Y-interception</th>
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</thead>
<tbody>
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<td></td>
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</tr>
<tr>
<td>C₀</td>
<td>-0,0139</td>
<td>0,1164</td>
</tr>
<tr>
<td>C₁</td>
<td>-0,0145</td>
<td>0,1406*</td>
</tr>
<tr>
<td>C₂</td>
<td>-0,0036*</td>
<td>0,0529*</td>
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<tr>
<td><em>Zilchiopsis collastinensis</em></td>
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<td></td>
</tr>
<tr>
<td>C₀</td>
<td>-0,0019</td>
<td>0,1368</td>
</tr>
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<td>C₁</td>
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<td>0,1859</td>
</tr>
<tr>
<td>C₂</td>
<td>-0,023</td>
<td>0,292</td>
</tr>
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* Slopes and Y-interceptions values statistically different from control (p < 0.05)
posed to endosulfan and control *Z. collastinensis* crabs (*p* > 0.05) (Table 2).

During the first four hours, OCR was similar in endosulfan concentrations and control group in *Z. collastinensis* (*p* > 0.05). In contrast, OCR of *D. pagei* was modified by the presence of endosulfan. Crabs exposed to C$_1$ had a significant increasing during the first four hours (*p* < 0.001 for hours 1, 2 and 3; *p* < 0.05 for hour 4) and a decrease during the first hour in crabs exposed to C$_2$ (*p* < 0.01). OCR at 22 hours was different only for crabs exposed to C$_1$, which had a higher rate compared with the control (*p* < 0.05). The oxygen consumption rate of *Z. collastinensis* was clearly higher than that of *D. pagei* (Fig. 1).

**FIGURE 1** - Mean values of oxygen consumption rate from control and exposed to endosulfan crabs.

**FIGURE 2** - Mean values of energy expenditure rate during the first four hours of control and exposed to endosulfan crabs.
In addition, Dilocarcinus pagei exposed to C₁ increased the energy expenditure rate as a response to endosulfan. Differences among control and exposed to 6 µg endosulfan l⁻¹ were significant in the first four hours (p < 0.05). Moreover, the energy expenditure rate was greater in Z. collastinensis (p < 0.001) because the individuals of this species were smaller than those of D. pagei crabs and had a higher oxygen consumption rate. Zilchiopsis collastinensis control and those exposed to endosulfan crabs had similar energy expenditure rates in the first four hours (p > 0.05) (Fig 2).

Another effect of endosulfan was the modification in the oxyregulation capacity of D. pagei, recognized by the a/b ratio. Compared with control crabs, C₁ caused a reduction in the oxyregulation capacity and an increase in the critical oxygen concentration (Cᵢ) (p < 0.05). The oxyregulation capacity and critical oxygen concentration of crabs exposed to C₂ were similar to control crabs (p > 0.05). The oxyregulation capacity and critical oxygen concentration were similar in both control and exposed Z. collastinensis crabs (p > 0.05) (Table 3).

The ammonia-N excretion rate of Z. collastinensis was higher than that of D. pagei in control and exposed to C₁ crabs (p < 0.001). Crabs of both species exposed to C₂ had a similar ammonia-N excretion rate (p = 0.917). This rate was similar in both control and exposed to endosulfan D. pagei crabs (p = 0.246). In contrast, Z. collastinensis crabs exposed to C₂ had a lower ammonia-N excretion rate than those exposed to C₁ and control crabs (p < 0.001) (Fig 3).

**TABLE 3 - Oxyregulation capacity (a/b ratio) and critical oxygen concentration (Cᵢ) from control and exposed crabs**

<table>
<thead>
<tr>
<th></th>
<th>D. pagei</th>
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<tr>
<td>a/b ratio</td>
<td>Cᵢ (ppm)</td>
<td>a/b ratio</td>
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<tr>
<td>Control</td>
<td>0.17</td>
<td>1.21</td>
</tr>
<tr>
<td>6 µg l⁻¹</td>
<td>1.22</td>
<td>3.22*</td>
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<tr>
<td>62 µg l⁻¹</td>
<td>0.96</td>
<td>2.85</td>
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* Differences statistically different from control (p < 0.05)
The O:N ratio was similar for both species in exposed to C1 and control crabs (p = 0.295; p = 0.055 respectively). In crabs exposed to C2, this ratio was higher in Z. collarastinensis crabs (p < 0.001). The O:N values were modified by the presence of endosulfan in Z. collarastinensis crabs exposed to C2 because the differences with control crabs were significant (p = 0.003). In D. pagei crabs, the O:N relation was similar among exposed and control crabs (p = 0.79), demonstrating no effects of endosulfan (Fig 4).

4. DISCUSSION

Environmental pollution by pesticides has been increasingly documented in rivers and lakes and poses a risk to the biota that inhabits those ecosystems. The exposure to endosulfan-containing products caused a shift in metabolism of D. pagei and Z. collarastinensis.

Endosulfan modified the oxygen consumption rate of D. pagei. Low concentrations resulted in an increase in the oxygen consumption rate, while a higher concentration caused a decrease of it. Endosulfan is a neurotoxic pesticide, which acts upon biota by blocking the chloride channels at the gamma-aminobutyric acid (GABA) receptor in the central nervous system, leading to neural excitation [23]. Also, it may have relevant sites of toxicological actions related with acetylcholinesterase inhibition [12, 24]. The increase in oxygen consumption rate could be related to those neurotoxic and physiological effects. The reduction in oxygen consumption of crabs exposed to 62 µg l−1 of endosulfan may be related with behavioral responses. Both species have the capacity of closing their gill chamber and isolate from the medium, which allows them to walk in terrestrial environments. When these crabs detect a stressor agent, they close their gill chamber, reducing their oxygen consumption and the intake of compounds present in the water. As the oxygen in the gill chamber water decreases and as the concentrations of excretion compounds increase, they replace this water for new oxygenated one, with the consequent influx of endosulfan. The net oxygen consumption rate is a balance between metabolic decrease caused by isolation and the increase related to acetylcholinesterase inhibition. Low concentrations of endosulfan caused an exiting stimulus in crabs, but the effect was not as strong as the one needed to activate behavioral defense mechanisms such as isolation.蒙特纳和科林斯 [12] reported a significant shift in oxygen consumption when individuals of the freshwater crab *Trichodactylus borellianus* were exposed to endosulfan. Similarly, shifts in oxygen consumption, as those observed in D. pagei crabs, were documented in the fishes *Labeo rohita* and *Geophagus brasiliensis* exposed to pesticides at different concentrations [25, 26].

In crustaceans, the metabolic pathways involved in nitrogen excretion are catabolism of amino acids and certain amides, degradation of nucleic acids as well as deamination of purine nucleotides and urea. Decapods crustaceans excrete nitrogen mainly as ammonia, and the most of it is excreted through the gill epithelium [27, 28]. The reduction in the ammonia-N excretion observed in Z. collarastinensis exposed to endosulfan might be related to a reduction in amino acid catabolism, suggesting that the pesticide interfere with the metabolism of this crab. Likewise a reduction in ammonia-N excretion was observed in the fish *Macrognathus aculeatum* when exposed to endosulfan [25].

Oxygen consumption could be combined with nitrogen excretion according to atomic equivalents. Information about the fuel used in metabolism and energy substrate type showed how environmental characteristics affect the metabolism of crabs. A high O:N ratio suggests an increase in lipid or carbohydrate metabolism, and a low O:N indicates an increase in protein metabolism [21]. In general protein consumption indicates as a stressful condition. However, in the present study, Z. collarastinensis crabs exposed to endosulfan showed a shift toward lipid consumption as energy substrate and a decrease in ammonia-N excretion. Because endosulfan causes histological damages in the hepatopancreas of crustaceans, proteins might be synthesized for cell and tissues production, with a decrease in amino acid catabolism and a reutilization of nitrogenous compounds. Therefore, the effects of sublethal endosulfan concentrations could not be strong enough to turn metabolic pathways to protein use in 22 hours, maintaining essentially lipid and carbohydrate use as fuel. The same behavior was observed in *T. borellianus* exposed to endosulfan [12].

In several shallow lakes of the Paraná-La Plata floodplain hypoxia is common [29]. Aquatic animals are able to regulate their oxygen consumption until the environment reaches a particular dissolved oxygen concentration called critical concentration (C). When the oxygen concentration falls below the C, animals cannot maintain a stable oxygen uptake and become oxyconformers. Endosulfan caused a shift in the oxyregulating capacity of D. pagei, increasing the C. Pesticide pollution, as a result of agricultural activities, make crabs, and probably crustaceans in general, more susceptible to environmental hypoxia. If the amount of oxygen needed for maintaining an aerobic metabolism is not achieved, there is a change to anaerobic metabolism, glycogenolysis of carbohydrates, lactic acid release and disturbance in acid-base balance, with several physiological effects [30]. A reduction in the oxyregulating capacity was reported for the shrimp *Penaeus aztecus* when exposed to napthalene [20]. Unfortunately, there is a lack of information about oxyregulation capacity and critical dissolved oxygen concentration in native species of the Parana-La Plata hydrosystems.

As other organic compounds, endosulfan is a hydrophobic biocide mainly associated with sediments of marine and freshwater environments. Crabs spend a large amount of time on the bottom, and any disturbance will promote the release of pesticides from sediments to the
immediate water phase. Hence, it is likely that crabs encounter endosulfan in the environment at similar concentrations to those used in the present study. When exposed to certain stressors, in this case endosulfan, *D. pagei* crabs controlled their oxygen consumption by getting isolated and avoiding a metabolism change as a defense mechanism. In contrast, *Z. collastinensis* crabs, which maintained the same oxygen consumption in all the treatments, suffered a shift in their metabolism, which caused several damages and the death of some individuals. This differential outcome could be related to the habitat of each species. *Dilocarcinus pagei* is a pleustonic crab that lives associated with roots of aquatic plants in rivers and ponds, where there is a high availability of dissolved oxygen. A defense mechanism related to oxygen consumption shifts and enzymes production could be developed by this crab because the availability of this gas is usually high. Instead, *Z. collastinensis* lives in burrows of the riverside, submerged in muddy water with low dissolved oxygen. A defense mechanism for this crab could be not related with oxygen consumption shifts because the typical availability of this gas is already low. Therefore, other physiological mechanisms, such as those concerning ammonia excretion shifts, might be employed by this species.

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**REFERENCES**


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