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APPLICATION OF A DISPERSION MODEL FOR SZEGED, A MEDIUM SIZED HUNGARIAN CITY: A CASE STUDY

András Zénó Gyöngyösi[1*], Tamás Weidinger[1] László Makra[2] and Györgyi Baranka[3]

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ABSTRACT

The aim of the study is the application of the CAR model to a medium-sized Hungarian city, Szeged. To study the sensitivity of the model, the concentration of pollutants as a function of distance from road axis, the effects of wind speed, road type and tree factor on the concentration as well as the concentration of the pollutants at different traffic speeds were analyzed and quantified. To summarize our results, main findings are as follows: the level of pollution increases with (i) increasing number of vehicles, (ii) decreasing speed in urban traffic (i.e., less than 50 $km \cdot h^{-1}$), (iii) larger fraction of heavy vehicles, (iv) increasing number of trees alongside the roads and (v) smaller mean annual wind speed. In addition, the model had been run on realistic input parameters, regional and city background concentration. Street geometry and traffic data for the period 1997-2007 at Szeged have been used. Model results have been compared to measurements showing good agreement with a slight overestimation of concentration due to the insufficient consideration of technical development of the vehicles; however, modelled data are showing smaller deviation than measurements.

KEYWORDS: traffic emission, air pollution, transport, statistical model, Szeged, Hungary.

INTRODUCTION

The majority (62.4%) of the population of Hungary lives in urban area. For this reason, both monitoring and modelling of urban air quality have great importance. The main source of pollution in cities – besides industry and households – is traffic. Though industrial and domestic emission is gradually decreasing year by year, road traffic is increasing continuously [1, 2]. Since it is virtually impossible to carry out fully comprehensive monitoring of pollution for all urban places, decision makers should use model results for the estimation of street air quality in many cases. There are three major approaches for street air quality models: (i) empirical approach; (ii) statistical approach and (iii) dynamical approach.

In Hungary, meteorological conditions for the development of poor air quality are most dominant from late autumn till spring time, in such cases when a well developed surface inversion fills the Carpathian Basin. Strong static stability usually occurs along with no significant wind conditions. This kind of situation occurs relatively frequently in the winter time during the development of a high pressure system after passing of a cold front over the Central European region. The stable layer inhibits pollutants to solute in the ambient atmosphere, so concentration of pollutants in urban area can rapidly increase.

Despite the progress made in controlling local air pollution, urban areas show ever increasing environmental stress. Safe comfortable urban environment and the risks of air pollution are of the major concerns. The importance of air quality problems depends on the size of the city, together with topographical, geographical and meteorological processes as well as with social factors [1].

The average annual variation of CO, NO, NO_2 and PM_{10} (with maxima in winter) are opposite to those of O_3 (with maxima in summer). The higher winter values are caused by atmospheric stability with frequent inversions. The lowest values in summer are due to dispersion caused by intensive vertical exchange in the atmosphere. The highest intensities of photochemical O_3 formation are observed during the early afternoon in summer. The very similar average weekly variations of CO, NO, NO_2 and PM_{10} show weekday maxima and weekend minima. Oppositely, those of O_3 show weekday minima and weekend maxima [1, 3, 4].

Study of the environmental impacts of any traffic management and control policies require not only analysis of average speeds but also other aspects of vehicle operation such as acceleration and deceleration [5]. Urban traffic is mainly characterised by stop-and-go driving cycles for vehicles joining the queue at traffic lamp junctions. The length of each cycle depends on the expected queue length at the

traffic lamp and the frequency of each cycle directly affects the level of vehicle emissions. The greatest percentage of emissions for a vehicle that stops at a traffic lamp is due to its final acceleration back to cruise speed after leaving the traffic lamp [6]. Another paper [7] deals with speed limits imposed by speed control traffic signals and the consequent emissions increase.

Furthermore, the shape of a city and the land use distribution determine the location of emission sources and the pattern of urban traffic. These factors together are affecting urban air quality. Accordingly, more compact cities with mixed land use provide better urban air quality compared to disperse and network cities [8].

Regulatory air pollution modelling has been carried out in Hungary since the early 1960s. Firstly Gaussian puff models were used, in which instability and boundary layer depths were calculated [9]. For the uniform application of transmission schemes the standardization of air quality models is crucial. This work ended in the early 1980s [10], when pollution of point, line and areal sources were modelled. The next step was the development of the Hungarian Standardized Model (HNS-TRANSMISSION) in the 1990s. This Gaussian transmission model can consider contribution of up to 50 sources. It is suitable to describe transmission processes from local to regional scales including the effect of orography [11]. The EPA AERMOD system has been implemented at the Hungarian Meteorological Service as a powerful tool for case study calculations [12, 13]. The development of a meteorological preprocessor for the model has also been performed.

For experimental and comparison purposes, numerical studies have been made with the Dutch CAR model [14], which has been applied in our experiments as well. The model is now used as a regulatory model for cities in the Netherlands and, as an international version, the CAR International [15] is also available. A parallel workstation version of the Finnish Meteorological Institute (the CAR-FMI) is a descendant of the Dutch model, which is able to calculate hourly concentrations and statistics (daily, monthly and annual means, percentiles, etc.) of inert (CO and NO_x) and reactive (NO, NO_2 and ozone) pollutants emitted by a network of sources (CAR-FMI web). CAR model has also been used by [16] in their estimation of pollution from traffic in Xian, China.

The aim of the study is to apply the CAR model to a medium-sized Hungarian city, Szeged. The measured concentrations of CO and NO_x are dominantly originated from traffic-related emissions [17]. In the CAR model, concentration data of CO and NO_2 are used. In order to convert NO_x to NO_2, an NO_2 submodel is also introduced.

Since our intention is mainly to determine annual means and percentiles of some pollutants (CO, NO and NO_2), and we want to analyze the effect of traffic on pollution on an annual basis, it is sufficient to use the original Dutch model, which requires much less computational resources than its descendants.

DESCRIPTION OF THE CAR MODEL

The Dutch **CAR** model (**C**alculation of **A**ir pollution from **R**oad Traffic) [14] uses an empirical approach for the estimation of mean annual concentrations of NO_2 and non-reactive pollutants (carbon-monoxide and benzene) in urban and rural areas. The relationship between street types, wind speed and concentrations of the pollutants considered was based on wind tunnel experiments [18]. The experiments considered 49 configurations of street dimensions (street width vs. height of obstacles aside, distances and shapes, etc.). Effect of trees along streets was also considered. Results were combined in the TNO Traffic model [19]. From TNO some distinct configurations were categorised and some modifications were performed. A source receptor function is specified for each street category as a function of distance from road axis (from 5 to 30 m). Annual averages and 1-, 8- and 24-hour 98 percentiles are the outputs of the model for each pollutant (Figure 1).

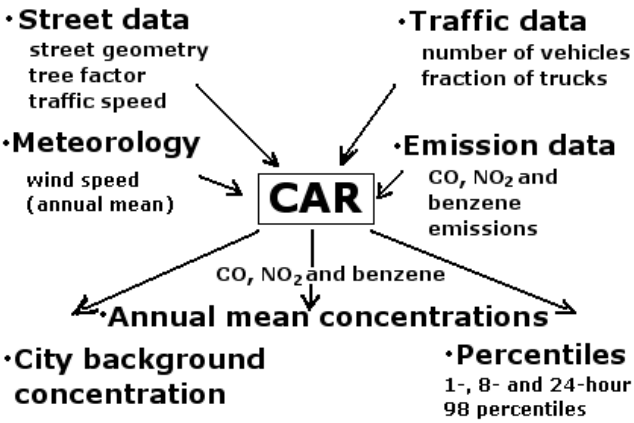

FIGURE 1 - Schematic diagram of the system parameter, input and output data of the CAR model [14].

The options of the model

One can choose from several street types for the calculations, as follows:

1. Road in open terrain, a few buildings or trees.

2. Base type, all roads different from type **1**, $\mathbf{3_a}$, $\mathbf{3_b}$ or **4**.

$\mathbf{3_a}$. Broad street canyon: building exceeding 3 m height on both sides of the road. Ratio of the height of the building vs. distance from road axis (*hb*) is between 1.5 and 3 on one side of the road and less than 3 on the other.

$\mathbf{3_b}$. Moderately narrow street canyon, *hb* ratio is less than 1.5 on both sides.

4. Building only on one side of the road, *hb* is less than 3.

The speed of road traffic can be categorised in four classes:

$\mathbf{V_a}$: Highway. Average speed is 100 km·h^{-1}.

$\mathbf{V_b}$: Road with maximum speed of 70 km·h^{-1}. Average speed is 44 km·h^{-1}.

$\mathbf{V_c}$: Regular city traffic. Average speed is 22 km·h^{-1}.

$\mathbf{V_d}$: Stagnating traffic. Flow of vehicles is not continuous. Average speed is 11 km·h^{-1}.

Emission factors can be adjusted to measurements in the model setup. The effect of trees along streets is considered for three types of vegetation:

1.00: Very few or no trees on either side of the street.

1.25: Trees on one side of the street, distance between trees is less than 15 m in the direction parallel to the road axis.

1.50: Trees on both sides of the street and tree tops touch each other over the street. More than one-third of the length of the street is covered by vegetation.

Calculation

Calculation is performed in the following steps:

1. Calculation of the city background concentration (C_b),

2. Assessment of the emission of the road traffic (E_t),

3. Calculation of the contribution by the configuration of the street (C_t),

Average concentrations are calculated at 1.5 m above surface from 5 up to 30 m away from the axis of the road.

The city background concentration ($C_b = C_r + C_c$) is obtained as a sum of the regional background concentration (C_r) and the size-dependent city contribution (C_c). The latter term ($C_c = \alpha \cdot R_c$) is a linear function of the radius of the city (R_c). The α coefficient has been determined by measurements. Diameter of the city equals to the diameter of the built-up area.

Two classes of traffic are considered: automobiles and trucks. Trucks are heavy vehicles (exceeding 3500 kg weight) and buses. Road traffic emission (E_t) is calculated as follows:

$$E_t = (1 - F_V) \cdot N \cdot E_p + F_V \cdot N \cdot E_V, \quad (1)$$

where F_V is the fraction of trucks in the traffic, N is the number of vehicles per day at the given location, furthermore E_p and E_V are the speed dependent emission factors of automobiles and trucks, respectively. It should be noted that emission factors used by the model have the dimension $\mu g \cdot m^{-1} \cdot s^{-1} \cdot vehicle^{-1}$, while the usual dimension of such parameters used by the official emission inventory is different ($g \cdot km^{-1}$). For this reason the emission parameters should be recalculated in the proper dimension (see Table 4 for the details and values used in the present calculation).

The contribution by the street configuration (C_t) is calculated using E_t road traffic emission factor and the street specific dispersion coefficient, which represents the effect of (i) wind speed, (ii) vegetation along the street and (iii) dilution during dispersion:

$$C_t = E_t \cdot \Phi_s \cdot F_r \cdot F_0, \quad (2)$$

where Φ_s is an empirical extinction (dilution) polynomial, a function of the distance from road axis. The dependent variable (x) of the polynomial is the distance from road axis. We use different Φ_s for different street types. F_r represents the ratio of the actual local annual mean wind speed to the national average. F_0 is the tree factor, which represents the effect of the trees on wind speed.

The 98 percentiles of the annual mean concentration for each pollutant (C_{pol}) is the sum of the city background concentration (C_b) and the street contribution (C_t) of CO, NO_2 and benzene:

$$C_{pol} = P_x \cdot C_t + C_b, \quad (3)$$

where P_x represents the ratio of the annual mean concentrations and the 98 percentiles of CO, NO_2 and benzene. P_x is a function of street type and can be adjusted to measurements in the model setup.

The above calculation is applicable only to inert gases. Since conversion of NO_x to NO_2 in streets can not be modelled in wind tunnel experiments, an NO_2 submodel – based on theoretical and empirical considerations – is introduced. The non-linear relation between NO_x and NO_2 is taken into account besides the direct emission of NO_2. The street contribution of NO_2 (C_{tNO2}) is calculated with the following correction factor:

$$C_{tNO2} = F_{NO2} \cdot C_{tNOx} + \frac{\beta \cdot C_{bO3} \cdot C_{tNOx}}{K + C_{tNOx}}, \quad (4)$$

where F_{NO2} is the fraction of emitted NO_2 of the total NO_x emission (that is a function of the traffic category and speed). So the first term represents the directly emitted NO_2 from traffic. The second term of the expression represents the ratio of NO_2 and NO_x at a certain ozone level (C_{bO3}). The β factor represents the fraction of background ozone concentration, which reacts with NO. K is a constant, based on measurements. C_{tNO2} , C_{tNOx} and C_{bO3} are the street contribution of NO_2, NO_x and background ozone concentrations, respectively [14].

SENSITIVITY STUDIES OF THE CAR MODEL

To study the effect of the input and system parameters on the calculated concentration, we performed model runs with arbitrary input data. The values of these parameters have been set to be close to their respective average or representative values for Szeged (Table 3 and 6). A city with a diameter of 4 km was considered. At an arbitrary site the fraction of trucks was put equal to 5 %, traffic was set to 20 000 vehicles per day with an average speed of 22 $km \cdot h^{-1}$ (**V_c** category) and tree factor was 1.25 in the standard run. Annual average wind speed was set to 2.5 $m \cdot s^{-1}$. Concentrations were calculated at 5 m from road axis. To assess the sensitivity, one parameter considered was modified, while the others remained constant.

In this chapter the effect of different input model parameters was studied to mean annual pollutants concentrations. Since for CO and benzene the results were identical (with different numerical values but same relative effects,

of course), we only present the results for CO. As NO_2 has a different behaviour, results for NO_2 are presented separately. However, if the type of pollutant is not mentioned, concentration in this chapter refers to annual mean concentration of CO.

We compared the concentrations calculated for both CO and NO_2 to their WHO and Hungarian Standard air quality limit values [20-22]. Limit values are given in three categories: Highly Protected (HP), Protected I. and Protected II. categories.

The effect of wind speed

To study the sensitivity of the model, the effect of wind speed was analysed. Calculations for different tentative annual mean wind speed were performed for all road types. Dependence of the pollutants concentrations on mean annual wind speed resulted in similar functions for all the pollutants considered (Figure 2). An obvious finding is that increasing wind speeds involve the decrease of pollutants levels.

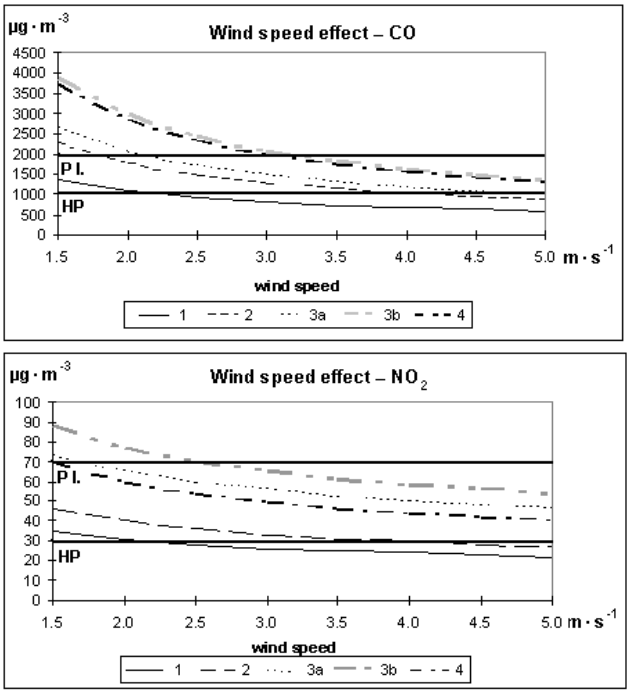

FIGURE 2 - The effect of mean annual wind speed on CO (upper panel) and NO_2 (lower panel) concentrations (µg·m^{-3}) 5 m away from road axis for different road types keeping other parameters constant: traffic 20 000 vehicles (5% trucks) per day, speed of vehicles 22 km·h^{-1} (V_c category), tree factor 1.25. Concentration limits: HP: Highly Protected; P I.: Protected I.

The highest CO concentrations (from 1 800 to 3 900 µg·m^{-3}, depending on road type) occurred at weak winds (at 1.5 m·s^{-1} mean annual wind speed), while strong winds (5 m·s^{-1}) resulted in the lowest concentrations (from 1 000 to 1 700 µg·m^{-3}). Wind effect, however, was more pronounced on CO than on NO_2. The ratio of maximum (at 1.5 m·s^{-1} mean annual wind speed) and minimum concentrations (at 5 m·s^{-1} annual mean) was 76 % for CO and 57 % for NO_2. The lowest concentration of CO for road type $\mathbf{3_b}$ is almost equal to its highest concentration for road type **1** (well below its limit value of Protected I. category: 2000 µg·m^{-3}). Its reason is that the effect of wind speed is less pronounced in a narrow street canyon, than in a broad street. It can be seen that the most significant decrease in mean annual pollutants levels with respect to the distance from the road axis appears at type $\mathbf{3_b}$. For road type **1** only small changes can be detected in the distance related concentrations; however, they do not exceed the limit value.

Pollutants concentration as a function of distance from road axis

Away from the axis of the road, lower concentrations are shown due to the dilution of the pollutants (Figure 3). In the standard run, CO levels close to the axis of the road were higher than the limit value for two of the five road types. However, concentrations of CO were below the limit at a distance exceeding 14 m for all the road types. According to other studies, the roadside concentrations of gaseous and $PM_{2.5}$ pollutants decrease with the distance from the road and the exposure to both gaseous and particle pollutants in the vicinity of the selected urban road sites is interrelated to on-road vehicle emissions [23].

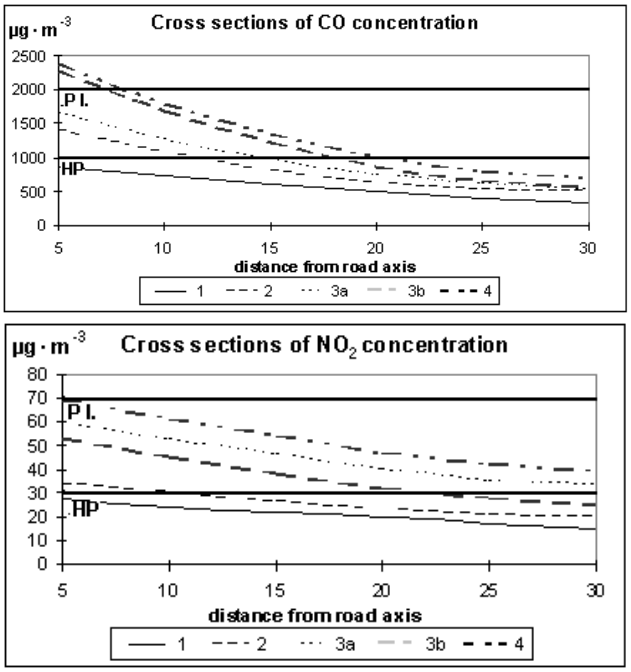

FIGURE 3 - Cross sections of CO (upper panel) and NO_2 (lower panel) concentrations (µg m^{-3}) for different road types. Annual mean wind speed: 2.5 m·s^{-1}, speed of vehicles: 22 km·h^{-1}, tree factor: 1.25. Concentration limits: HP: Highly Protected; P I.: Protected I. Distance from road axis is given in meters.

The effect of the road type and tree factor on the pollutants concentrations

The concentrations are the highest for road type $\mathbf{3_b}$, while those for road type **4** are only slightly lower. On the other hand, the lowest levels are detected for type **1**. Dif-

ferent circulation patterns in each road type canyon result in different mean annual concentrations by constant tree factors (Table 1). More trees aside result in higher concentrations, since trees near the road reduce wind speed and, hence, dilution of the pollutants is also reduced. Furthermore, it is obvious that the tree effect on pollutants levels is as large as the effect of buildings close to the street: by tree factor **1.50** at road type $\mathbf{3_a}$ concentration is equal to that at road type $\mathbf{3_b}$ by a tree factor value of **1.00**. Tree effect indicates the highest impact on concentrations for road types $\mathbf{3_b}$ and **4** (Table 1).

TABLE 1 - Concentrations of CO (μg·m^{-3}) for different road types and tree factors (F_0) 5 m away from road axis. Regional wind speed, traffic and speed of vehicles were put equal to 2.5 m·s^{-1}, 20 000 vehicles per day and category V_c, respectively. Relative contribution of trees is given in brackets (%).

Road type	F_0 = **1.00**	F_0 = **1.25**	F_0 = **1.50**
1	790	924 (17%)	1 058 (34%)
2	1 220	1 461 (20%)	1 703 (40%)
$\mathbf{3_a}$	1 411	1 701 (21%)	1 990 (41%)
$\mathbf{3_b}$	1 990	2 424 (22%)	2 858 (44%)
4	1 916	2 331 (22%)	2 747 (43%)

Pollutants concentrations at different traffic speeds

The CAR model can handle 4 different traffic speeds. Results for the most important speed categories are presented for calculation, assuming a tree factor of F_0 = 1.00 (Table 2).

TABLE 2 - Concentrations of CO and NO_2 at 5 m away from road axis for different traffic speeds. An annual mean wind speed of 2.5 m·s^{-1} was considered. (V_b: Road with maximum speed of 70 km·h^{-1}, average speed is 44 km·h^{-1}. V_c: Regular city traffic, average speed is 22 km·h^{-1}. V_d: Stagnating traffic, flow of vehicles is not continuous, average speed is 11 km·h^{-1}).

Road type	V_b	V_c	V_d	V_b	V_c	V_d
	[CO; μg·m^{-3}]			[NO_2; μg·m^{-3}]		
1	534	924	1 228	28	28	30
2	759	1 461	2 010	36	36	39
$\mathbf{3_a}$	859	1 701	2 358	59	59	63
$\mathbf{3_b}$	1 161	2 424	3 410	70	70	75
4	1 122	2 331	3 276	54	54	59

At all speeds the highest concentrations were taken for road types $\mathbf{3_b}$ and **4**. For CO, the mean annual concentration increases significantly with decreasing average traffic speed, since at lower average speed vehicles perform more speed change cycles especially in the lowest speed category, when vehicles perform several stop and go cycles. However, for NO_2 it is not the case. This is because vehicles are not the only sources of NO_2 generation. Nitrogen-dioxide can be formed due to chemical interaction of gases that are present in the urban air. Annual mean CO levels vary from 27 % to 171 % of the *Protected I.* limit value (2000 μg·m^{-3}) (Table 2). Concentrations of NO_2 occur within a much closer interval than those of CO. Its concentrations vary from 40 % to 107 % of the *Protected I.* limit value (70 μg·m^{-3}) (Table 2). As traffic speed decreases, pollution reaches the unhealthy level for several road types. At speed $\mathbf{V_b}$, levels of both CO and NO_2 are under (or equal) the *Protected I.* limit values in all cases. At speed $\mathbf{V_c}$ for type $\mathbf{3_b}$ and **4,** concentration of CO is over the *Protected I.* limit value, while at $\mathbf{V_d}$ traffic speed, pollution is moderate only on streets with open area. For NO_2 different results were obtained: on road type $\mathbf{3_b}$ concentration of NO_2 is at the limit and for roads $\mathbf{3_a}$ and **4** it is close to the limit for all categories. Further calculations showed that a doubling in the traffic (i.e. double number of vehicles) results in 71 % increase in the CO concentration. Neither the effect of trees nor the increasing traffic speed can compensate the effect of a double truck fraction. The fraction of trucks has a great impact on the NO_2 concentration. Heavy duty vehicles may contribute to about 60 % of the total NO_x-emissions [24].

THE CASE STUDY

The CAR model has been applied to input data collected in a medium size Hungarian city, Szeged. The results have been compared to the measurements and to the air quality limit values of the pollutants considered. In this section – after a short site description – the input data are introduced and the results of the model calculations are discussed.

Site description

Szeged is a medium sized city with a population of about 155 000 inhabitants in the south-eastern part of Hungary (20º06'E; 46º15'N). The built-up area of the city is 46 km^2. This is the largest town in the southern part of the Great Plain, at the confluence of rivers Tisza and Maros. The annual mean temperature is 11 ºC, while the annual mean precipitation total is about 570 mm. The prevailing wind direction is westerly to north-westerly and the annual mean wind speed is 3.2 m·s^{-1}. As the major industrial area is found north-west to the city, air currents transport polluted air downtown [25, 26].

The traffic of Szeged is overcrowded. Though the order of magnitude of road traffic did not change in the period 1995-2000 but a slight increase in the daily number of vehicles can be experienced. On the other hand, structure of the traffic changed considerably. Majority of the vehicles have already been equipped with exhaust catalysers, so emission has significantly decreased despite the stagnating traffic: levels of road traffic emissions of CO in year 2000 were 35-40 % of those in year 1990 [27].

As a comparison, despite the rapid increase of the vehicles in Beijing, China by 60 % between 1998 and 2003, total vehicular emissions have not increased. Improvement of fuel quality (banning lead, reducing sulphur), introduction of CNG and LPG in buses and taxis, as well as fiscal incentives such as tax deductions for new vehicles meeting enhanced emission standards to encourage their sales, significantly improved the environmental quality of the Chinese capital [28]. Traffic regulations introduced by policymakers in Delhi, India, resulted in similar conclusions [29].

Due to highways M5 (Budapest – Szeged – Röszke, Hungarian-Serbian border; completed in 2005) and M43 (Szeged – Nagylak, Hungarian-Romanian border; construction started in 2009 and its completion is planned in 2012), which will drive transit vehicles outside the inhabited area of Szeged, will result in a significant drop in the traffic and hence, traffic related air pollution. This study is going to be a reference to the effects of the here-mentioned highways, as well.

The input data

Traffic census has been processed at 9 different sites in the city (Figure 4; sites 1 to 9 are from top left to right centre and left bottom). Both the average daily number of motor vehicles passing through each location and the air pollution data were considered for the 11-year period 1997-2007. Mean daily number of vehicles for the period considered is indicated for each location (Figure 4), furthermore, temporal course of mean daily number of vehicles at two different sites (Site 4 and 9) is also presented (Figure 4, bottom right). An increasing trend is present for the urban average traffic for the annual means taking into account all sites (~970 vehicles per day per year growth rate for the urban average during the 8 years period). Model results from two different type of urban sites are analysed: An air quality monitoring station is located at site 4 which is a typical dense urban area not so far from the city centre (with an average of 18 181 vehicles per day for the 8-year period 1997-2004), while site 9 is an open suburban site with an average of 4 676 vehicles per day.

Vegetation type and traffic speed categories for each site were estimated at a field trip experiment performed by the authors. According to this survey, vegetation types are **3a** (i.e. 'broad street canyon') and **2** ('base type'), furthermore, traffic speed categories are $\mathbf{V_c}$ (average traffic speed is 22 $km \cdot h^{-1}$) and $\mathbf{V_b}$ (average traffic speed is 40 $km \cdot h^{-1}$) for site 4 and 9, respectively. Tree factors of these locations were **1.25** (trees on one side of the street) and **1.00** (very few or no any trees), respectively (Table 3).

City diameter (4 km) was calculated for the area of Szeged using a circular model for the city. Concentrations were calculated at 5 m away from the road axis. This is the closest location to the source where concentrations can be obtained with the CAR model. Calculated concentrations are the highest here so the effects of the input parameters and the difference between each site are not attenuated by dilution.

The emission factors were changed from their default values according to the inventory of the Automotive Engineering Environmental and Energy Division at the Institute of Transport Sciences (web of the Ministry of Environment and Water, Hungary). Note that the dimension used in the CAR model ($\mu g \cdot m^{-1} \cdot s^{-1} \cdot vehicle^{-1}$) differs from the one used in other sources ($g \cdot km^{-1}$). In Table 4 the parameters are shown in both dimensions.

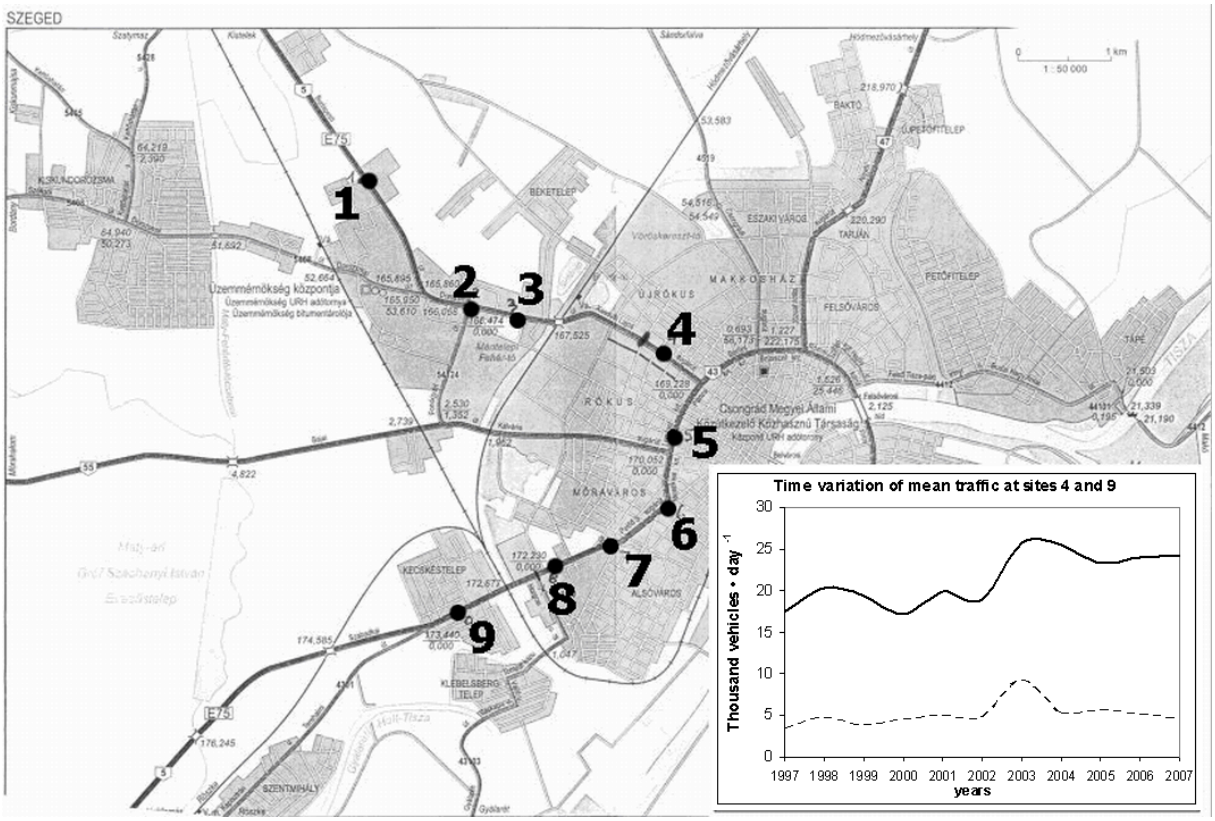

FIGURE 4 - Map of Szeged with the location of the measurement sites. Bottom right panel: time variation of daily number of vehicles at two locations (sites 4 and 9) for each year (1997–2007).

TABLE 3 - Road types, traffic speed and tree factor data at each site. V_b: Road with maximum speed of 70 km·h^{-1}, average speed is 44 km·h^{-1}. V_c: Regular city traffic, average speed is 22 km·h^{-1}. V_d: Stagnating traffic, flow of vehicles is not continuous, average speed is 11 km·h^{-1}.

Site	Road type	Traffic speed type	Tree factor
1	**2**	V_b	**1.00**
2	**4**	V_b	**1.00**
3	**4**	V_b	**1.00**
4	**3_a**	V_c	**1.25**
5	**3_a**	V_c	**1.25**
6	**3_a**	V_c	**1.25**
7	**3_a**	V_b	**1.25**
8	**4**	V_b	**1.25**
9	**2**	V_b	**1.00**

TABLE 4 - Emission factors for cars and trucks at different speed categories in different units. Parameters were taken from the official emission inventory of the Automotive Engineering Environmental and Energy Division at the Institute of Transport Sciences (KTI). (source: web of the Ministry of Environment and Water, Hungary).

Speed type	Speed (km·h^{-1})	CO		NO_2	
		g·km^{-1}	µg·m^{-1}·s^{-1}·vehicle^{-1}	g·km^{-1}	µg·m^{-1}·s^{-1}·vehicle^{-1}
		Cars			
V_a	13	30.57	0.354	1.38	0.016
V_b	22	21.00	0.243	1.33	0.015
V_c	44	11.72	0.136	1.40	0.016
V_d	100	6.40	0.074	2.45	0.028
		Trucks			
V_a	13	21.26	0.246	8.01	0.093
V_b	22	15.75	0.182	6.75	0.078
V_c	44	10.74	0.124	6.06	0.070
V_d	100	8.86	0.103	11.28	0.131

The background concentration data

In lack of onsite measurements background values can be determined by regional and urban scale air quality modelling. EMEP model activity includes transboundary air pollution modelling of main pollutants like (S, N, O_3 and PM) using actual emissions and meteorological condition to get spatial distribution of them over Europe [30]. In the area of Szeged the regional background intervals according to the EMEP calculation are shown in Table 5. Furthermore, the background concentrations of ozone are also published because of its important role in NO_x chemistry using by road models.

TABLE 5 - Summary of different measured and calculated background concentration values at Szeged.

2006 annual averages (µg·m^{-3})	Urban background	Regional background	
	measured at Kossuth str	measured at K-puszta	calculated by EMEP
NO_2	34.2	1.78	3.3 - 6.6
CO	687.0	-999.9*	-999.9*
Benzene	2.2	-999.9*	-999.9*
O_3	31.9	48.00	60 - 70

* -999.9: values are not available

Regional background concentrations have been measured at three stations in Hungary. Sites are located in areas of low population density, which are as far as possible from major roads, populated and industrial areas. The closest station to Szeged called K-puszta has a central location in the country and its measurements have been taken into consideration during EMEP model simulations and verifications. Measured annual averages of NO_2 and O_3 at K-puszta in 2006 are also shown in Table 5.

In the same way, local and actual urban background values can be examined by using an urban scale dispersion model (e.g.: ADMS-Urban). If this kind of evaluation for Szeged is not available, annual average values of an urban site would be accepted as background concentration in the measuring site, which is far away from sources and, which is, therefore broadly representative of city-wide background conditions, e.g. elevated locations, parks and urban residential areas. Only one monitoring site is operating at Szeged (Kossuth Lajos Avenue 89), annual averages of which are given in Table 5.

RESULTS AND CONCLUSIONS

Model integrations for all 9 locations (Figure 4) were performed using traffic data for each year in the period considered. The statistics (11-year averages, standard and relative deviations) of the input traffic data and output CO concentrations (Table 6), as well as temporal course of CO and NO_2, annual mean and 1 h 98 percentile concentrations are presented for site 4 (large traffic) and site 9 (small traffic) (Figure 5), respectively.

TABLE 6 - Model results of 11-year integration at 9 sites in Szeged for CO. 11-year averages, standard deviations and relative deviations are given for traffic and CO concentrations, respectively. Average fraction of trucks and city background concentrations (C_b) are also given. (Input sets of road type, traffic speed and tree factor data at each site are presented in Table 3.)

Site	Fraction of trucks, %	traffic (number of vehicles per day)			CO concentration (µg·m^{-3}); C_b = 254 µg·m^{-3}		
		11-year average	Standard deviation	Relative deviation (%)	11-year average	Standard deviation	Relative deviation (%)
Site 1	0.13	28 219	8 652	31	976	225	23
Site 2	0.08	26 011	6 471	25	1 398	273	20
Site 3	0.06	22 887	4 109	18	1 253	176	14
Site 4	0.04	21 408	3 188	15	740	92	12
Site 5	0.05	17 419	2 672	15	1 829	380	21
Site 6	0.04	10 849	3 280	30	1 234	340	27
Site 7	0.07	11 741	1 649	14	701	253	36
Site 8	0.05	8 851	1 156	13	735	58	8
Site 9	0.08	5 063	1 520	30	383	38	10

Major findings of the study are as follows.

Traffic increased with time at all locations during the period 1997-2007 (Figure 4).

[i] The pattern of traffic (spatial distribution) did not change during the period of time considered. Annual mean daily number of vehicles was the largest at site 1 and the smallest at site 9 in all year (Table 6).

[ii] 1 h 98 percentile concentration values of CO at a site with high traffic (e.g. site 5) are approximately 4 times higher than values at a site with low traffic (e.g. site 9).

[iii] Mean annual concentration of CO is less then 40% of the limit for *Highly Protected* category (1 000 $\mu g \cdot m^{-3}$) at site 9 and around 74% at site 4, while 1 h 98 percentile values are around the limit for *Protected I.* category (2 000 $\mu g \cdot m^{-3}$) at site 9 and above it at site 4.

[iv] Results obtained for NO_2 are similar to those for CO (Figure 5).

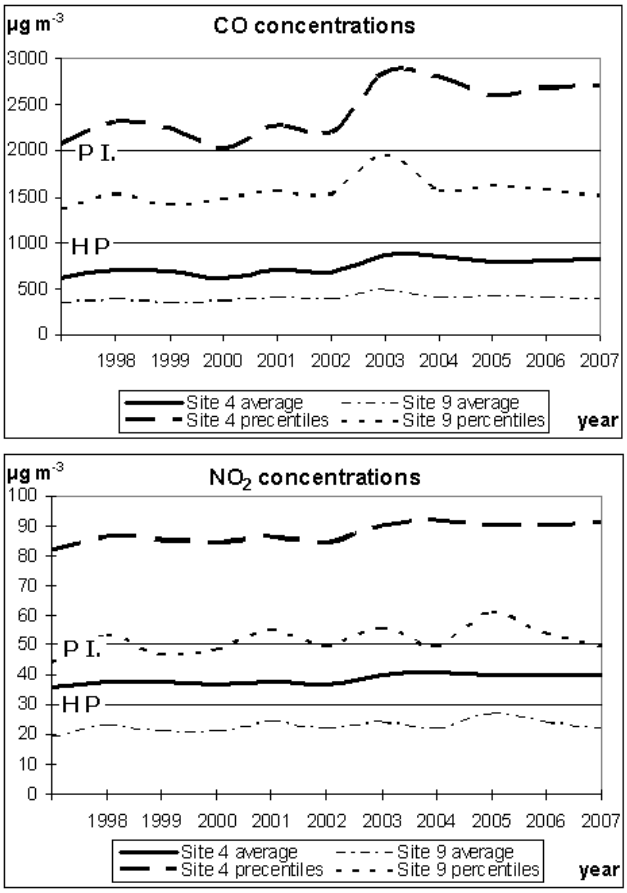

FIGURE 5 - Calculated annual mean and 1h 98 percentile CO (upper panel) and NO_2 (lower panel) concentrations ($\mu g \cdot m^{-3}$) at sites 4 and 9. Concentration limits: HP: Highly Protected; P I.: Protected I.

For the year 2001 a test calculation of concentration cross section at Site 4 on a monthly basis was performed (Figure 6). Seasonal variation of the tree factor and monthly mean wind speeds were taken into account. There was a significant variation in the output concentrations, although a seasonal variation in the traffic itself was not considered. According to the results, much higher concentrations occur in the summer than in the winter. This is due to the fact that wind speed is the least from late summer till early autumn (2.7-2.9 $m \cdot s^{-1}$, from July till November) and vegetation has more effect on the wind speed in summer and autumn than in the winter (Figure 6).

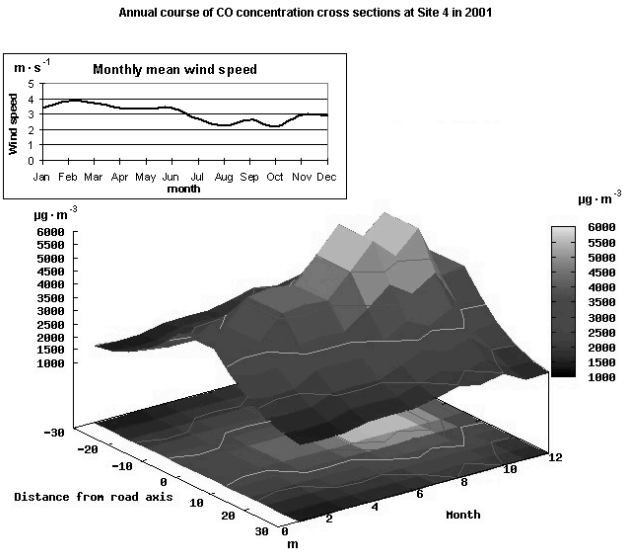

FIGURE 6 - Cross sections of CO 1 h 98 percentile concentrations for each month in 2001. (Monthly mean wind speed (top left) and seasonal variation of tree factor are considered.)

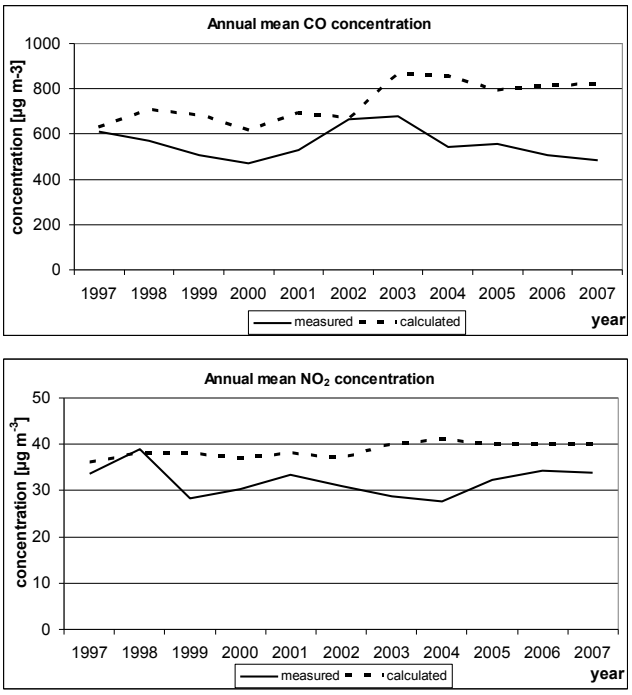

FIGURE 7 - CO (upper panel) and NO_2 (lower panel) annual mean concentrations ($\mu g \cdot m^{-3}$) measured near site 4 (solid line) and calculated (dotted line) for site 4, respectively.

Furthermore, it was detected that a doubling in the traffic (i.e. double number of vehicles) results in 71 % increase in the CO concentration. Neither the effect of trees nor the increasing traffic speed can compensate the effect of a double truck fraction. The fraction of trucks has a great impact on the NO_2 concentration.

Concentration data collected at the air quality monitoring station (near site 4) were compared to the above model output. Concentration data showed slight growth for CO and a gradual decrease for NO_2 (6.88 $\mu g \cdot m^{-3} \cdot year^{-1}$ and –0.62 $\mu g \cdot m^{-3} \cdot year^{-1}$, respectively) in the period considered (1997-2004) (Figure 7).

Measured data were slightly lower than the modelled ones. In the model results a smaller deviation is present from the average than in the measurements. These discrepancies arise from the fact that emission parameters have been taken constant, although the structure and technical quality of the transportation system in Szeged is improving considerably.

It should be noted that in the present work we only wanted to demonstrate the behaviour of the CAR model, and did not want to fit them to measurements, although we did not get good agreement between the modelled and measured concentrations. In order to use the model by decision makers for environmental prediction, the emission parameters used for the calculations should be updated on a regular basis.

To summarize the results, main findings are as follows: concentrations of the pollutants increase with the (i) increasing number of vehicles, (ii) decreasing speed of road traffic, (iii) larger fraction of heavy vehicles, (iv) increasing number of trees alongside the roads and (v) smaller mean annual wind speed.

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TWO NEW NICKEL HYPERACCUMULATORS FROM THE GREEK SERPENTINE FLORA

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ABSTRACT

Nine *Centaurea* and three *Viola* species, predominately or exclusively found on serpentine in Greece were investigated, to examine their soil-tissue relations for Ni. Nickel content and distribution within the leaves showed that two species could be characterized as nickel hyperaccumulators. Leaves from wild grown plants were examined and x-ray analyzed with a Scanning Electron Microscope equipped with an x-ray microanalysis system. 3830±312 μg and 1023±79 μg nickel g^{-1} of leaf dry mass was found in *Centaurea thracica* and *Viola vourinensis* correspondingly after atomic absorption spectroscopy. In both species the metal was accumulated in large epidermal cells. *Centaurea thracica*, a member of the Greek serpentine flora, is reported for the first time as a nickel hyperaccumulator. *Viola vourinensis*, a Greek serpentine endemic, though accumulating lower metal quantities than *C. thracica*, could be also characterized as nickel hyperaccumulator, since the threshold proposed for nickel hyperaccumulators is 1000 μg of the metal g^{-1} dry mass.

KEYWORDS: *Centaurea,* EDX-ray microanalysis, nickel hyperaccumulators, serpentine flora, *Viola*.

INTRODUCTION

Ultramafic soils are characterized by high Mg, Fe, heavy metals such as Ni, Cr and Co and nutrient imbalances, specifically, a Ca:Mg quotient <1. The soils often provide harsh conditions for plant growth yet support a specialized flora often endemic to the substrate [1-5]. Extensive ultramafic outcrops are found in the Balkan Peninsula, distributed particularly in central Bosnia, west and central Serbia, north-central and south-eastern Albania and north-western and central Greece. Most of the *c*. 335 Balkan serpentine endemic taxa are found in Greece. Mountainous regions of northern Pindos and Vourinos of the Greek mainland, as well as the island of Evvia constitute important 'hot spots' that harbour many endemic taxa [6, 7].

Though in many cases plant resistance is due to metal exclusion from the protoplast [8], there are species which actively take up metal elements resulting in accumulation of extremely high levels of metal [9, 3]. Species accumulating high quantities of metals are characterized as metal hyperaccumulators [9, 10]. The threshold defining hyperaccumulation depends on the element involved [11]; thus, the threshold for Co, Cr and Ni is 1000 μg g^{-1} of dry leaf mass [12].

Nickel hyperaccumulation is prevalent and more often reported than the accumulation of any other metal. More than 50 Ni-hyperaccumulating taxa belong to the genus *Alyssum* [12, 13]. Though certain members of Asteraceae have been also characterized as Ni-hyperaccumulators [14], *Centaurea* species have not been referred to as hyperaccumulators until recently. Reeves and Adigüzel [15] found 13 plants within the genus *Centaurea* from the Turkish flora that hyperaccumulate nickel. Some of them appear to be serpentine endemics whereas some others grow on a variety of substrates. *Centaurea* species from the Greek serpentine flora have not been yet investigated from this viewpoint.

Viola species are known as Zn-hyperaccumulators. The best known example is *Viola calaminaria* Lej., a species recognized as metal accumulator since 1885 [16]. Significantly higher than normal Ni values were recorded for the western North American serpentinophyte *Viola cuneata* [17]. Horie et al. [18] reported two species, namely *Viola yubariana* Nakai and *Viola sacchalinensis* Boissieu, endemics of ultramafic soils of Hokkaido area of Japan, which show strong Ni-accumulation. Some other tropical representatives of Violaceae, such as *Hybanthus* spp., have been known as Ni-hyperaccumulators also [19]. Again, Greek serpentine endemics of the genus *Viola* have not been yet examined.

The "unusual appetite" of plants for metal uptake [20] has been an interesting subject of scientific research [11 and literature there-in]. Much research has focused on identifying new hyperaccumulators [e.g. 13, 21, 22] and examining the physiological mechanisms underlying metal tolerance and accumulation [e.g. 23-25]. A third part of research is dealing with metal phytoextraction, either for phytom-

ining, i.e. the use of plants to extract metals from soils with high metal concentration [e.g. 26] or the so-called phyto-remediation of contaminated sites [e.g. 27]. Recently, the ecology of metal hyperaccumulation has also gained much attention [11, 28]. Concerning the role of hyperaccumulation, it has been hypothesized that is multiple [summarized by 11, 29, 30]. The most attractive hypothesis is the defense of the metal accumulating plant against herbivores or pathogens.

The phenomenon of nickel hyperaccumulation raises interesting questions concerning the mechanism of metal uptake, sequestering and toxicity avoidance [3, 24, 31, 32]. The distribution of the metal in the plant body has received much attention. Leaves seem to be the main sinks for nickel [3, 33]. According to the present knowledge, nickel is concentrated in leaf epidermis [25, 34-36]. Guard cells possess lower metal quantities than the rest of epidermis [25, 36]. Ni seems to be accumulated either in the vacuoles of epidermal cells or in outside cell walls [25, 37, 38].

Nine *Centaurea* and three *Viola* species from the Greek serpentine flora have been investigated to examine their soil-tissue relations for Ni. Here, two new Ni-hyperaccumulators from Greece are presented.

MATERIALS AND METHODS

Plant material

Preliminary SEM energy dispersive x-ray (EDX) microanalysis experiments were carried out using plant material kept in the Herbarium of Patras University (UPA), Greece. A list of the herbarium specimens examined is shown in Table 1. The preliminary trials showed that only two species, namely *C. thracica* and *V. vourinensis* accumulate nickel. In order to avoid damage of herbarium specimens additional plant material of *C. thracica* and *V. vourinensis* was collected in 2004 from ultramafic soils of Domokos in central Greece (39° 06´N, 22° 11´E) and of Mt Vourinos (40° 11´N, 21° 43´E) correspondingly. Leaves from at least five individuals were used for the study. All leaf samples of both herbarium and field specimens were washed by careful agitation for 2 min in de-ionized water and left to air dry.

Microsccopy and microanalysis

Leaves were mounted on stubs with double sided carbon adhesive tape, sputter coated with carbon and x-ray analyzed with a JEOL 6300 Scanning Electron Microscope (SEM; JEOL, Tokyo, Japan). Energy dispersive x-ray microanalysis was carried out using a Link Pentafet system (Oxford Instruments, model 6699; Oxford, UK), connected to the SEM. The system is equipped with a Si(Li) detector and a thin window for elements from Be to U. Voltage was 20 kV, probe current 10 nA and collection time 50 s. Energy dispersive spectra and dot mapping were processed using a Link ISIS software (series 300, Oxford, UK), which enables the system to give directly the elemental composition for each selected element [36]. Typical detection limit for the EDS detector is 0.1 wt % [39]. Elemental concentrations were estimated excluding carbon from normalized percentages. Micrographs as well as x-ray dot maps were taken in digital form.

Atomic absorption spectroscopy

Nickel content was measured with a Perkin-Elmer 3110 atomic absorption spectrometer (Boston, USA). For this purpose, 0.2 g of dry leaves from each sample was digested with HNO_3 and HCl in an autoclave. Leaf material was sampled from five individuals from each species and was independently analyzed.

TABLE 1 - List of the herbarium specimens examined with their provenance. All the material originates from Greece and is kept at University of Patras (UPA).

Name	Provenance
Centaurea attica Nyman subsp. *megarensis* (Halácsy & Hayek) Dostál	Central Greece, Mt. Gerania, the western and south-western slopes of Korifi summit.
C. charrelii Halácsy & Dörfl.	Central Greece, along the road from the city of Lamia to Domokos, very close to the crossing towards Omvriaki village
C. ebenoides S. Moore	Evvia Island, close to the village of Limni
C. euboica Rech. f. Subsp. *euboica*	Evvia Island, Mt. Kandili, close to the village of Prokopi
C. euboica subsp. *intermedia* Phitos & T. Georgiadis	Evvia Island, 2 km north of Pappades village
C. grisebachii (Nyman) Heldr. subsp. *occidentalis* E. Gamal-Eldin & Wagenitz	Northern Greece, Mt. Vasilitsa, close to ski center
C. ptarmicoides Halácsy (= *C. ptarmicifolia*)	Northern Greece, Mt. Smolikas, eastern slopes
C. rupestris subsp. *finazzeri* (Adamović) Hayek	Northern Greece, 6 km N of Ano Garefi village
C. thracica (Janka) Gugler	Central Greece, from the village of Kedros village to Loutra Smokovou
Viola albanica Halácsy	Northern Greece, Mt. Grammos, close to Skirtsa summit
V. dukadjinica W. Becker & Košanin	Northern Greece, the upper parts of Mt. Mavrovouni
V. vourinensis Erben	Northern Greece, Mt. Vourinos, eastern slopes close to the summit

RESULTS

Centaurea thracica

As presented previously, drying procedure of plant material does not cause any substantial nickel redistribution [36]. Data presented here are obtained, therefore, from air dried plant material. Examination of a leaf cross section of *Centaurea thracica* (Fig. 1a) under the analytical SEM revealed that nickel is accumulated in the epidermis of both leaf surfaces; this is shown in the x-ray dot map of the metal distribution (Fig. 1b). Repeated microanalysis trials with the leaf surface showed a moderate nickel concentration; nickel concentration measured in the area shown in Fig. 1c, was 0.52 %. The energy dispersive spectrum obtained is given in Fig. 1d. Further microanalysis experiments revealed that the metal is not uniformly distributed in the various epidermal cell types. A series of spot microanalyses is presented for the epidermis area shown in Fig. 1e. Nickel elemental percentage of guard cells (Fig. 1e, point 1) was found to be 0.11 %. By contrast, nickel elemental percentage was found to be much higher in the large epidermal cells (point 2: 1.48 %, point 3: 1.49 %, point 4: 1.58 %) than in guard cells. The low accumulation of the metal in guard cells was verified in line scan microanalyses; such a line scan experiment is shown in Fig. 1f. The signal for nickel presence along the dashed line in the micrograph of Fig. 1f is given in the insert. A steep decrease of the metal signal is obtained from guard cells, whereas the metal signal from the large epidermal cells in both sides of the stoma is increased. The metal accumulation in the large epidermal cells and its absence from guard cells can be further confirmed from the x-ray dot map of the epidermis area of Fig. 1g, shown in Fig. 1h. No metal accumulation was observed in the other eight *Centaurea* species examined.

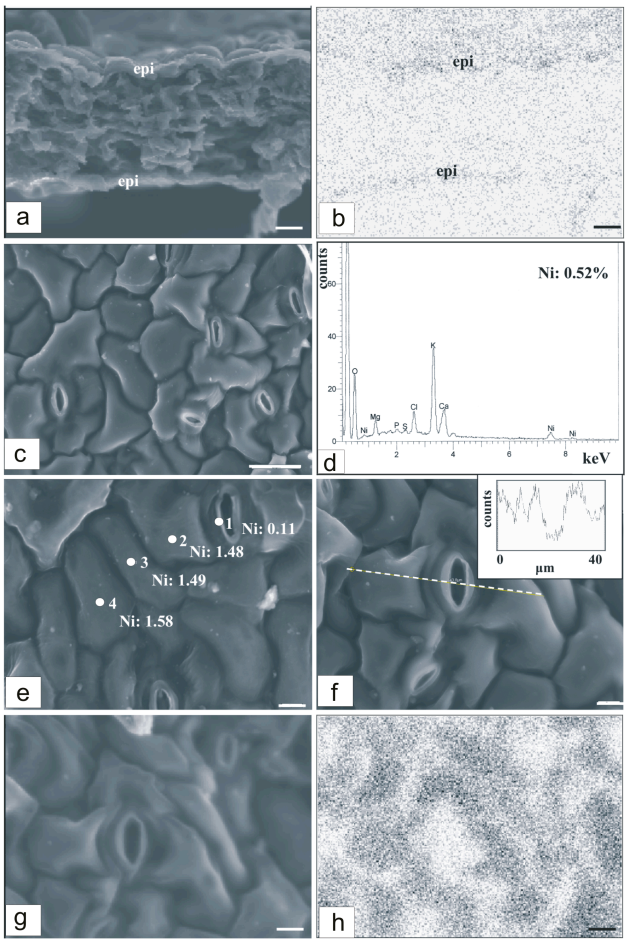

FIGURE 1 - SEM micrographs, x-ray microanalysis dot maps and ED spectrum of *Centaurea thracica* leaves. a, b, cross sectioned leaf (a), and the corresponding dot map of nickel distribution (b). Note that nickel signal is elevated in both epidermises (epi). c, Leaf surface view; the corresponding x-ray microanalysis gave nickel concentration 0.52%. The corresponding ED spectrum is given in d. e, Spot microanalyses on leaf epidermis showed low nickel content in guard cells (point 1) and high in large epidermal cells (points 2, 3, 4). f, A scan analysis across the dashed line gave weak nickel signal in the area of the stoma (insert). g, h, Micrograph and the corresponding dot map of nickel distribution of an epidermis area with a stoma. Nickel signal is strong in large epidermal cells away of the stoma. Bars in a and b represent 50 µm, in c 20 µm and in e-h 5 µm.

Viola vourinensis

X-ray microanalyses of *Viola vourinensis* leaves revealed a similar distribution of nickel within the leaf, i.e. metal is accumulated in upper and lower epidermis. Nickel concentration in this species was found to be lower than in *C. thracica.* For example, in the area shown in Fig. 2a nickel concentration was found to be 0.20 %. Again, spot microanalyses (Fig. 2b) showed that the metal concentration is low in guard cells (point 1, Ni: 0.10 %) and in trichomes (point 2, Ni: 0.18 %), but is high in large epidermal cells (point 3: 1.15 %). No metal was found in the other two *Viola* species examined.

Nickel content of dry leaves of both species was determined using atomic absorption spectroscopy. The results confirmed the data obtained from the SEM x-ray microanalysis method. Thus, Ni content was found to be much higher in *C. thracica* (3830±312 µg g^{-1} leaf dry mass) than in *V. vourinensis* leaves (1023±79 µg g^{1} leaf dry mass; Table 2).

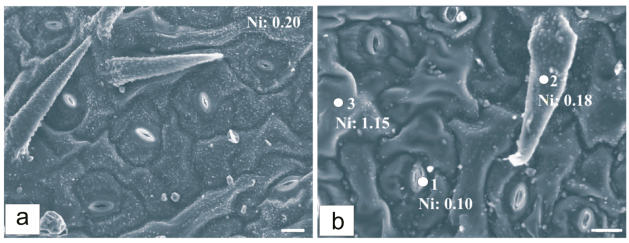

FIGURE 2 - *Viola vourinensis* leaves under SEM. X-ray microanalysis of the area shown in a, gave a nickel concentration 0.20%. Spot microanalysis trials in several points of the leaf surface shown in b gave low nickel content in guard cells (point 1: 0.10 %) and trichomes (point 2: 0.18%) but high nickel content in large epidermal cells (point 3: 1.15%). Bars represent 20 µm.

TABLE 2 - Mean nickel content (±SE) in leaves of five individuals each of *Centaurea thracica* and *Viola vourinensis*

Species	μg g^{-1} dry mass mean ±Se	range	n
C. thracica	3830±312	2688-4771	5
V. vourinensis	1023±79	748-1208	5

DISCUSSION

The investigation of selected Greek serpentine species of the genera *Centaurea* and *Viola* revealed two new Ni hyperaccumulators, namely *Centaurea thracica* and *Viola vourinensis*. Nickel content detected in *C. thracica* leaves (3830±312 μg g^{-1} of dry mass) clearly classifies the species among Ni-hyperaccumulators. By contrast, metal content of *Viola vourinensis* leaves (1023±79 μg g^{-1} of dry mass) is just above the threshold (1000 μg g^{-1} of dry mass) proposed for nickel hyperaccumulators [10]. However, it is worthy to note that the metal accumulating *Viola* species reported so far are mainly zinc hyperaccumulators [14, 40]. The nickel accumulating species reported by Horie et al. [18] from Japan have been characterized just as strong accumulators.

The discovery of two taxonomically unrelated nickel hyperaccumulators in Greece is particularly interesting, as only members of the Brassicaceae known to hyperaccumulate Ni in the Mediterranean region, most of which belong to *Alyssum* sect. *Odontarrhena.* A notable exception is the *Centaurea* species found recently in Turkey [15]. The new evidence that two extra families, Asteraceae and Violaceae, are involved in hyperaccumulation events of the Mediterranean flora offers strong support to the assumption that this process, much like serpentine tolerance, has evolved independently and more than once in the area, within members of different families. Similar conclusions have also been reached by investigators working in different groups and/or in other parts of the world [e.g. 41-43].

Reeves and Adigüzel [15] have already determined 13 *Centaurea* species as Ni- hyperaccumulators and these, together with *C. thracica* have been assigned to their taxonomic sections in Table 3. Although the genus *Centaurea* in its wider sense comprises more than 240 species in Greece and Turkey, classified to at least 33 sections, those species that have been recognized to hyperaccumulate nickel appear to concentrate in specific sections, and half of them belong to *C.* sect. *Cynaroides*. Members of this section are vigorous biennials or perennials that may reach up to 2 m. Only four additional sections appear to share the same property of Ni-hyperaccumulation: *Cheirolepis*, *Microlophus*, *Pseudophaeopappus* and *Pseudoseridia*. The development of a Ni-hyperaccumulation potential in specific sections of *Centaurea* explains why our screening test applied to several obligate serpentine members of the genus in Greece belonging to the sections *Acrocentron, Acrolophus* and *Phalolepis* failed to identify any further hyperaccumulators.

TABLE 3 - Assignment of *Centaurea* taxa known as Ni-hyperaccumulators to sectional categories [15]

Centaurea species	Section
C. aladaghensis Wagenitz	*Cynaroides*
C. amanicola Hub.-Mor.	*Cynaroides*
C. antitauri Hayek	*Pseudophaeopappus*
C. arifolia Boiss.	*Cynaroides*
C. cataonica Boiss. & Hausskn.	*Cynaroides*
C. cheirolopha (Fentzl) Wagenitz	*Pseudoseridia*
C. ensiformis P.H. Davis	*Cheirolepis*
C. haradgianii Wagenitz	*Cynaroides*
C. ptosimopappa Hayek	*Ptosimopappus*
C. ptosimopappoides Wagenitz	*Ptosimopappus*
C. sericea Wagenitz	*Cheirolepis*
C. spicata Boiss.	*Cynaroides*
C. thracica (Janka) Hayek	*Microlophus*
C. tomentella Hand.-Mazz.	*Cynaroides*

Within *Viola,* Ni-hyperaccumulation deserves further attention and it seems premature to comment on any relationship between the taxonomic construction of the genus and the ability of certain species to hyperaccumulate.

As revealed by our SEM x-ray microanalysis investigation, nickel is accumulated in leaf epidermis in both species. This is in agreement with previous reported data for other species [25, 34-36, 44, 45]. Metal distribution is also higher in large epidermal cells than in guard cells, which is also in line with previously reported data [25, 36-38]. Nickel presence in leaf epidermis is in line with the defense theory of hyperaccumulation [30, 46, 47], as leaf epidermis is the first tissue attacked by possible herbivores.

Ideas concerning phytoextraction [5, 48] of metals from metal rich soils, either for phytomining [26, 49], or phytoremediation [50-53] are attractive. Phytomining involves growing of a metal hyperaccumulator species, harvesting the biomass and ashing it to recover the metal. A species useful in this direction should be fast-growing, producing considerable biomass. In that respect, *V. vourinensis*, being a small rosette plant does not fulfill this condition. One of the fast growing species proposed for possible use is another member of Asteraceae, namely *Berkheya coddii* [54]. According to data presented here, *Centaurea thracica* and members of *C.* section *Cynaroides* showing relatively high biomass could play an important role in phytoextraction/ phytoremediation of Ni-contaminated soils.

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EVALUATION OF GENOTOXIC EFFECTS OF ODIUM SULPHITE, POTASSIUM SULPHITE AND CALCIUM SULPHITE ON THE ROOT MERISTEM CELLS OF *Allium cepa*

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ABSTRACT

The effects of different treatments with food preservatives sodium sulphite (SS), potassium sulphite (PS) and calcium sulphite (CS) on the cytology, DNA content and interphase nuclear volume (INV) of *A. cepa* were investigated. Four concentrations of these additives (50, 100, 150, and 200 ppm) were applied for 24, 48, and 72 h. All concentrations of these chemicals showed an inhibitory effect on cell division in root tips of *A. cepa* and caused a decrease in mitotic index values. Additionally, all treatments changed the frequency of mitotic phases as compared with the control groups. These compounds increased chromosome abnormalities, among them c-mitosis, micronuclei, anaphase bridges, laggards, stickiness, vacuolated nuclei and unequal distributions. The nuclear DNA content and interphase nuclear volume (INV) decreased when compared with control groups.

KEYWORDS: Sodium sulphite, potassium sulphite, calcium sulphite, genotoxic effect, DNA content, interphase nuclear volume.

INTRODUCTION

All commercial food products have preservatives, and every manufacturer adds preservatives to food at any time during processing. The purpose is generally to avoid spoilage during the transportation time. Since food is so important for survival, food preservation is one of the oldest technologies used by mankind. There are thousands of additives used by the food industry for a variety of purposes. In fact, when food additives are given to organisms in excessive amounts, they may cause toxic reactions. There have been many studies on the genotoxicity and carcinogenicity of several food additives in plants, mice, and human peripheral blood lymphocytes [1-3]. However, there are many food preservatives whose possible genotoxic effects are un-known.

Sodium sulphite (SS) (E221), potassium sulphite (PS) (E 225), and calcium sulphite (CS) (E 226) are sodium, potassium, and calcium salts of sulphurous acid. SS, PS and CS have become the leading preservatives in the food sector throughout the world over the past 30 years. They are used for the preservation of pastries, margarine, cheese, in prepared soup tins, beverages, fruit sausages, fishes, sweets and ground beef [4].

The Ministry of Agriculture of Turkey (General Directorate of Protection and Control) [5] has suggested that these preservatives may be used at 200 mg/l in food. No studies have been carried out, to our knowledge, on the cytological effects of PS and CS in plant systems, despite the fact that they are commonly used.

Most studies on sulphites toxicity in plants have focused on the mitotic activity and chromosomes [1, 6, 7], but there are not many reports on the effects of SS, PS and CS on the activity of nuclear DNA and interphase nuclear volume (INV).

Previous studies have shown that plant chromosomes are excellent materials, as they are large and sensitive to changes in the environment. Since Levan [8] introduced the first Allium test, various chemicals have been tested for their cytogenetic activities using this test [6, 9]. This test is very comfortable as it is easy to make preparations of onion roots. They contain rather homogenous meristematic cells, having only 16 chromosomes, which are very long, well visible and get stained easily. The test is a fast and inexpensive method, allowing the investigation of universal mechanisms for meristematic plant cells and extrapolation on animal cells. Plant bioassays, which are sensitive and simple in comparison with animal bioassays, have been validated in international collaborative studies under the United Nations Environment Program (UNEP), World Health Organization (WHO) and US Environmental Protection Agency (US EPA), and proven to be efficient tests for genotoxic monitoring of environmental pollutants [10]. Therefore, the study of the effect of several chemicals on plant mitosis may provide valuable information in relation to possible genotoxicity in mammals and especially in humans.

In the present report, we have therefore considered it of interest to investigate the effect of SS, PS and CS on cell division, chromosomes behaviour, *in situ* DNA content and interphase nuclear volume in root tip cells of *A. cepa*.

MATERIALS AND METHODS

In this study, root tip cells of *A. cepa* (2n=16) were used as the test system and the food preservatives sodium sulphite (CAS no: 7757-83-7), potassium sulphite (CAS no: 10117-38-1) and calcium sulphite (CAS no: 10257-55-3) as the test substances. The substances were obtained from Sigma-Aldrich.

Bulbs of *A. cepa* were placed in small jars with their basal ends dipping in distilled water and germinated at room temperature (25±2 ^{o}C). When the newly emerged roots were of 1.00-2.00 cm in length, they were used in the test. Roots of *A cepa* were treated with a series of concentrations of the three salts, i. e. 50, 100, 150 and 200 ppm diluted with distilled water for 24, 48, and 72 h. Control groups were grown in distilled water. Root tips were placed in a solution of ethanol (99 %) and glacial acetic acid (3:1) for 24 h, washed with distilled water three times, and then stained using aceto-orcein. The squash technique was applied for the study of the mitotic index (MI) and chromosomal aberrations. Three replicates were performed for each treatment and scoring was made from the 3 bulbs and 3 roots of each replicate, and a minimum 1500 mitotic cells were counted from each slide. The MI was calculated for each treatment as a number of dividing cells /100 cells. The cytological abnormalities were scored in the mitotic cells.

For Feulgen cytophotometric estimation of 2C nuclear DNA contents, the fixed root-tips were hydrolyzed in 1 N HCl for 12 minutes at 60 ^{0}C and stained in Feulgen solution for one hour. The stained root-tips were washed in three changes of SO_2 water for 10 minutes each and dried briefly on absorbent paper. Darkly stained root-tips were squashed in a drop of 45 % glacial acetic acid. Scoring was done from the cells of 20 different root tips using a Reichert-Zetopan microspectrophotometer, at a wavelength of 550 nm. On average, 100 2C telophase nuclei were measured in each of three replicates in every dose of SS, PS and CS [11].

For the study of INV, the mean of two diameters of each selected Feulgen-stained nucleus, observed at right angles to each other and measured under oil-immersion objectives was obtained. The INV was calculated using the formula, $4/3\pi r^3$, where r is the radius of the nuclei [12].

Analysis of variance of the data was done with SPSS computer program. Dunnet t (2-sided) Multipli Range test was employed to determine the statistical significance of differences among the means. The differences were considered as statistically significant at $P<0.05$.

RESULTS AND DISCUSSION

The effects on mitotic index (MI) and the frequency of mitotic phases are given in Tables 1-3 for the treatments with SS, PS, and CS, respectively. As can be seen, these food preservatives significantly decreased MI in the treatment groups compared with the control at all concentrations and treatment periods.

From the results in Table 1, it was noted that the reduction in the mitotic activity was increased when exposure time increased from 24 to 72 h. The 150 and 200 ppm concentrations of SS were toxic after the 72 h-treatment period, and no dividing cells were found. MI was reduced significantly at the 200 ppm concentration at the 24 h treatment, and at 150-200 ppm concentrations at the 48 h treatment period.

Table 2 shows results for PS; there were statistically significant differences between 24, 48 and 72 h for 50, 100, 150 and 200 ppm. Additionally, it was observed that 200 ppm concentration of PS was toxic after 72 h treatment period, there was no cell division.

TABLE 1 - Cytogenetic analysis of *A. cepa* root tips exposed to different concentrations of sodium sulphite for different periods.

Time of treatment(h)	Concentrations (ppm)	Examined total cells	Total mitosis	Pro. %	Met. %	A-T %	M. I. (M.±S.E.*)	A. B.	S.	C-M.	L.	M.	U. D.	V.N.	Total abnormalities* (%)
	Control	1537	163	59.34	17.83	22.83	10.60 ± 0.31	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	50	1518	167	62.76	17.57	19.67	11.00 ± 0.28	0.00	1.41	4.44	0.93	0.82	1.23	0.00	8.83 *
24 h	100	1535	159	53.16	21.31	25.53	10.35 ± 0.15	2.24	0.70	3.72	1.27	0.00	1.32	0.00	9.25 *
	150	1543	127	59.88	17.39	22.73	8.23 ± 1.22	0.00	0.00	3.44	1.33	3.48	2.00	0.00	10.25 *
	200	1500	110	56.80	18.40	24.80	7.33 ± 1.00 *	3.13	0.00	9.95	0.72	4.21	2.13	0.00	20.14 *
	Control	1503	152	55.56	21.86	22.58	10.11 ± 0.98	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	50	1527	157	55.66	19.26	25.09	10.28 ± 0.24	1.74	1.35	7.97	0.58	1.28	0.00	0.00	12.92 *
48 h	100	1567	145	43.90	21.55	34.55	9.25 ± 0.17	3.00	1.64	13.14	3.82	1.32	1.27	0.00	24.19 *
	150	1552	93	48.91	21.19	29.90	5.99 ± 0.82 *	6.37	0.00	11.93	0.00	1.74	0.00	0.00	20.04 *
	200	1539	89	51.85	21.23	26.92	5.78 ± 0.56	5.42	0.00	10.28	4.15	2.41	0.00	2.18	24.44 *
	Control	1548	146	46.15	25.21	28.64	9.43 ± 0.67	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	50	1591	139	35.89	27.12	36.99	8.73 ± 0.38	1.68	1.97	1.74	2.13	0.00	0.00	3.21	10.73 *
72 h	100	1514	124	43.29	25.52	31.19	8.19 ± 0.45	2.35	0.00	12.11	4.00	4.17	0.00	4.19	26.82 *
	150	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic
	200	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic

A. B., Anaphase bridges; S., Stickiness; C-M., C-Mitosis. L., Laggards; M., Micronucleus.; U. D., Unequal distiribution; V. N., Vacuolated nuclei.
* Significant at $P< 0.05$ level.

TABLE 2 - Cytogenetic analysis of *A. cepa* root tips exposed to different concentrations of potassium sulphite for different periods.

Time of treatment (h)	Concentrations (ppm)	Examined total cells	Total mitosis	% Pro.	% Met.	% A-T	M. I. (M.± S.E.*)	A. B.	S.	C-M.	L.	M.	(%) Total abnormalities *
	Control	1525	276	74.57	7.25	18.18	18.09 ± 0.28	0.00	0.00	0.00	0.00	0.00	0.00
	50	1518	260	74.98	6.53	18.49	17.12 ± 0.13	0.00	3.00	3.52	2.18	0.13	8.83 *
24 h	100	1550	252	73.43	5.33	21.24	16.25 ± 0.95	2.17	2.13	4.35	0.00	0.00	8.65 *
	150	1522	212	71.18	4.47	24.35	13.92 ± 0.33 *	2.13	1.56	1.63	4.51	3.28	13.11 *
	200	1543	170	66.72	6.58	26.70	11.01 ± 1.12 *	3.68	1.27	3.70	2.11	3.35	14.11 *
	Control	1538	315	76.38	4.48	19.14	20.48 ± 0.89	0.00	0.00	0.00	0.00	0.00	0.00
	50	1575	333	75.50	4.41	20.09	21.14 ± 0.45 *	1.27	0.28	4.35	2.22	5.23	13.35 *
48 h	100	1520	258	76.92	7.54	15.54	16.97 ± 0.64	3.37	2.16	3.92	1.34	3.38	14.17 *
	150	1571	249	74.13	9.81	16.06	15.84 ± 0.38	2.18	2.35	7.56	3.15	3.27	18.51 *
	200	1591	237	72.21	10.19	17.60	14.89 ± 0.15	0.00	1.22	9.21	0.00	7.54	17.97 *
	Control	1508	268	77.85	10.67	11.48	17.77 ± 0.69	0.00	0.00	0.00	0.00	0.00	0.00
	50	1523	217	71.23	12.13	16.64	14.24 ± 1.35 *	1.69	3.18	7.82	0.00	7.48	20.17 *
72 h	100	1517	183	70.70	13.95	15.35	12.06 ± 0.47 *	.52	2.97	6.13	2.19	6.51	18.32 *
	150	1535	140	63.41	16.28	20.31	9.12 ± 0.82 *	2.46	0.00	7.00	4.56	5.24	19.26 *
	200	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic

A. B., Anaphase bridges; S., Stickiness; C-M., C-Mitosis.; L., Laggards; M., Micronucleus.
* Significant at P< 0.05 level.

TABLE 3 - Cytogenetic analysis of *A. cepa* root tips exposed to different concentrations of calcium sulphite for different periods.

Time of treatment(h)	Concentrations (ppm)	Examined total cells	Total mitosis	% Pro.	% Met.	% A-T	M. I. (M.±S.E.*)	A. B.	S.	C-M.	L.	M.	(%) Total abnormalities *
	Control	1528	172	41.11	32.23	26.66	11.25 ± 1.13	0.00	0.00	0.00	0.00	0.00	0.00
	50	1531	157	49.16	23.45	27.39	10.25 ± 1.02	0.43	1.38	0.00	0.00	0.70	2.51 *
24 h	100	1513	160	51.70	25.38	22.92	10.57 ± 0.88	0.00	0.00	1.06	0.96	0.22	2.24 *
	150	1515	141	64.22	28.27	7.51	9.30 ± 0.28	0.00	0.00	1.98	1.83	1.53	5.34 *
	200	1524	156	63.56	26.00	10.44	10.23 ± 0.65	0.00	0.00	1.46	0.65	3.12	5.23 *
	Control	1532	176	43.58	31.28	25.14	11.48 ± 0.74	0.00	0.00	0.00	0.00	0.00	0.00
	50	1500	165	51.20	23.70	25.10	11.00 ± 0.53	1.27	0.88	0.00	0.00	0.63	2.78 *
48 h	100	1521	141	54.38	25.35	20.27	9.27 ± 0.28	0.00	2.18	3.11	1.33	1.53	8.15 *
	150	1548	144	57.00	23.00	20.00	9.30 ± 0.15	0.00	2.75	1.33	1.14	5.00	10.22 *
	200	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic
	Control	1508	171	44.22	25.43	30.35	11.33 ± 0.39	0.00	0.00	0.00	0.00	0.00	0.00
	50	1524	130	58.35	29.39	12.26	8.53 ± 0.48 *	2.58	2.23	3.56	1.84	4.24	14.45 *
72 h	100	1522	128	62.23	16.31	21.46	8.40 ± 0.52 *	3.45	1.56	1.79	1.48	8.96	17.24 *
	150	1519	110	57.78	22.33	19.89	7.24 ± 0.19 *	4.26	6.97	5.00	6.28	5.34	27.85 *
	200	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic

A. B., Anaphase bridges; S., Stickiness; C-M., C-Mitosis. L., Laggards; M., Micronucleus.
Significant at P< 0.05 level.

According to Table 3 - results for CS - the reduction in the MI was significant at the 50, 100 and 150 ppm concentrations for 72 h treatment period. In roots treated with the highest concentration (200 ppm) of CS at 48 and 72 h treatment periods mitosis had completely ceased.

The biochemical aspects of sulphite effects are well known. In aqueous solution sulphite is oxidized to sulphate, a process considerably retarded by glucose. Sulphite inhibits the activity of dehydrogenases, amylases and ATPase as well as that of a variety of cations including sodium, potassium and magnesium. Genetic investigations have re-vealed that sulphite has a strong affinity for nucleotides, and sulphite has shown as mutagenic in T_4 phage, lambda phage, *E. coli*, and yeast. Sulphite inhibits mitosis in L cells and in human lymphocytes [1]. Njagi and Gopalan [1] stated that sodium benzoate and sodium sulphite inhibit DNA synthesis, induce anaphase bridges, and cause premature chromosome condensation leading to pycnotic nuclei and chromatin erosion in interphase nuclei in roots of *Vicia faba*. Meng and Zhang [13] reported that sodium bisulphate caused sister chromatid exchanges and micronuclei in human blood lymphocytes in a dose-dependent manner. Sodium metabisulphite, a food preservative, in-duced chromosome aberrations and sister chromatid ex-changes, and also decreased replication index and mitotic index in cultured human lymphocytes [3]. Sodium metabisulphite significantly decreased mitotic index and increased the mitotic abnormalities dose dependently in root tip cells of *A. cepa* [6]. Gömürgen [7] reported that potassium metabisulphite and potassium nitrate increased mitotic abnormalities and decreased the mitotic index in *A. cepa*.

In the present work, the antimitotic activities of the SS, PS and CS have been confirmed. The treatment of root tips of *A. cepa* with these food additives caused reduction or inhibition of meristematic cell divisions. It has been reported that many of the food additives inhibit mitotic activity [3, 7, 9]. Njagi and Gopalan [1] indicated that sodium benzoate and sodium sulphite are cytotoxic in *V. faba* root meristems. Reduction in the mitotic activity could be due to inhibition of DNA synthesis or a blocking in the G_2- phase

of the cell cycle, preventing the cell from entering mitosis [14].

The food preservatives SS, PS and CS caused a change in the frequencies of different stages of mitosis (Tables 1-3). The frequency of prophases generally decreased after treating with SS and PS when compared to the control. The frequency of metaphases increased after treating with SS and PS, but this frequency was reduced after treating with CS. The frequency of anaphase-telophase stages increased after treating with SS and PS, while these stages decreased in general after treating with CS at all concentrations and treatment periods. These changes in the stage of mitosis indicate that SS, PS and CS affect the relative duration of each stage as compared with the control. The general effect of SS and PS was to increase the percentage of cells at metaphase and ana-telophase stages. In our opinion, this may be explained on basis of a requirement for spindle formation of active protein synthesis to ensemble microtubules. These results are in line with the results of many scientists who examined the effects of different chemicals on root meristems [7, 9].

SS, PS and CS induced very few abnormal cells in the *A. cepa* root tips, and the main effect appears to be clastogenicity. The chromosome aberrations results obtained for SS, PS and CS are summarised in Tables 1-3 and Figure 1. Their percentages increased as the concentration of the applied preservatives and the duration of treatment increased when compared with the control groups. There were statistically significant differences between controls and treated groups in chromosome abnormalities. The most frequent chromosome abnormalities were C-mitosis, micronuclei, anaphase bridges, laggards, stickiness, vacuolated nuclei and unequal distribution (Fig. 1).

For all food preservatives the most frequent type of abnormalities was C-mitosis (Fig. 1 a) Large numbers of C-mitotic metaphases indicates that SS, PS and CS act as a potent spindle inhibitor. C-mitosis was first described by Levan [8] in root tips of *A. cepa* as an inactivated of the spindle followed by the random scattering of the condensed chromosomes. Such C-mitotic cells have been also reported to be induced by the treatments with various other preservatives [3, 6, 7, 9]. The action of the preservatives like colchicine may be explained by causing certain disturbances in the protein tubulin. C-mitosis is considered as index of mitotic poisoning.

As shown in Tables 1-3, significant increases in micronucleus (MN) (Fig 1. b) frequency were detected in *A. cepa* roots after exposure to 200 ppm PS for 48 h, and 100 ppm CS for 72 h. Additionally, MN frequency generally increased in all treatments at other concentrations of SS, PS and CS when compared with the control groups. The two basic phenomena leading to the formation of MN in mitotic cells are chromosome breakage and dysfunction of the mitotic apparatus. MN are formed from acentric chromosome or chromatid fragments and whole chromosomes or chromatids that lag behind in anaphase and are left outside the daughter nuclei in telophase. MN can cause cell death due to the deletion of primary genes, have mutagenic effects and there is a strong correlation between the ability of a chemical to cause chromosome aberrations and point mutations [15]. MN were reported by many investigators following treatment with different food additives [2, 7, 9, 13]. MN formation is undoubtedly an important mechanism for chromosome loss.

Another interesting abnormality found in the present study is the appearance of anaphase bridges in root tips of *A. cepa* (Tables 1-3) (Fig. 1c). Such chromosome bridges were also reported to be induced by several other food additives. Njagi and Gopalan [1] reported that the food preservatives sodium benzoate and sodium sulphite caused anaphase bridges in *V. faba*. Gömürgen [7] showed that potassium metabisulphite and potassium nitrate caused anaphase bridges in *A. cepa*. Türkoğlu [9] also showed that exposure of root tips of *A. cepa* to high concentrations of boric acid, citric acid, potassium citrate and sodium citrate led to formation of anaphase bridges. The formation of bridges could be attributed to chromosomal stickiness and to chromosome breakage and illegitimate reunion. Induction of bridges may lead to loss of genetic material [16].

Laggards in meta-and telophase stages were also observed in *A. cepa* roots in a very low percentage after some treatments with SS, PS and CS concentrations (Tables 1-3) (Fig. 1d). Lagging chromosomes were the result of failure of the chromosomes to move to either of the poles. Additionally, acentric chromosome fragments remain as laggards. Gömürgen [7] and Türkoğlu [9] also reported the induction of laggards following treatments with food additives.

Stickiness of chromosomes at metaphase was observed following treatments with SS, PS and CS (Tables 1-3) (Fig. 1e). However, as can be seen from the tables, chromosome stickiness was observed in a low percentage. The stickiness of the chromosomes at metaphase caused inability of normal movement of the chromosomes at anaphase. Similar aberrations were reported by Rencüzoğulları et al. [3, 6], Gömürgen [7], Türkoğlu [9] following treatments with other food additives in human lymphocytes and *A. cepa* root tips. Stick chromosomes reflect highly toxic effects, usually of an irreversible type probably leading to cell death. Chromosomal stickiness has been attributed to an action on the chromosome proteins.

In addition to the above-mentioned type of abnormalities, vacuolated nuclei and unequal distribution were observed in the present study. The vacuolated nuclei were only observed in the group treated with SS at 200 ppm for 48 h and 50 and 100 ppm for 72 h (Table 1) (Fig. 1f). Vacuolated nuclei may be the result of a deficit of DNA in the nucleus. The precocious movement of the chromosomes might have been caused by the early terminalisation, stickiness of chromosome and/or because of the movement of the chromosome ahead of the rest during anaphase [17]. In prophase, the occurrence of vacuolated nuclei is regarded as physiological effect of SS on the chromosomes.

Similar results to the present study were also reported by El-Ghamery et al., [18] when they *Nigella sativa* and *Triti-*

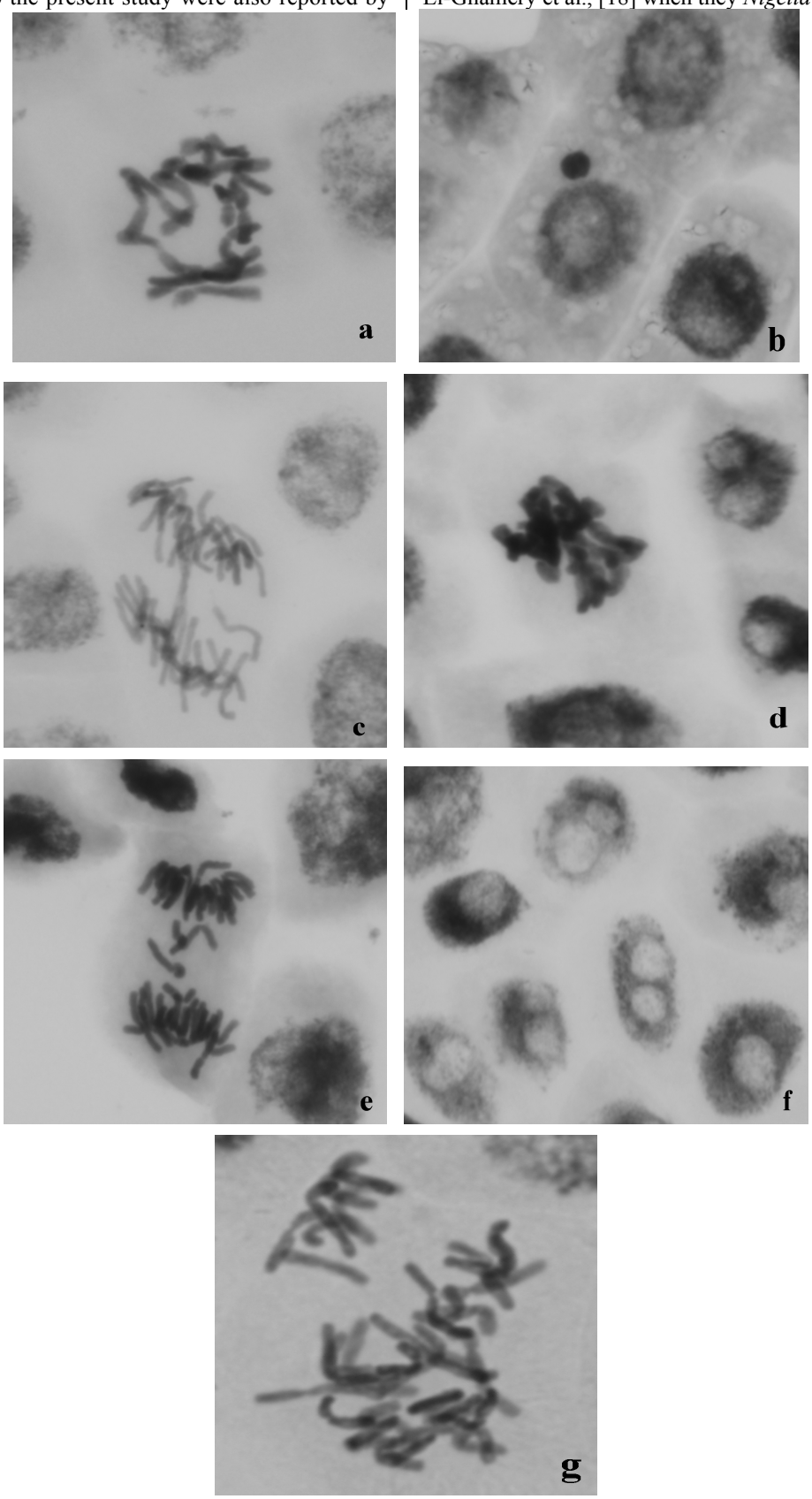

FIGURE 1 - Differnt types of aberrations induced by the food additives in *Allium cepa* root tips. a. C-mitosis (treated with SS, PS and CS), b. Micronucleus at interphase (treated with SS, PS and CS), c. Anaphase bridge (treated with SS, PS and CS), d. Sticky metaphase (treated with SS, PS and CS), e. Laggards (treated with SS, PS and CS), f. Vacuolated nuclei (treated with SS), g. Unequal distribution (treated with SS).

cum aestivum roots with different chemicals. Unequal distribution of chromosomes was also observed after SS treatment. This abnormality was observed in a few treatment groups (Table 1) (Fig. 1g), and it could beattributed to the interference of the food additives with tubulin and/or polymerization of the microtubule subunits forming the spindle apparatus. Unequal distributions were reported in both plant and animal cells as a result of chemical treatment [7, 9].

The types of abnormalities produced by SS, PS and CS may be grouped in three classes. The first is often referred to as mitotic abnormalities and is the result of an action on the mitotic apparatus. The class includes C-mitosis, multipolarity, polyploidy and chromosome lagging. The other class of anomalies observed is chromosomal stickiness, which is generally regarded as a physiological effect on chromosomes during division and has been considered as a category of chromatid aberration. The third class is chromosome aberrations, including chromosome breaks, bridges, fragments, sister union and micronuclei. In this respect, the chromosomal aberrations (clastogenic type) are considered as reliable indicators of mutational change that are used as reliable evidence for screening the mutational activity.

The effects of the different treatments of SS, PS and CS on the amounts of DNA and INV are shown in Table 4. Increased concentration and/or prolonged periods of treatment resulted in increased reductions in the amounts of DNA and INV.

The reduction in mitotic activity was accompanied with a negative effect on the amounts of DNA and INV. This inhibition could be due either to blocking of G_1 suppressing DNA synthesis or, most likely, a blocking in G_2 checkpoint preventing the damaged cells from entering mitosis [14]. Most studies on food additives toxicity in several test materials have been focused on the cytotoxic effects, but there are not many reports on the effects of food additives on the activity of nuclear DNA and INV. Njagi and Gopalan [1] reported that sodium benzoate and sodium sulphite inhibited DNA synthesis in *V. faba* root meristems. The investigations on the DNA contents were reported after treatment with other chemicals [19, 20]. Additionally, Mohanty et al. [20] and Bennet et al. [21] suggested that a low dose of aluminum decreased INV.

In conclusion, from the present study, it appears that, SS, CS, and PS which are used commonly in the food industry have clear chromotoxic effects. The results of studies using sulphite, sodium sulphate and sodium metabisulphite on human blood lymphocytes showed that mitotic index was decreased and chromosomal abnormalities occured [3, 30, 31]. Similar results were obtained in this study using *Allium cepa* root meristem cells. For this reason it is necessary to be careful when using these chemicals in foods as preservatives, and it is necessary to find new safe substances as alternatives to SS, CS, and PS.

TABLE 4 - Effect of different concentrations of calcium sulphite, potassium sulphite and sodium sulphite on DNA contents and interphase nuclear volumes in *A. cepa* root tips after treatments for different durations

		Sodium sulphite		Potassium sulphite		Calcium sulphate	
Time of treatment	Concentrations (ppm)	DNA content (pg ± S. E *)	Nuclear volume (μm^3± S. E *)	DNA content (pg ± S. E *)	Nuclear volume (μm^3± S. E *)	DNA content (pg ± S. E *)	Nuclear volume (μm^3± S. E *)
	Control	65.70 ± 0.23	198.33 ± 0.31	91.98 ± 0.29	195.15 ± 0.88	67.28 ± 1. 13	193.21 ± 0.30
	50	60.75 ± 0.27	137.98 ± 0.25 *	84.93 ± 0.31	174.54 ± 0.10	54.87 ± 0.15	183.27 ± 0.18
24 h	100	53.72 ± 0.24	128.00 ± 0.47 *	75.20 ± 0.02	152.15 ± 0.71 *	47.41 ± 0.33	162.81 ± 0.12 *
	150	49.79 ± 0.37	112.88 ± 0.44 *	67.91 ± 0.10	160.48 ± 2.97 *	40.38 ± 0.11 *	150.93 ± 0.05 *
	200	43.72 ± 0.39	92.82 ± 1.18 *	61.20 ± 0.09 *	130.14 ± 0.87 *	38.04 ± 0.60 *	134.61 ± 0.34 *
	Control	66.08 ± 0.28	188.87 ± 2.04	92.52 ± 0.09	194.34 ± 01.06	67.48 ± 0.82	199.00 ± 0.24
	50	56.59 ± 1.13	118.49 ± 1.99 *	79.22 ± 0.12	173.51 ± 0.51	46.71 ± 0.10	170.77 ± 0.28 *
48 h	100	50.42 ± 0.98	105.88 ± 1.50 *	70.58 ± 0.09	165.18 ± 041 *	41.35 ± 0.96 *	152.62 ± 0.33 *
	150	45.14 ± 0.50	94.80 ± 1.23 *	63.19 ± 0.06 *	144.57 ± 1.19 *	38.58 ± 0.32 *	137.48 ± 0.19 *
	200	37.07 ± 1.25 *	79.19 ± 0.18 *	51.90 ± 0.75 *	120.77 ± 1.07 *	Toxic	Toxic
	Control	66.31 ± 0.75	190.33 ± 0.98	92.83 ± 0.51	199.06 ± 1.94	67.55 ± 1.12	199.13 ± 0.20
	50	51.32 ± 0.48	95.16 ± 0.75 *	71.85 ± 0.73	145.27 ± 2.10 *	43.53 ± 0.19 *	153.79 ± 0.12 *
72 h	100	47.18 ± 0.41	83.60 ± 0.61 *	66.05 ± 0.91	141.49 ± 0.45 *	37.61 ± 0.23 *	142.22 ± 1.49 *
	150	Toxic	Toxic	49.99 ± 0.26	119.16 ± 1.30 *	31.22 ± 0.37 *	131.90 ± 0.19 *
	200	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic

Significant at $P< 0.05$ level.

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LEAD TOXICITY INDUCED ANTIOXIDANT ENZYME AND PHENYLALANINE AMMONIA-LYASE ACTIVITIES IN *Jatropha curcas* L. RADICLES

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ABSTRACT

In vitro embryo culture was designed to investigate the effects of lead on superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and phenylalanine ammonia-lyase (PAL) activity in *Jatropha curcas* radicles. Radicles biomass decreased progressively with rising lead concentrations up to 800 μM. SOD and POD activity increased gradually with increasing lead concentrations, the highest value observing at 800 μM. PAL activity was induced significantly at all lead concentrations, and the peak increment is observed at 200 μM. However, CAT activity showed no significant change under lead stress. Patterns of SOD and POD isoenzymes suggested that different isoenzymes are differentially regulated by lead stress. The changes of staining intensity in these isoenzyme bands are consistent with the changes of these enzyme activities as assayed in solution. These findings support that increased SOD, POD and PAL activity are involved in the defense mechanism of *Jatropha curcas* radicles against lead toxicity.

KEYWORDS: Toxicity element, *in vitro* embryo culture, *Jatropha curcas* L., ROS-scavenging enzymes.

INTRODUCTION

Heavy metal pollution is one of the most important ecological problems due to its impact on human health through the food chain [1]. Among heavy metals, lead (Pb) is a nonessential nutrients element, and the exposure to relative low concentrations results in serious toxicity. Lead can cause growth inhibition and even plant death due to its detrimental effect on many physiological processes [2]. Moreover, Pb also can induce the intracellular overproduction of reactive oxygen species (ROSs), thus damaging plant cells [3, 4]. To prevent oxidative damage, plant cells have evolved comprehensive and integrated antioxidant defense system that comprises a variety of antioxidant molecules and enzymes. The main antioxidant enzymes include superoxide dismutases (SOD), peroxidases (POD) and catalases (CAT), which are capable of removing ROSs and their products and/or repairing oxidative damage [5]. In several plants, Pb-induced changes in ROS-scavenging enzymes, including SOD, POD and CAT, have been detected [6-8]. Therefore, it is imperative to compare ROS-scavenging enzyme activity among plant species with different Pb tolerance, and it will tell us the intrinsic role of antioxidant enzymes in Pb tolerance.

Jatropha curcas L., belongs to the family *Euphorbiaceae*, and is cultivated as a medicinal plant in many tropical and subtropical countries. Various parts of the plant hold potential for use as a source of oil, animal feed, medicinal preparations [9]. In the present study, *Jatropha curcas* embryos were germinated and grown *in vitro* with different lead concentrations, to investigate the effects of Pb on biomass, antioxidant enzyme and phenylalanine ammonia-lyase (PAL) in radicles. Information from this study will be helpful to identify toxic critical values of lead in soils based on *Jatropha curcas*'s biochemical responses.

MATERIAL AND METHODS

Plant materials and chemical

Mature *Jatropha curcas* seeds were collected in August, 2007 from more than 10 individual wild trees in Panzhihua, Sichuan provice, China. Seeds were oven dried, selected and stored in a plastic box with labeled (No. 20070822) were deposited at 4 °C until processing. Nitro blue tetrazolium (NBT), L-phenylalanine and methionine were purchased from Sigma (St. Louis, MO, USA). Other reagents used were of grade or higher.

Embryo germination and seedlings growth

Seeds were surface sterilized in 70 % ethanol for 30 s, and then in 0.1 % mercuric chloride for 8 min. The seed rinsed with distilled sterile water and soaked in sterile water for 24-36 h in a culture room. Thus, each embryo was dissected from the seeds in clean bench. Murashige and Skoog (MS) medium pH was adjusted to 5.8 ± 0.1 prior to autoclaving at 121 ± 2°C for 15 min, with 30 g L^{-1} sucrose and 6 g L^{-1} agar powder. Thus, two experiments were carried out. Experiment 1 was performed with four levels of

Pb treatments containing MS medium: 100, 200, 400 and 800 µM Pb for 7 days *in vitro* culture. Solution Pb^{2+} was supplemented as $Pb(NO_3)_2$. Experiment 2 was performed with MS medium. Rotten and contaminated embryos were removed promptly. When the cotyledons of seedlings had developed, radicles were harvested and washed with distilled water, blotted and immediately frozen in liquid nitrogen or stored at -70°C for analysis. Three sets of seedlings were analyzed for each Pb concentration, with 15 embryos per set.

Protein extraction

Fresh radicles were homogenized in 50 mM sodium phosphate buffer (pH 7.0, 1/10, w/v) containing 0.5 mM EDTA and 150 mM NaCl. The homogenate was centrifuged at 12 000 rpm for 10 min, and resulting supernatant was stored frozen at -70°C for assaying of enzyme activities later.

Assay of antioxidant enzymes

SOD activity was determined by measuring its ability to inhibit photochemical reduction of NBT [10]. One unit of SOD was defined as the amount of enzyme that caused 50 % inhibition of the photo-reduction of NBT under the assay condition. POD activity was determined by the Sakharov and Bautista method, using guaiacol as substrate [11]. The reaction mixture contained 3.8 mL 3 % guaiacol (50 mM sodium phosphate buffer, pH 7.0), 100 µL 2 % H_2O_2 and 100 µL enzyme extract. One enzyme unit was defined as the amount of enzyme that produced the change of 1 absorbance per min at 470 nm. CAT activity was measured following the change in absorbance of the reaction mixture at 240 nm. The assay solution contained 2.8 ml sodium phosphate buffer (50 mM, pH 7.0), 100 µL H_2O_2 (1 %) and 100 µL of crude extract [12]. One unit of CAT activity was defined as the amount of enzyme, which reduces 1 µmol H_2O_2 per minute. SOD, POD and CAT activity was expressed in unit per gram fresh weight (U g^{-1} FW).

Enzyme extraction and PAL activity assay

PAL activity was assayed from the reaction product *trans*-cinnamate of L-Phe decomposition as measured by the increase of absorbance at 290 nm [13]. The reaction mixture contained 20 mM L-Phe and 100 µL enzyme extract in a final volume of 3 mL of 50 mM Tris-HCl (pH 8.8). The mixture was incubated at 30°C for 30 min, and was terminated by the addition of 0.5 mL of 10 % trichloroacetic acid. One unit of enzymatic activity was defined as the amount of enzyme needed to increase in absorbance of 0.01 per min. PAL activity were expressed as unit per gram fresh weight (U g^{-1} FW).

Polyacrylamide gel electrophoresis (PAGE)

Native gel electrophoresis for isoenzymes was carried out with 7 % acrylamide gel. SOD isoenzyme activity was determined by the Beauchamp and Fridovich method [14]. Gels were equilibrated with 50 mM phosphate buffer (pH 7.5) containing 2.8×10-5 mol riboflavin, 28 mM N,N,N,N-tetramethyl ethylenediamine (TEMED) for 30 min, then washed in distilled water for 1 min and resubmerged in the same buffer containing 2.45 mM NBT for 10-20 min with gentle agitation in the presence of light. Enzyme bands ap-peared as colorless bands on a purple background. For detection POD isoenzymes, gels were soaked in deionized water for 5 min, and then incubated in 0.03 % H2O2, 0.2 % (w/v) benzidine and 0.1 % (v/v) acetic acid for 3-5 min. When maximum contrast was achieved, the reaction was stopped by rinsing the gel with deionized water [15].

Statistical analysis

All treatments were arranged in a completely randomized design with three replicates. All data were expressed as means ± SD. Statistical significance was evaluated with Student's t-test, and differences were considered significant if P values were < 0.05.

RESULTS AND DISCUSSION

As shown in Figure 1, biomasss in *Jatropha curcas* radicles decreased gradually with increasing Pb concentration up to 800 µM. Decreasing biomass was related to morphological responses of radicles exposed to different lead concentration (Data not shown). Decrease of growth under heavy metal excess is usually explained as the consequence of increased lignin synthesis, thus reducing extensibility of the cell walls. There is also strong time difference between redox-active and redox-inactive metals in terms of their inductive effect on lignin synthesis [16]. Amongst heavy metals, Pb is generally considered to be a highly toxic element and its negative effects on plant development and growth have been frequently observed in previous studies. Increasing Pb level can decrease the root length and fresh weight of *Lythrum salicaria* and *Oryza sativa* [2,7]. Thus, the present study showed that elevated lead concentration can inhibit the normal growth and development of *Jatropha curcas* radicles.

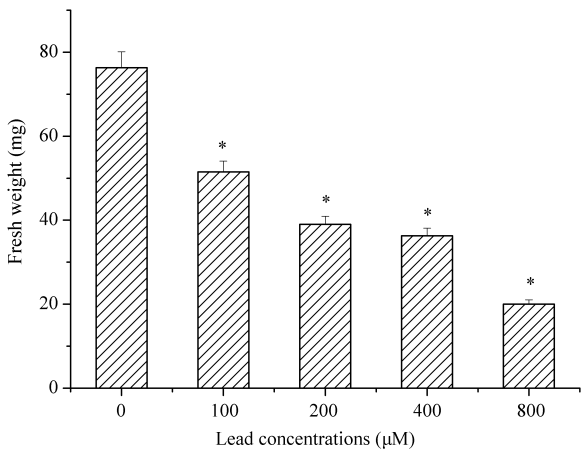

FIGURE 1 - Effects of different lead concentrations on fresh weights of *Jatropha curcas* radicle. The values and standard errors (vertical bars) of three replicates are shown. Significant difference (P< 0.05) is denoted as asterisk (*) between control and lead treatments.

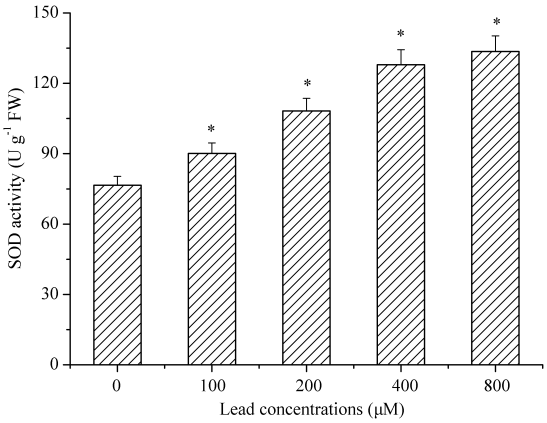

FIGURE 2 - Effects of different lead concentrations on superoxide dismutase (SOD) activity in *Jatropha curcas* radicles. The values and standard errors (vertical bars) of three replicates are shown. Significant difference (P< 0.05) is denoted as asterisk (*) between control and lead treatments.

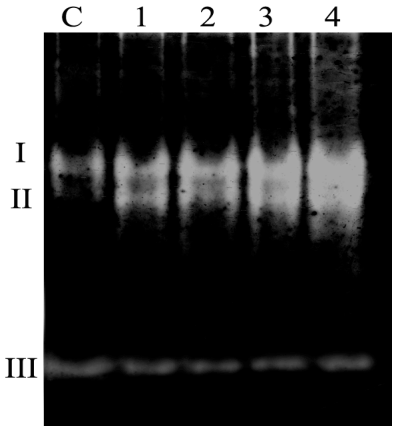

FIGURE 3 - Patterns of superoxide dismutase (SOD) isoenzymes in *Jatropha curcas* radicles. Lanes from left to right were 0, 100, 200, 400 and 800 μM, respectively.

The main function of SODs is to scavenge ROS generated in various physiological processes, thus preventing the oxidation of biological molecules, either by the radicals themselves, or by their derivatives [5]. In this study, SOD activity increased gradually with rising Pb concentration up to 800 μM, and the largest activity increased by 74.4 % compared to the control (Figure 1). A number of heavy metal stresses can lead to enhanced production of ROS within plant tissues, and SOD has been proposed to be important in plant stress tolerance [3,8]. Our results suggested that SOD activity in all treated radicles have markedly higher activity overall compared to the control. Increased SOD activity in the radicles could be due to either increased production of ROS or it could be a protective mechanism employed by *Jatropha curcas* plants against oxidative damage. Electrophoresis analysis suggested that at lest three SOD isoenzyme bands are detected, and different patterns are found (Figure 3). The staining intensity of isoenzyme bands (I and II) enhanced gradually with increasing lead concentrations, but the staining intensity of isoenzyme band III show no significant change. Changes of SOD isozyme patterns in some plant species has been suggested to be due to increased requirement against heavy metal stresses [17]. Our results indicate that these SOD isoenzymes (I and II) might be used as molecular probes to study the oxidative stress that occurs specifically in plant as a result of environmental adversity. The diverse responses of these SOD isoenzymes reported in these studies reveal that each condition produces its own unique effects, and further study of these should help to clarify the overall importance of oxidative stress phenomena in *Jatropha curcas*.

PODs are widely distributed in higher plants where they are involved in various processes, such as hormone regulation, defence mechanisms, and lignin biosynthesis. Induction of POD activity in plant cells has been correlated with development of increased tolerance to a variety of chemical compounds and physical stresses, including lead stresses [4, 6]. Therefore, PODs have often served as a parameter of metabolism activity during growth alterations and environmental stress conditions. According to Figure 4, POD activity increased by 23 %, 52 %, 230.3 % and 417.6 % compared to the control, respectively. Enhanced POD activity might potentially increase oxidative stress due to increased production of ROS. Earlier results showed that lead toxicity induced POD activity in some plant species [18,19], which are correlated with growth reduction (Figure 1 and Figure 4). Moreover, strong enhancement of POD activity is often matched by an increase in SOD and/or CAT and/or PAL activities under heavy metal stress [4,18]. Our results showed a positive correlation with SOD and POD (Figure 2 and Figure 4), although the significance of such induction was not clear. The present study lends further support to those findings. Results of isoenzyme pattern showed that at least five POD isoenzyme bands in all tested radicles are detected (Figure 5). Increased POD activity mainly comes from the changes of the staining intensity of isoenzyme bands (I, III and IV). Our findings here show a complex regulation of steady-state POD activity at a temporal and spatial level, as well as provide evidence for showing a correlation between Pb concentrations and the changes of POD isoenzyme pattern. Multiple POD isoforms reported for some plants were detected in the same tissue under different Pb concentrations stress, suggesting that different POD isoenzymes might be involved in distinct processes [17,19]. Moreover, the changes of staining intensity of iso-enzyme bands were consistent with the changes of activity assayed in solutions (Figure 4 and Figure 5).

CAT plays important role in the protection of cells from the toxic effects of hydrogen peroxide. In oily seeds, CAT is particularly important in the early events of seedling growth because it removes H_2O_2 produced during β-oxidation of the fatty acids [6]. The present study suggested

that CAT activity in all treated radicles show no significant change, and the highest activity only increased by 14.7 % compared to the control. Our findings suggested, at least here, that CAT appear not to be an effective ROS-scavenger exposed to Pb toxicity.

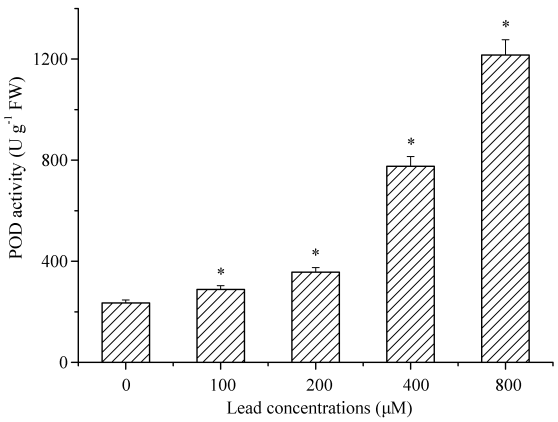

FIGURE 4 - Effects of different lead concentrations on peroxidase (POD) activity in *Jatropha curcas* radicles. The values and standard errors (vertical bars) of three replicates are shown. Significant difference (*P*< 0.05) is denoted as asterisk (*) between control and lead treatments.

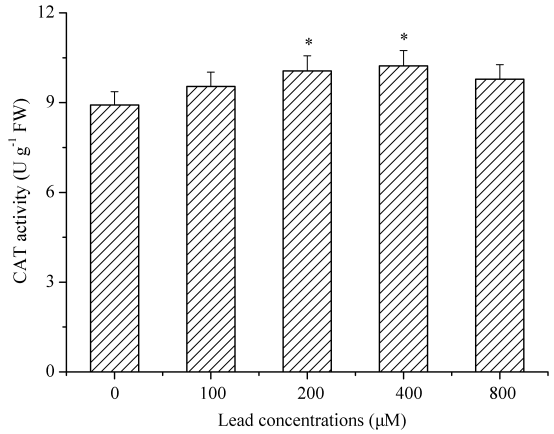

FIGURE 6 - Effects of different lead concentrations on catalase (CAT) activity in *Jatropha curcas* radicles. The values and standard errors (vertical bars) of three replicates are shown. Significant difference (*P*< 0.05) is denoted as asterisk (*) between control and lead treatments.

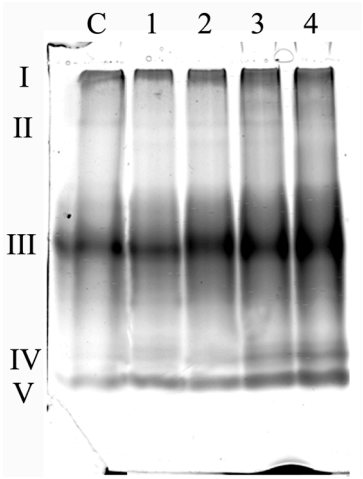

FIGURE 5 - Patterns of peroxidase (POD) isoenzymes in *Jatropha curcas* radicles. Lanes from left to right were 0, 100, 200, 400 and 800 μM, respectively.

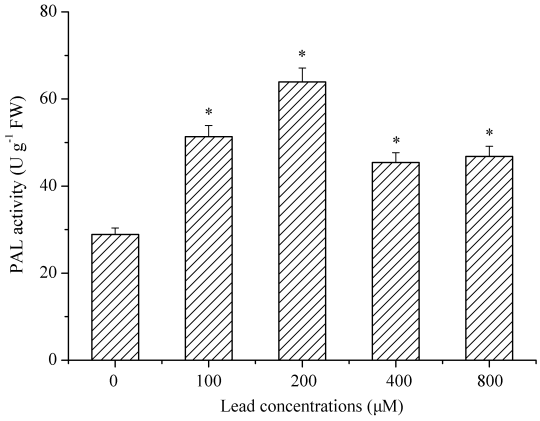

FIGURE 7 - Effects of different lead concentrations on phenylalanine ammonia-lyase (PAL) activity in *Jatropha curcas* radicles. The values and standard errors (vertical bars) of three replicates are shown. Significant difference (*P*< 0.05) is denoted as asterisk (*) between control and lead treatments.

PAL, a key enzyme of plant phenylpropanoid metabolism, is involved in the biosynthesis of a wide variety of secondary metabolities that are important for normal growth and for responses to abiotic and biotic stresses [20]. Thus, PAL is generally recognized as a marker of environmental stress in different plant species. As shown in Figure 6, PAL activity increased gradually with increasing Pb concentration up to 200 μM, and the largest activity is 2.21 higher than that of the control. Increased PAL activities have been investigated in several plant species under heavy metal stress [18,21]. Thus, it seems that, in *Jatropha curcas* radicles, the enhancement of PAL activity could be related to the implication of this enzyme in the plant response to lead stress.

Our findings strongly support the hypothesis that SOD, POD and PAL in *Jatropha curcas* radicles are believed to be ones of the most important factors of the plant biochemical defense against lead toxicity, which is actively involved in the self-regulation of plant metabolism. The role of each particular component in the overall antioxidant defense mechanisms in *Jatropha curcas* radicles, especially low molecular mass antioxidants, are unclear and should be further investigated.

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RESPONSE OF BULK DEPOSITION TO CHANGES IN INDUSTRIAL ACTIVITIES IN A MEDITERRANEAN URBAN REGION

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ABSTRACT

Bulk deposition samples were collected monthly at six sampling points in the highly industrialized city of Cartagena, Mediterranean coast of Spain, during a period of 13 years. The study has focused particularly on the effects on dustfall composition caused by the end of two industrial activities, a lead smelter and a fertilizers plant which shut down in March of 1992 and December of 2001 respectively. Both factories were located within the urban area. This fact meant an enormous impact on the population. Not only because of the pollutant concentration released by such activities but on people's perception. The detailed observation of the results stresses the influence that these factories had on the air quality over this period of time. This study was initiated in 1991, before the facts described, and finished in year 2003, allowing us to observe the variation of deposit concentration.

The samples were obtained following the protocol of the British Standard Norm 1747, which establishes the analytical and valuation techniques for chemical pollutants present in the atmosphere. In this paper the results of total deposition weight, water-soluble and water-insoluble fractions and pH, from 1991 to 2003, are presented. During the study period the aerosol deposit has suffered a significant decrease, and the pH value of the soluble fraction has increased. These changes are related with the cease of the industrial activities described.

KEYWORDS: Bulk deposition, soluble and insoluble fractions, pH, industrial activity.

INTRODUCTION

Atmospheric aerosols play an important role in climate and atmospheric chemistry and harmful effects on human health [1]. Usually the studies focus on airborne particulate matter, and on smaller fractions because of their interaction with human health. Dustfall comprises the coarse particles from atmospheric aerosol, responsible for the dust accumulated in the vicinity of the industrial facilities [2], because of their greater settling velocity [3].

The composition of atmospheric aerosols varies markedly, depending upon sampler location, the proximity of significant sources of aerosols and their gaseous precursors, and meteorology [4]. Human activities have had a major effect on the emission and subsequent deposition of constituents, e.g. acid deposition through combustion of fossil fuels. Accordingly, it is important to assess emissions and deposition trends and their relations, particularly as human activities change [5].

Cartagena has undergone an important industrial development from 60's onwards, producing a singular concentration of large chemical and metallurgical factories [6]. During the nineties Cartagena´s industrial scene changed dramatically. First in 1992, a lead smelter (point C in Figure 1) situated in the outskirts ceased its activity. In relation to this new situation, in July of the same year a sulphuric acid plant was also shut down. This sulphuric acid plant was part of a fertilizer plant (point A in Figure 1) which was also located very close to the city, it finally finished its activity in December of 2001. In addition to these plants, there is an electrolytic zinc plant situated in the outskirts of the city (point B in Figure 1). This study was initiated in 1991 before the facts described and finished in 2003 after them, so it is possible to observe the variation in the deposit. We have determined deposition total weight, pH in soluble fraction and soluble and insoluble fractions weight in all samples.

MATERIALS AND METHODS

Settleable particulate matter sampling

The study of the settleable particle matter is usually based on the gravitational sampler [7]. In Cartagena, there are installed six horizontal funnels of known area, based on

the dustfall standard gauge described in the British Standard Norm 1747 [8]. Three of them are situated in areas affected by the factories, both by their geographic proximity to the industrial area, and, more importantly, winds that blow in these locations. These sampling points are in Los Mateos, and the railway stations RENFE and FEVE (sampling points 1, 2 and 3). Sampling point number 6 is located in Media Legua. This sampling point is especially interesting because its position, although it is very close to the aforementioned industries, it is out of the principal wind profiles of the area. Sampling point number 5 is the San Agustín square, and represents the harbour situation. The last sampling point, number 4, is in España square. This is the less polluted area of the city. Figure 1 shows the distribution of the sampling stations on a Cartagena map.

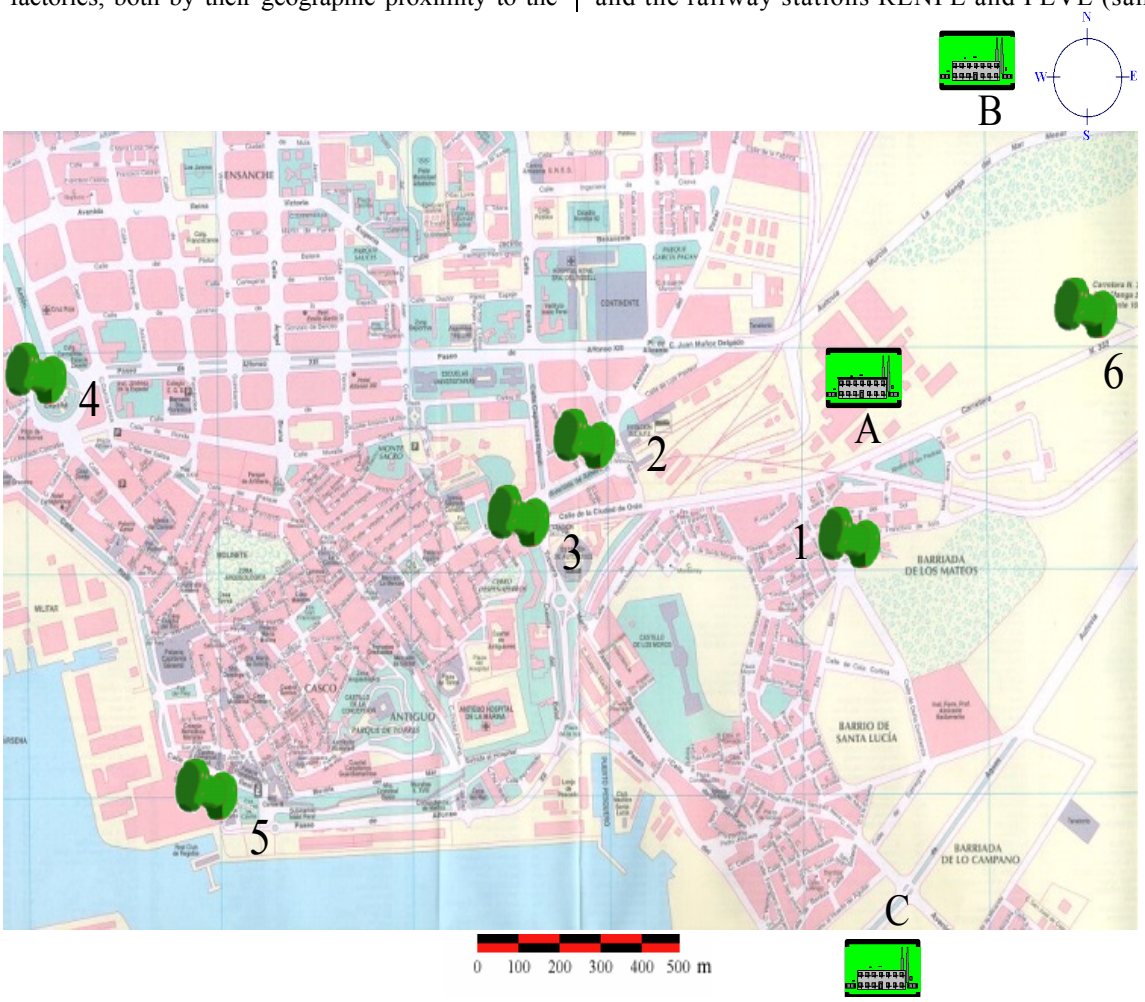

FIGURE 1 - Map of Cartagena. Settleable particulate matter sampling points and main industries location. A.- fertilizer plant (shut down on December 2001). B.- Electrolytic Zinc plant (active). C.- Lead smelter (shut down on March, 1992).

The sampling period started in February 1991 and finished in December 2003. The samples were collected monthly, except the sample from August that was collected together with the one from September due to logistics. This makes a total of eleven samples per year. Both wet and dry depositions were collected. All sampling conditions were registered. The sampling procedure is described on the Ministerial Order from August 10, 1976 [9]. The funnel where particulate matter settled during the sampling period was rinsed with 500 ml of distilled water. The particles that might be adhered to the surface of the gauge were brushed off to assure that the entire sample was collected. Finally, the deposit containing the sample was replaced with a clean one and the next sampling period started. Sampling information was stored in the sampling log-book (date, time, sampling point, rinse water volume and remarks, if any).

Sample Analysis

The content of the deposit was taken to the laboratory where it was first screened by a 1.5 mm strainer. The goal of this screening was to eliminate insect, leaves or bird feathers or any debris. Subsequently, the pH was measured (Crison Micro pH 2000).

The sample was centrifuged (P Selecta Macrotonic) and the soluble and insoluble fractions were separated. The insoluble fraction was dried in the oven at 105ºC (Heraeus E-42) and weighted (Mettler AJ100). The supernatant cor-

responded to the soluble fraction. The volume of this fraction was recorded, and an aliquot was dried and weighted. A crucible was weighted both when empty and after the aliquot had been dried. The total mass was calculated by sum of these two fractions. The results were expressed in $mgm^{-2}d^{-1}$.

Statistic data treatment

The results were saved on Excel file and switched to .sav extension. This format corresponds to the statistical package SPSS 12.0 (SPSS Inc., Chicago, IL.) used in this study. The effect of various factors on the data was studied, these factors are period studied and City area. Period factor is divided in three categories, the first category shows the data of settleable matter obtained from February 1991 until the closure of the lead smelter in March 1992 (period 1). Into the second category we have classified the data collected from April 1992 until December 2001, when the fertilizer plant shut down (period 2), and the third group reflects the results from years 2002 and 2003 (period 3).

The City area factor is also classified in three different categories [7]. Category 1 is formed by sampling points 1, 2 and 3. These three stations represent the so-called industrial area. The category 2 represents the urban area, and includes the sampling points 4 and 5. Finally, sampling point number 6 is within category 3, and it is considered as an intermediate urban-industrial area.

RESULTS

In this study we analyzed 687 bulk deposition samples corresponding to 13 years study, in these samples we determined four parameters, therefore, we processed 2748 data by means of statistics. The data were initially surveyed focusing on the characterization of the type of distribution constituted by each of the variable under study. We carried out this analysis in both direct and lognormal variables, the results of this analysis showed that only pH direct variable fitted a Normal distribution, the others variables adapted to a Lognormal distribution (Kolmogorov-Smirnov and Shapiro-Wilk`s tests, $p < 0.01$).

Table 1 presents the statistics, arithmetical mean, geometrical mean and standard deviation of the pH, total weight and soluble and insoluble fractions weights for each of the categories (Period 1,2,3 and City area 1,2,3) in which they were classified.

We must indicate that in all of cases the sampling point situated in España square (sampler 4) registered higher pH values and lower weights results during the studied years.

In the present work, the pH of the soluble fraction was determined, finding that samples with pH lower than 5.6 occurred only very occasionally. The lowest values were found in 1997, 1998 and 1999 for the overall city. Since that time a pH value increase took place, 2002 and 2003 registered the highest pH values.

With the aim of studying the spatial and temporal differences in the studied variables, we carried out a statistical test, using the Period and City area factors classifications explained. Firstly, we applied Levene test to check

TABLE 1 - Statistics, Arithmetical mean, geometrical mean and standard deviation of the pH, total weight (T.) and soluble (S.) and insoluble (I.) fractions weights for the city and for each of the categories of the city area (industrial, urban and intermediate) in the three time periods and in the total time period.

		City				Industrial area (1)				Urban area (2)				Intermediate area (3)			
		pH	T.	S.	I.	pH	T.	S.	I.	pH	T.	S.	I.	pH	T.	S.	I.
P.1	N	55	55	55	55	27	27	27	27	19	19	19	19	9	9	9	9
	Mean	6,5	439	222	217	6.0	616	319	298	7.1	250	115	135	6.8	301	154	148
	G. M.	6.5	345	166	167	5.9	541	275	256	7.0	211	92	107	6.8	251	127	118
	S. D.	0.9	327	185	159	0.9	357	208	169	0.6	158	84	99	0.5	198	106	110
P.2	N	542	543	543	543	277	278	278	278	181	181	181	181	84	84	84	84
	Mean	6.2	407	250	157	5.9	566	365	20	6.5	226	122	104	6.5	271	147	125
	G. M.	6.2	313	170	122	5.8	468	278	161	6.5	194	96	88	6.5	229	112	101
	S. D.	0.7	361	287	153	0.7	422	346	189	0.5	144	105	72	0.5	190	146	96
P.3	N	89	89	89	89	44	44	44	44	30	30	30	30	15	15	15	15
	Mean	7.1	253	115	136	7.0	272	121	151	7.3	213	98	116	7.1	274	132	142
	G. M.	7.1	221	94	119	7.0	241	101	132	7.2	186	80	99	7.0	244	107	129
	S. D.	0.6	142	75	83	0.6	151	75	92	0.6	124	64	71	0.4	143	94	71
Total	N	686	687	687	687	348	349	349	349	230	230	230	230	108	108	108	108
	Mean	6.4	390	231	159	6.1	533	331	202	6.7	226	119	108	6.6	274	145	129
	G. M.	6.3	301	157	125	6.0	436	244	163	6.7	194	94	91	6.6	233	113	106
	S. D.	0.7	342	265	147	0.8	405	326	180	0.6	142	99	74	0.5	183	137	94

TABLE 2 - Level of significance for the Kruskal-Wallis test (factor Period 1 to 3 and factor City area 1 to 3) carried out for the transformed logarithmic data, except in pH case.

	Factor: Period			
Variable	pH	Total Weight	Soluble Weight	Insoluble Weight

Test	KrusKal-Wallis	KrusKal-Wallis	KrusKal-Wallis	KrusKal-Wallis
Level of significance	0.000	0.000	0.000	0.010
		Factor: City area		
Variable	pH	Total Weight	Soluble Weight	Insoluble Weight
Test	KrusKal-Wallis	KrusKal-Wallis	KrusKal-Wallis	KrusKal-Wallis
Level of significance	0.000	0.000	0.000	0.010

homogeneity of variance and according to its results, variances were not equal across groups, the non-parametric Kruskal-Wallis test was used. Table 2 shows the results of the Kruskal-Wallis test.

Figures 2 to 5 reflect the average values and 95 % confidence interval of the analytical parameters pH, total weight, soluble fraction weight and insoluble fraction weight for each category of the sampling stations (industrial area, urban area and intermediate area) for each time period.

Table 3 presents the percentages of the soluble and insoluble fractions of the total amount deposited in the city per each studied year and for the total time period.

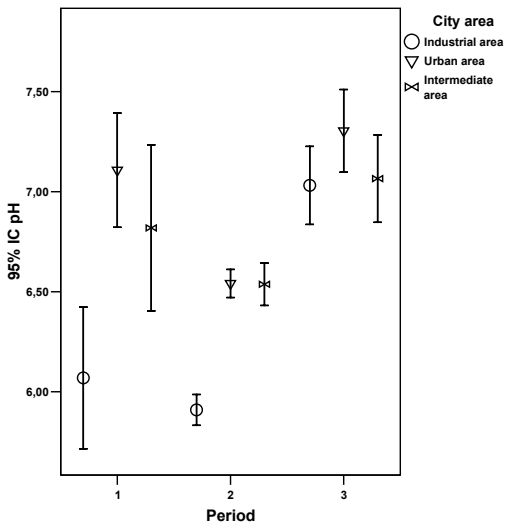

FIGURE 2 - Average values and 95 % confidence interval of the analytical parameter pH for each category of the City area (industrial, 1, urban, 2 and intermediate, 3) in the three time periods. (pH units).

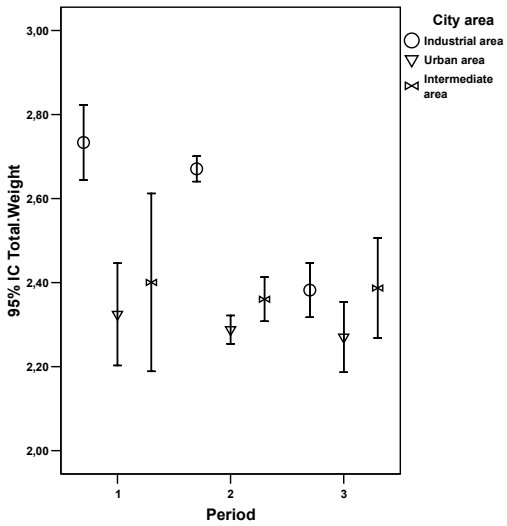

FIGURE 3 - Average values and 95 % confidence interval of the analytical parameter logarithm of total weight for each category of the City area (industrial, 1, urban, 2 and intermediate, 3) in the three time periods. ($mgm^{-2}day^{-1}$).

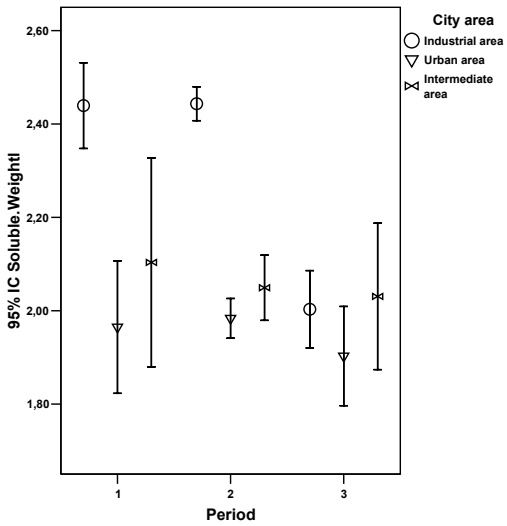

FIGURE 4 - Average values and 95 % confidence interval of the analytical parameter logarithm of soluble fraction weight for each category of the City area (industrial, 1, urban, 2 and intermediate, 3) in the three time periods. ($mgm^{-2}day^{-1}$).

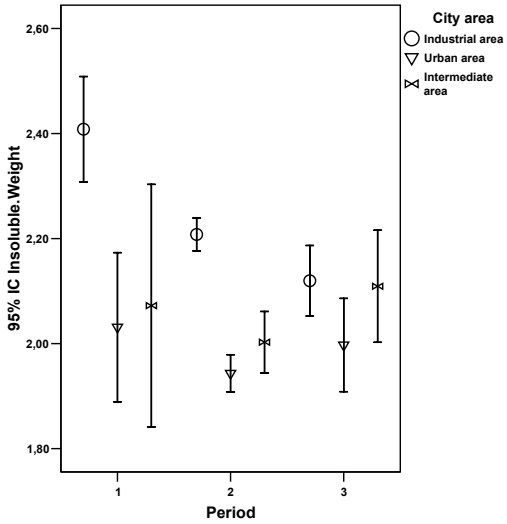

FIGURE 5 - Average values and 95 % confidence interval of the analytical parameter logarithm of insoluble fraction weight for each category of the City area (industrial 1; urban 2 and intermediate 3) in the three time periods. ($mgm^{-2}day^{-1}$).

TABLE 3 - Percentages of the soluble and insoluble fractions of the total amount deposited in the city per each studied year and for the total time period.

	Total	
Year	**% Soluble**	**% Insoluble**
1991	47.11	52.89
1992	54.86	45.14
1993	62.70	37.30
1994	58.50	41.50
1995	56.65	43.35
1996	64.39	35.61
1997	65.62	34.38
1998	68.43	31.57
1999	70.38	29.62
2000	46.11	53.89
2001	61.07	38.93
2002	43.17	56.83
2003	42.88	57.12
Total	59.17	40.83

TABLE 4 - Correlations between the rainwater collected in the studied time period, water collected by the sampler, pH of the soluble fraction and deposited weights (total, soluble and insoluble weights (lognormal variables)).

		Rain water	**Water collected**	**pH**
	Pearson's Coeff	0.390(**)	0.467(**)	-0.456(**)
Logsoluble	Bilat. Sig.	0.000	0.000	0.000
	N	579	687	686
	Pearson's Coeff	0.159(**)	0.221(**)	-0.084(*)
Loginsoluble	Bilat. Sig.	0.000	0.000	0.028
	N	579	687	686
	Pearson's Coeff	0.355(**)	0.428(**)	-0.390(**)
Logtotal W.	Bilat. Sig.	0.000	0.000	0.000
	N	579	687	686

*The correlation is significant at the 0.05 level (bilateral)
**The correlation is significant at the 0.01 level (bilateral)

Table 4 shows the correlation between rainfall data, water accumulated in the sampler, pH of the soluble fraction and deposited weights (total, soluble and insoluble weights).

DISCUSSION

Qualitative and quantitative assessment of atmospheric deposition is essential for understanding regional variations, determining when the critical loads are exceeded, and demonstrating, by analysis of pluriannual trends, the efficiency of emissions reduction policies on the regional scale [5, 10].

As indicated in the introduction, the city of Cartagena suffered a profound change in its industrial scene from 1991 to 2003. While in the beginning of this period a great industrial activity was present between the city limits, as well as, a great harbour activity (see figure 1). Most of this activity has moved to neighbouring harbour of Escombreras. These facts are clearly noticeable in the settleable particulate matter characteristics.

According to the results the variables statistic study was carried out using transformed logarithmic data, except in pH variable case, which was used directly. Lognormal distribution is the most usual distribution in environmental variables [11].

In the atmosphere, we can find acid compounds as a product of complex chemical, physical, and photochemical and oxidation reactions. These compounds are removed from the atmosphere by deposition and end up on the ground surface. The acid deposition is a phenomenon responsible for damaging the ecosystems and deteriorating buildings [12], therefore the value of pH of soluble fraction deposited is a very important parameter. The pH values lower than 5.6 are considered a consequence of anthropogenic emission, therefore pH values higher than that value are not influenced by human activities, or the neutralization by dust alkaline components might have taken place [13].

In Cartagena important acidic anthropogenic emissions have been reported, Casado and Martínez-Mena [14] assessed SO_2 and NO_x emissions of 92900 t and 9200 t respectively, in the 1980s decade. However, in this study only occasionally found pH values lower than 5.6.

We considered the neutralization capacity is produced by alkaline components derived not only from natural sources, $CaCO_3$ from our soils or calcareous dust load in the air coming from remote areas, like dust of the Sahara desert [15-18], but also from anthropogenic origin, such as NH_3 and metallic oxides, Casado and Martínez-Mena [14] assessed 3455 t of NH_3 and 0.5 t of ZnO emissions in Cartagena.

On the other hand, when pH data are evaluated for each sampling point category (industrial, 1; urban, 2 and intermediate, 3) for each period, the results show that the industrial area pH values are lower than other areas, and this parameter increases in the Period 3 in all of them, after the fertilizers plant closed, and the statistical differences between areas disappear in this period (kruskal-Wallis test $p > 0.01$), see Figure 2.

Deposited ambient dust is mainly important as a source of nuisance [2]. Regarding public health, particulate matter not only can be inhalated, but also play a role in the ecological route, entering into the food chain [3]. The atmospheric deposition contributes greatly to this last route. The input of persistent pollutants into vegetation and soils predominantly occurs via deposition of dust particles. The accumulation of metal compounds in soil can cause problems by increased uptake and contamination of plants. Moreover, dust deposits also cause contamination of aboveground plant parts. This can enhance transfer to humans via contact (children having contact with plants, bare soil or objects and take up heavy metals via contaminated hands) or by the consumption of vegetables [19].

In the present study we carried out the determination of the total weight in all of the samples. The annual mean of this parameter for the overall city was above 300 $mgm^{-2}d^{-1}$ (see table 1) until year 2001, limit established in the Decree 833/1975 [20], Spanish legislation. Currently, Spanish laws do not collect any reference value for deposited dust, the Royal Decree 1073/2002 [21] derogated the aforementioned deposit maximum value. In the Period 3 (2002 and 2003) lower values were registered.

Figure 3 shows total weight trends by City area categories and by period. The industrial area registered the highest values during periods 1 and 2, being statistically different (Kruskal-Wallis test $p < 0.01$ in both periods). In period 3 (after fertilizers plant closure), industrial area suffered an important decrease and no significant differences between the various areas were noticed (Kruskal-Wallis test $p > 0.01$), constituting a homogenous group. Urban and Intermediate areas keep values without significant differences during the three periods.

The annual means in our study are higher than values reported by Vallack [22] in Selby, North Yorkshire. Irvine et al. [23] registered in Hamilton, Canada, mean values of 240 $mgm^{-2}d^{-1}$ in an area called industrial area, this result is comparable with the values of the urban and intermediate areas of our study. In the Dead sea values around 20-450 $mgm^{-2}d^{-1}$ were found by Singer [24], the highest values in such study are lower than our study's results for the industrial area until 2001. In Varanasi, India, an annual mean value of 520 $mgm^{-2}d^{-1}$ was recorded by Tripathi et al. [25], the values registered in the sampling point of maximum deposit are as high as the Industrial area results until 2001.

The soluble and insoluble fractions weights were determined in all samples. When these variables were studied for area categories and for period 1, 2 and 3, both showed a different performance. Soluble fraction weight registered the lowest values in the years 2002 and 2003, period 3, throughout the city (see table 1), but it was noticed mainly in the Industrial area, where an acute decrease for this parameter was observed in the period 3 (see Figure 4), as well as a decrease in the deposit total weight, disappearing the statistical differences between the areas for both parameters (Kruskal-Wallis test $p > 0.01$)

The insoluble fraction weight had a similar performance, the values registered in urban and intermediate areas were stable during the three periods. The industrial area values were fluctuating, an acute decrease was observed in the period 2, after the lead smelter was shut down (Figure 5). The statistical differences between areas disappeared in the period 3 (Kruskal-Wallis test $p > 0.01$), after the closure of the fertilizers plant, like the other weights measured.

Likewise, the percentages of the soluble and insoluble fractions in the total deposit were determined in each studied year (Table 3). For the deposition total weight, the soluble and insoluble fractions represented 60% and 40% respectively. 1991, 2000, 2002 and 2003 had a percentage of insoluble fraction above 50%, while in 1993, 1997, 1998 and 1999 this percentage was lower than 35%. Others studies report that, generally speaking, insoluble fraction is higher than soluble fraction. Tripathi et al. [25] reported insoluble fraction percentages higher than 60% in Varanasi, India. Nasralla et al. [26] found values above 86% in El Cairo, and Ganor et al. [27] in Lake Kinneret, registered average values for hydrological years of total deposit and insoluble fraction, the mean percentage value reported was 52%.

Soluble fraction increase can be affected by rainfall and pH decrease. Table 4 reports the bivariate correlations between rainfall collected during the sampling periods, water accumulated in the collector, soluble fraction pH values, total weight and soluble and insoluble fractions weights. There are positive correlations at a level of significance of 99% between water collected in the sampler (collector washing water is not included) and the rest of parameters. An increment of the total deposit is registered when water collected increases, it affects the soluble fraction more than the insoluble fraction. There are high and negative correlations at a level of significance of 99% between pH and soluble fraction. It might be explained because the solu-

bility of some compounds included in the deposit increases when pH decreases [28].

The increment of the total deposit in response to the increase of rainfall is explained by the washing effect caused by the rain. Another cause might be the increment of the sampling efficiency, because rainwater washes down the particles deposited on sampler surfaces into the collector bottle, avoiding the blow-out of collected particles, quite the opposite of samplers with sticky surface, which is covered by a layer of dust and its retentive powers are reduced so further deposited dust may not be retained on the surface, likewise if any amount of rain falls deposited dust may be washed off [29]. These problems can be avoided restricting the period sampling in the first case and using a collecting bottle in the second.

It is important to remark that the region climate characteristics (semi-arid climate, vegetation, soils composition, etc.) also determine the values of the settleable particulate matter. Likewise, when air masses over the Sahara desert move northward over Spain may originate the Sahara red rains, it is due to particles scavenging by rainfall [16]. The frequency of African dust outbreaks over the Southern Iberian Peninsula is relatively high, with a mean occurrence of 16 to 19 episodes/year [30], although they are not always coupled with rain events.

CONCLUSIONS

The deposit values found in Cartagena show an acute spatial and temporal variability, although spatial variability decreased at the end of the study period. The bulk deposition total weight values were high in general.

This study shows the influence of industrial activity on bulk deposition values. During the sampling period, a decrease of bulk deposition was observed. The industrial situation in our city has influenced the characteristics of the deposit. The shut down of the lead smelter in year 1992, brought a reduction in the insoluble fraction present in the settleable particulate matter samples. In spite of that fact, this reduction did not influence that much the overall mass settled. On the other hand the closure of the fertilizers plant in year 2001 represented a significant reduction in the total mass settled, and an important change in the acidity of the settleable particulate matter to a more neutral pH values. These changes were more evident in the Industrial area.

The analysis of correlations carried out, allows us to conclude that the decrease of the pH affects the values of soluble fraction of deposition.

In spite of the acute decrease, the deposit values are high at the end of the study. The high natural loads in the Iberian Peninsula must be taken into account when aerosol studies are carried out in this region.

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A STUDY OF THE DISTRIBUTION OF THE ANNUAL EXTREME DAILY PRECIPITATION TOTALS IN ATHENS, GREECE

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ABSTRACT

The objective of the study is to investigate the probability distribution function of the annual extreme daily precipitation totals over Athens province. In particular, the series of annual extreme daily precipitation totals at the National Observatory of Athens, for a 115-years period (1891-2005), is analyzed in order to define the appropriate probability distribution model. The candidate distributions are the 3-parameter Gamma distribution and the Generalized Extreme Value (GEV) or Jenkinson's distribution.

The various parameters of candidate distributions are estimated with the method of maximum likelihood, the method of moment estimator and the probability-weighted method. Chi-squared test is utilized for testing the fit of the two candidate distributions to the sample. The 3-parameter Gamma distribution is rejected, and several validation methods are utilized in order to decide whether the Gumbel or the Fréchet distribution fits better to the data.

KEYWORDS: Extreme rainfall, 3-parameter gamma distribution, generalized extreme value distribution, Athens.

INTRODUCTION

The enhancement of the greenhouse effect is expected to generate serious climatic changes. Precipitation trends may provide an additional indicator in the evaluation of climatic change. Precipitation variability in space and time is one of the most relevant characteristics of Mediterranean climate. Moreover, extreme precipitation events in this part of Eastern Mediterranean have significant environmental consequences which cause considerable damages in urban as well as in rural areas.

Many researchers have carried out studies on precipitation variability in Greece [1-7]. The incidence and the time and space variability of monthly and annual precipitation in Athens province has been studied by a number of researchers using precipitation data measured at the National Observatory of Athens (NOA) [4, 8-12].

Analyses of changes in extreme precipitation have used parametric methods. The parametric methods usually involve fitting a suitable distribution to the data and estimating distribution's parameters. Various studies have been carried out on frequency distributions of extreme precipitation events. In many cases, the Gamma distribution is considered to be the most suitable [10, 13-15], nevertheless, in some other studies, the Fréchet or the Inverse Gaussian distributions appear to be better [16]. Houssos and Bartzokas [6] have studied extreme precipitation events in NW Greece from a meteorological point of view. In this paper, we consider as "extreme daily precipitation value of a year" the maximum value of the daily precipitation totals of that year.

The aim of this study is to find the best fitted probability model to the annual extreme precipitation totals over Athens province in order to assess the corresponding results to the future climate models findings. In particular, 115 values time series of the longest available record of annual extreme daily precipitation totals in Greece, at the NOA, which is located near to the centre of Athens ($\lambda = 23^0\ 43'$ E, $\varphi = 37^0\ 58'$ N, h = 107 m a.m.s.l.), for the period 1891-2005, is analyzed in order to define the appropriate probability distribution function for that region.

RESULTS AND DISCUSSION

The statistical analysis technique, we shall use, depends on the properties of the annual extreme daily precipitation totals series $x_1, x_2, ..., x_n$. Therefore, the series must be examined thoroughly.

In order to examine the time series data for any possible periodicity, auto-correlation technique was applied. The Mann-Kendal trend analysis [17] was applied in order to reveal a trend in time series and its significance. Moreover, we utilize the following none parametric techniques for testing hypotheses concerning some characteristics of the investigated time series. The change-point test is testing the "null" hypothesis of the homogeneity of the data, and the use of run tests below and above the median test, tests the "null" hypothesis that the data are independent and identically distributed. All the above statistical tests used are in level of significance a = 0.05.

The analysis of the data showed that the maximum annual extreme daily precipitation total (189.5 mm) was observed during the year 1895, but the minimum one (17.2 mm) during the year 1898. The overall mean of the entire time series was found to be 49.88 ± 26.82 mm. Moreover, the median value of the examined time series was 42.8 mm.

Figure 1 shows the auto-correlation function and the maximum time lag is 23 years. This correlogramm does not present any periodicity.

Figure 2 shows the time series of the annual extreme daily precipitation totals during the investigated period. The applied Mann-Kendal trend analysis [17] showed that a statistically insignificant decreasing overall trend is evident (b-coefficient = -0.0688 mm/year, significance level = 0.213).

The change-point test that we applied does not reject the hypothesis of homogeneity of the data (|z| = 1.78<1.96). The use of test runs below and above the median test does not reject the "null" hypothesis that the data are independent and identically distributed (|z| =0.922<1.96).

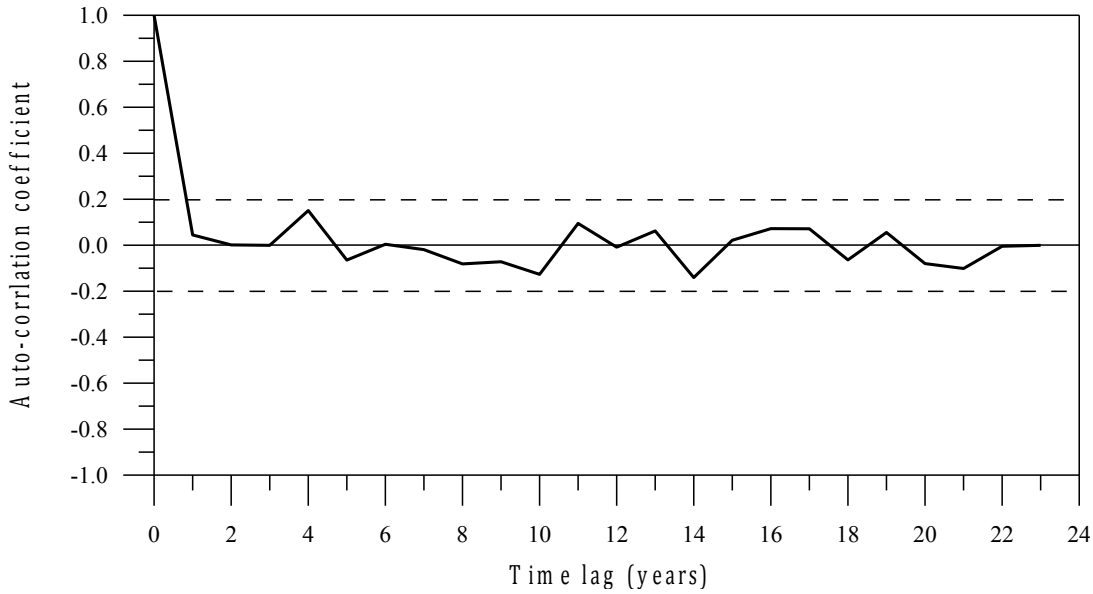

FIGURE 1 - Correlogram of the annual extreme daily precipitation totals at NOA for the period 1891-2005. Dashed lines represent 95% confidence limits for the auto-correlation coefficient.

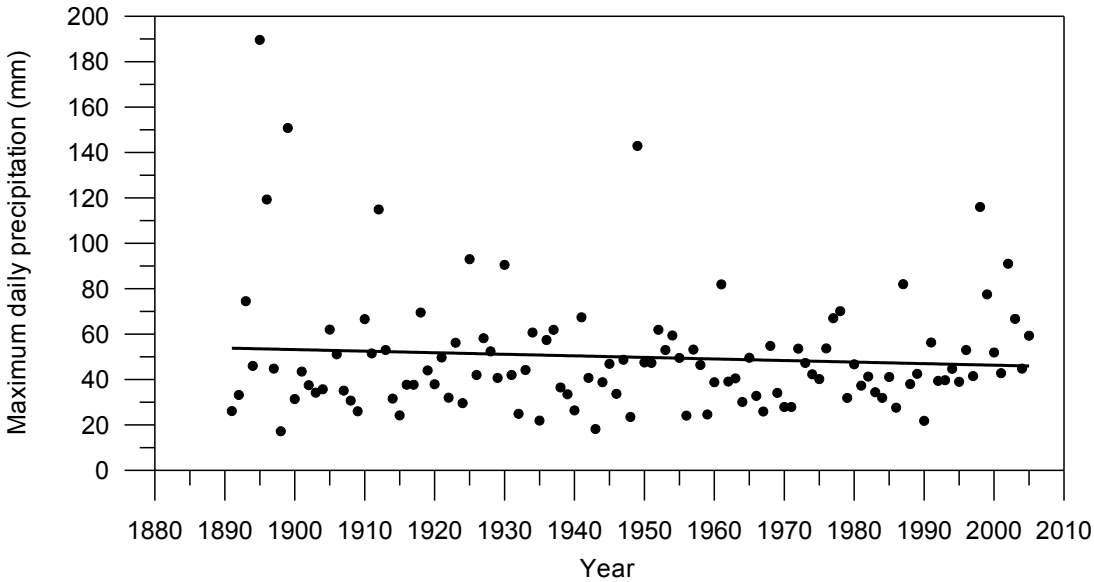

FIGURE 2 – Time series of the annual extreme daily precipitation totals at NOA for the period 1891-2005. The straight line (y = a + bx) is the least square fit.

Thus, we can claim that the time series under consideration shows no auto-correlation, does not exhibit any trend and, moreover, the data are independent observations of the same distribution. Therefore, we can proceed in fitting the data with the following candidate distributions.

THE CANDIDATE DISTRIBUTIONS

The Jenkinson distribution

Jenkinson's distribution is considered as distribution of the extremes [18-19] and is given by the relation (1).

$$G(x) = \Pr[X \le x] = \exp\{-[1+\alpha(\frac{x-\gamma}{\beta})]^{-1/\alpha}\} \quad (1)$$

where the support is the set $\{x : 1 + a(x-\gamma)/\beta > 0\}$ and $-\infty < \gamma < \infty$, $\beta > 0$, $-\infty < \alpha < \infty$.

The GEV distribution includes the Fréchet, the Weibull and the Gumbel distribution if a>0, a<0, or a=0, respectively.

The 3-parameter Gamma distribution

The probability density function of the 3-parameter Gamma distribution is

$$f(x;\alpha,\beta,\gamma) = \frac{(x-\gamma)^{a-1}}{\beta^{\alpha}\Gamma(\alpha)}\exp\{-\frac{(x-\gamma)}{\beta}\} \quad x > \gamma, \beta > 0, \gamma > 0 \quad (2)$$

This distribution was proposed for fitting to the logarithms of flood flow peaks by the US Water Research Council [20].

We will try to fit this distribution to the annual maximum precipitation totals as well as the logarithmic values of this. Bobee [21], instead of fitting the above distribution to the logarithmic values, attempted to fit the original data to the Log-Pearson type 3 distribution.

ESTIMATIONS

Several methods have been proposed for the estimation of the unknown parameters. The distributions parameters are estimated with the use of maximum likelihood method, the method of moments, as well as the method of probability-weighted moments. Provided it exists, the maximum likelihood estimator (MLE) is preferred because of its good asymptotic properties. Some alternative ways are the method of moments and the weighted method of moments.

Estimation for the Jenkinson distribution

The MLE demands the solution of non-linear system of three equations with three unknown parameters. The estimation of the parameters are treated separately for $\alpha = 0$ and $\alpha \neq 0$.

The MLE is the solution of the system of the log likelihood equations, and it can be found with numerical techniques only. Hosking [22] and Macleod [23] have provided the Fortran subroutine MLEGEV, that facilitates the calculation of the MLEs. When the subroutine does not converse, then either α is close to zero or the MLE does not exist. The existence of the MLE is the major concern here.

Smith [24] proved that when α<-1 the MLE does not exist, while when –1<α<-1/2, there may be problems. The regularity assumptions hold for α>-1/2, however, when α> 1/2 the second and higher moments do not exist. Fortunately, the condition $-1/2 < \alpha < 1/2$ is satisfied almost always.

When the MLE does not exist, then we can use the method of probability-weighted moments (PWM) [25].

Apart of the possible loss of efficiency compared to MLE, another drawback of the PWM is that it can lead to infeasible estimates in the sense that the estimated distribution has an upper bound, which is exceeded by some data values [26].

The MLEGEV algorithm converges to $\hat{a}$= 0.266 with a confidence interval (-0.00545, 0.537). Since the confidence interval includes the value α=0, the case α= 0 (Gumbel distribution) must be treated separately. Table 1 illustrates the results extracted by the application of MLE method on the original data for the GEV and the Gumbel distribution models.

TABLE 1 - Results from the GEV and the Gumbel distribution models for the estimation of α, β and γ parameters by the application of MLE method.

Method (distribution model)	$\hat{\alpha}$	$\hat{\beta}$	$\hat{\gamma}$
MLE GEV	0.266*	13.403	37.230
MLE Gumbel		16.725	39.972

(*): 95% confidence interval of α is (- 0.00545, 0.537).

Estimation for the 3-parameter Gamma distribution

The parameters α, β and γ of the 3-parameter Gamma distribution are estimated with the MLE, as well as with the method of moment estimator (MME). Table 2 illustrates the results extracted by the application of MLE and MME methods on the original data, as well as to the logarithmic values of the data.

TABLE 2 - Results from the 3-parameter Gamma distribution model for the estimation of α, β and γ parameters by the application of MLE and MME methods.

Data	Method	$\hat{\alpha}$	$\hat{\beta}$	$\hat{\gamma}$
For the original data	MLE	2.106	16.031	16.114
	MME	33.071	0.651	28.319
For the logarithmic values of original data	MLE	16.034	0.105	2.126
	MME	0.143	8.941	2.519

It is obvious that the MME method gives unacceptable estimators because, for the original data, we have $\hat{\gamma}$ greater than the minimum value of the sample, whereas for the logarithmic values, we obtain $\hat{\alpha} < 1$. The latter case is unacceptable because for $\hat{\alpha} < 1$ the distribution has the shape of reversed J distribution, which is something that does not agree with the histogram of the data (Figure 3).

Goodness of fit

The Chi-squared test is used for testing the goodness of fit of the candidate models. Table 3 illustrates the results

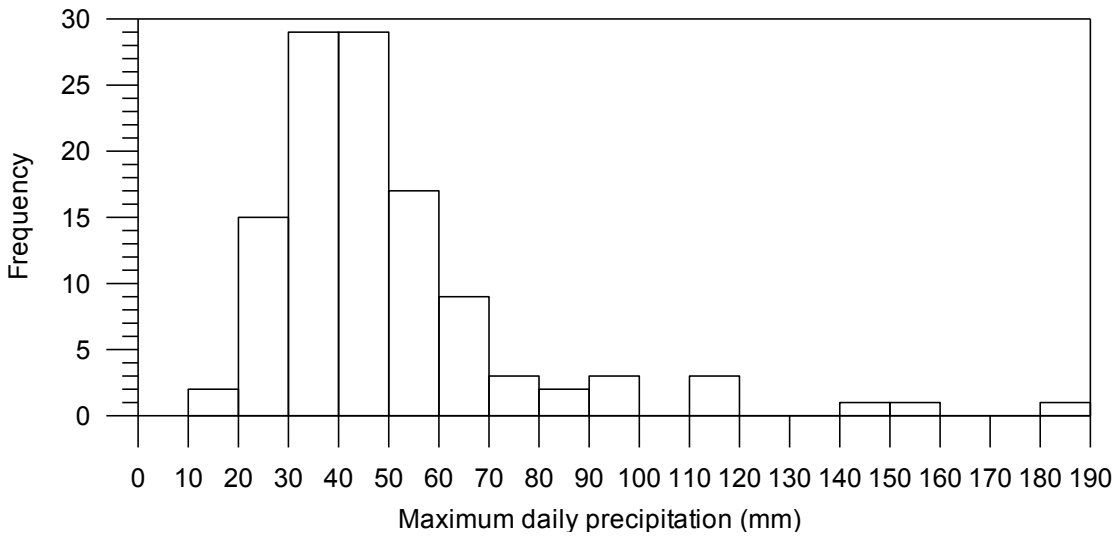

FIGURE 3 - Histogram of the annual extreme daily precipitation totals at NOA for the period 1891-2005.

TABLE 3 - Results of Chi-squared test by the application of goodness of fit for each distribution model.

Distribution model	χ^2	p-value
3-parameter Gamma for the logarithmic values	15.3185	0.0090
3-parameter Gamma for the original data set	9.5973	0.0825
GEV (Fréchet)	3.6928	0.5944
GEV (Gumbel)	4.4890	0.4813

extracted by the application of goodness of fit for the used models with the application of the above test.

As we can see from the p-values, the GEV distribution models fit better compared to the Gamma distribution ones, and the problem focuses on the type of the Jenkinson distribution (Fréchet or Gumbel). The following tests have been proposed for testing the "null" hypothesis $H_0 : \alpha = 0 \; vs \; H_\alpha : \alpha \neq 0$.

Likelihood ratio (LR) test [27], a modification of the likelihood ratio (LR*) test [28], and the Wald (W) test [29] are utilized for testing the hypothesis that the shape parameter of the Jenkinson distribution is zero. In this way, we discriminate between the Gumbel and Fréchet distributions.

All the above-mentioned tests are asymptotically equivalent as well as optimal, which under H_0 follow the X_1^2 distribution. Table 4 illustrates the results of the application of the above three tests.

TABLE 4 - Values of the test statistics.

LR	LR*	W
0.62	0.61	0.41

A comparison with the critical value 3.84 from the X_1^2 tables shows that the "null" hypothesis cannot be rejected at level of significance α = 0.05.

SOME VALIDATION METHODS

The P-P plot

The validation methods used for supporting our results are the P-P plot, the mean excess function and the return period.

The usual validation method for the adequacy of a model is the P-P chart. This plot consists of the points

$$\left\{ \left(\hat{F}(x_{(i)}), \frac{i}{n+1} \right) : i = 1, \ldots, n \right\},$$

where $\hat{F}$ is the estimated distribution. The closer the points to the diagonal, the better the fit of the distribution to the data.

The correlation coefficients r for Gumbel and Fréchet distribution are 0.920 and 0.997, respectively. Both correlation coefficients are very high. A more careful look at the P-P plots (Figure 4) for the Gumbel distribution reveals that the lack of fit corresponds to the large values of the sample. In other words, any possible discrimination between these two models will occur because of the different behavior of the distributions at the tails. This will become more apparent when we will study the return period.

Mean excess plot

The mean excess *e(y)* is given by the formulae (3)

$$e(y) = E(X - y \mid X > y) = \frac{\sigma + \alpha y}{1 - \alpha} = \frac{\sigma}{1 - \alpha} + \frac{\alpha}{1 - \alpha} y \qquad (3)$$

From this expression, we have that if the annual precipitation data follow the GEV distribution, then the *e(y)* function must be linear in *y*.

In practice, the mean excess function is estimated by

$$\hat{e}_n(a) = \frac{\sum_{i=1}^{n} x_i I_{(a,\infty)}(x_i)}{\text{number of } x_i > a} - a \qquad (4)$$

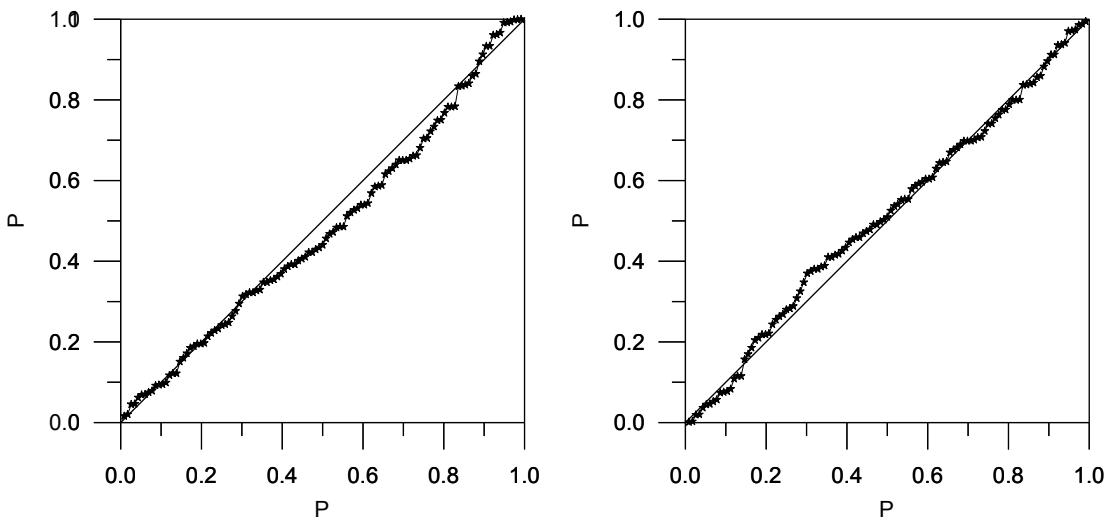

FIGURE 4 - P-P plots for the Gumbel distribution (left panel) and for the Fréchet distribution (right panel).

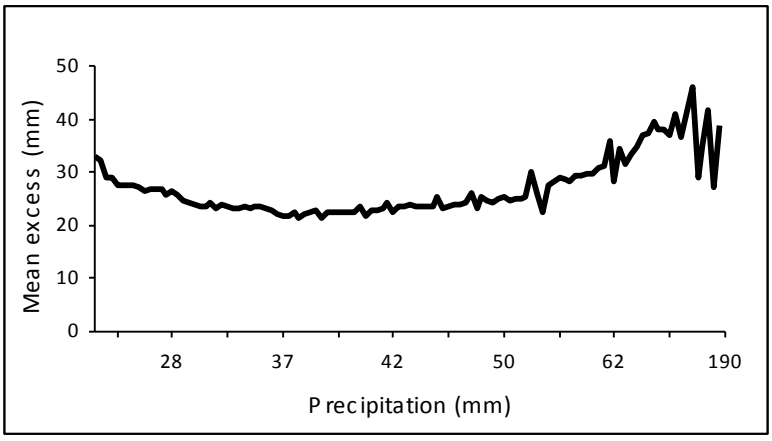

FIGURE 5 - Mean excess function.

The empirical function $\hat{e}_n(a)$ is plotted at the values $\alpha = x_{(n-k)}$ (k=1,…, n-1) which is the (k+1)-largest observation.

As we can see from Figure 5, the excess function is almost constant. In fact, the slope is 0.095. From the slope of the graph, we can have a quick estimate of the shape parameter α. Indeed, from the equation $\frac{\alpha}{1-\alpha} = 0.095$, we can obtain $\alpha = 0.102$. This value of α is another indication that the Gumbel distribution is a serious candidate model for the extreme precipitation events.

Return period

If F(x) is the cumulative distribution function of the extreme daily precipitation totals per year X, the return period r(t) of the event {X>t} is $r(t) = \frac{1}{1-F(x)}$ years, and it represents the expected number of years for the event {X>t} to occur for first time.

Table 5 illustrates the results from return period for Fréchet and Gumbel distribution models, and the corresponding return levels. As we can see from Table 5, the return period for the Gumbel distribution increases in an unacceptable level.

TABLE 5 - Return period values for Fréchet and Gumbel distribution models.

Return level (mm)	Return period (years)	
	Fréchet	Gumbel
50	2.87	2.36
100	21.44	36.7
150	83.27	720
180	156.79	4326

This difference in the behavior will become clearer with the help of the return level plot. With the use of Delta method, we can construct the corresponding 95% confidence intervals. Figure 6 reveals the return level plots and 95% confidence interval for Gumbel and Fréchet distributions.

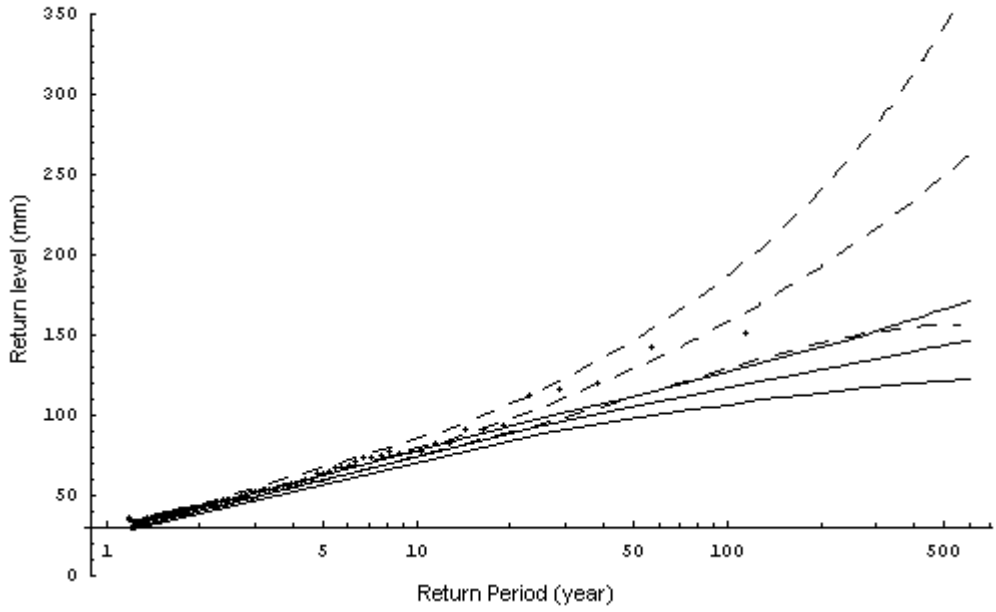

FIGURE 6 - Return period (years) and 95% confidence interval for Gumbel (solid lines) and Fréchet (dotted lines) distributions.

As we can see, the 95% confidence interval for the Gumbel distribution return level is shorter compared to that for the Fréchet distribution and, therefore, it extrapolates some points.

CONCLUSIONS

All the tests and the validation methods agree that the extreme daily precipitation totals per year in Athens during the 115-years period (1891-2005), follow the GEV distribution.

However, we obtain for the shape parameter α that it is close to zero and we cannot distinguish between Gumbel or Fréchet distribution, because none of the statistical tests rejects the Gumbel model. The Gumbel distribution gives shorter confidence intervals, which means that it is more accurate. However, the excessive values observed in Athens, three to four times that of the mean annual extreme daily totals, as well as the devastated events that took place in Venezuela in 1999, where in one day the precipitation amounts were at least three times higher than any value recorded before [30-31], make us not reject the Fréchet distribution so easily. Future climate models could be based on the outputs of the suggested distribution model.

The decision is subjective. If we want to be more accurate in our predictions, we should choose the Gumbel distribution. However, the Gumbel distribution model extrapolates some values (Figure 6), and especially the largest values of the sample. This deviation between the Gumbel model and the large values of the sample is also shown in Table 5. If we want to be more conservative in our decisions, we should choose the Fréchet distribution.

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AFLATOXIN M1 LEVEL OF HUMAN BREAST MILK SAMPLES IN SAMSUN AND NEIGHBOUR PROVINCES, TURKEY

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ABSTRACT

Aflatoxins are a group of toxic compounds produced by certain strains of the fungi *Aspergillus flavus* and *A. parasiticus*. This study was carried out to determine the AFM1 levels of human milk samples in Samsun province, Turkey. The breast milk samples were obtained from 50 healthy lactating women whose ages ranged between 18-38 years. Samples were analysed for AFM1 by microtitre plate enzyme-linked immunosorbent assay (ELISA), and 33 mothers (66%) were found to be contaminated with AFM1. Contamination levels were ranging from 0.38 to 9.43 ng/L (median 4.26±0.38 ng/L). AFM1 contamination levels of all samples were lower than the maximum tolerance limits of European Commission, Codex Alimentarius and United States for infant and animal milk, for human consumption. The study showed that whereas AFM1 levels were found to be low, contamination was found high in breast milk samples of mothers living in Samsun province. Although it seems that the mother's milk is not at risk for aflatoxins, adverse effects of AFM1 in exposed infants and children should be considered. Therefore, routine monitoring of AFM1 should be performed and the study must be extended to both mothers and infants of the other cities in Turkey.

KEYWORDS:
Aflatoxin M1, breast milk, ELISA

INTRODUCTION

Aflatoxins are a group of toxic compounds produced by certain strains of the fungi *Aspergillus flavus* and *A. parasiticus*. Food and feed contamination with aflatoxins is a great problem worldwide, because they are highly toxic, mutagenic, teratogenic and carcinogenic for animals and humans, especially for children. The major aflatoxins of concern are designated as B1, B2, G1 and G2. These toxins are usually found together in various foods and feeds in varying proportions. Aflatoxins M1 (AFM1) and M2 are oxidative metabolic products of aflatoxin B1 (AFB1) and B2, respectively, produced by animals and usually excreted into the milk, urine and faeces of dairy cattle and other mammalian species that have consumed aflatoxin-contaminated food or feed [1, 2].

The toxicity of AFM1 is about one order of magnitude less than that of AFB1. AFM1 could be detected in milk, 12-24 h after the first AFB1 ingestion. When the intake of AFB1 is finished, the AFM1 concentration in the milk decreases to an undetectable level after 72 h. Also, humans could be ingesting AFM1 directly from dairy products. AFB1 and AFM1 were classified by the International Agency for Research on Cancer (IARC) as human carcinogens class 1 and 2B, respectively [3, 4].

Foetal and early childhood environments are considered critical for growth and disease risk in later life. Breast milk, which contains immunoglobulins, is the best food for babies; but, mothers must have good nutritional status and good food during pregnancy and lactation period, because various xenobiotics, such as aflatoxins, may be very harmful for mothers and their babies [5, 6].

This study was carried out to determine the AFM1 levels of healthy lactating women breast milk by enzyme-linked immunosorbent assay (ELISA), and evaluate the potential risk for mothers and newborns from AFM1 ingestion in Samsun province, Turkey.

MATERIALS AND METHODS

Samples and Instruments

Samples of breast milk were obtained from 50 healthy lactating women, between 18-38 years old. The mothers have been living in Samsun and neighbouring cities. Approximately 30 ml milk was collected from each mother in coloured glass bottles. The samples were stored at -20 ^{o}C until analysis. ELISA was performed on a Digital and Analog Systems microplate reader (Rome, Italy). A Nuve NF800 R Refrigerated Centrifuge (Ankara, Turkey) was used for centrifugation of the samples.

Determination of AFM1 by ELISA

Milk samples were centrifuged (10 min at 3500g and 10 °C) for degreasing. After centrifugation, the upper cream layer was removed completely by aspiration through a Pasteur pipette. Skimmed milk (defatted supernatant) was used directly in the test (100 µl per well).

RIDASCREEN Aflatoxin M1 ELISA kits (R-Biopharm AG, Darmstadt, Germany) were used to determine AFM1. The samples were analysed as described by the manufacturer. The basis of the test was the antigen-antibody reaction. The wells in the microtiter strips were coated with specific antibodies to AFM1. The measurement was made photometrically at 450 nm and absorption was inversely proportional to the AFM1 concentration in the sample.

RESULTS AND DISCUSSION

AFM1 was detected in 33 samples (66%), and contamination levels were ranging from 0.38 to 9.43 ng/L ($\bar{X} \pm S\bar{x}$ = 4.26±0.38 ng/L). Mostly, AFM1 levels were found between 5 and 10 ng/L (16 samples, 32%). AFM1 levels of 17 samples (34%) were determined to be lower than 5 ng/L (Table 1).

TABLE 1 - AFM1 concentrations in breast milk samples.

AFM1 level (ng/L)	*n*	%
Not detected	17	34.00
<5	17	34.00
5-10	16	32.00
Total (*n*)	50	100.00

Distributions of AFM1 contamination among the breast milk samples obtained for different cities are shown in Table 2. The highest mean concentrations were seen in Ordu and Samsun, 4.95 and 4.90 ng/L, respectively. Maximum AFM1 level (9.43 ng/L) was detected in Ordu.

TABLE 2 - Distributions of AFM1 contamination of breast milk among cities in Turkey.

City	Age	*n*	Detected samples	Range (ng/L)
Samsun	18-37	22 (44%)	15	0.71-7.24 (4.90±2.63)
Ordu	22-37	10 (20%)	6	2.23-9.43 (4.95±0.91)
Amasya	23-38	6 (12%)	3	1.27-5.12 (3.65±0.83)
Sinop	23-27	5 (10%)	5	0.38-3.89 (2.23±1.30)
Tokat	22-32	2 (4%)	0	-
Others	20-30	5 (10%)	4	0.38-5.73 (3.85±0.81)

Although the detection limit of the test kits was 5 ng/L, the used ELISA Reader Instrument was calculating these values according to the standards (0, 5, 10, 20, 40, and 80 ng/L). Because of this, the results, which are lower than 5 ng/L, were accepted to be positive. Furthermore, we could see colour changing of the micro-wells in the ELISA plate. Also, similar with our study, Sadeghi *et al.* [7] determined AFM1 level to be lower than 5 ng/L, with the same test kits and method.

The European Commission, Codex Alimentarius and United States prescribe a limit of 25 ng/kg AFM1 for infant milk. The maximum level of AFM1 is 10 ng/kg for infant milk in Austria and Switzerland [7]. In addition, the European Commission and Turkish Government have set limits for maximum AFM1 as 50 ng/L for animal milk [8, 9]. In the present study, AFM1 contamination levels of all samples were lower than all the accepted maximum tolerance limits.

Aflatoxin contaminations and levels of human breast milk samples have been determined in various studies from 1980's. Wild *et al.* [10] reported development, validation and application of an ELISA to the detection of AF in human breast milk. Fifty-four samples were collected from women in rural villages in Zimbabwe and 6 samples (11%) were found to be positive for AF in ELISA with levels up to 50 pg/ml. However, no positive samples were detected out of 42 samples obtained from France.

Various contamination rates and concentrations of AFM1 were reported in different studies. For example, AFM1 contamination was determined in 99.5% of 445 milk samples (from 2 pg/ml to 3 ng/ml) in Abu Dhabi, UAE [11]; 24 of 124 breast milk samples from Bangkok and Khon Kaen (median 20 and 23 ng/L, respectively) in Thailand [5]; 36% of 388 milk samples (median 13.5 pg/ml) in Egypt [12] and 157 of 160 human breast milk samples (median 8.2 ng/kg) in Tehran, Iran [7].

Aflatoxins have been implicated in human diseases including liver cancer, Reye's syndrome, Indian childhood cirrhosis, chronic gastritis, kwashiorkor and certain occupational respiratory diseases in various parts of the world [4].

Abdulrazzaq *et al.* [1] investigated the correlation between neonates' morbidity and mothers' aflatoxin ingestion. AFM1 concentrations in newborn infants correlated with those of their mothers, and it was determined whether the presence of AFM1 in cord blood was associated with an increase in morbidity in the newborns. A strong correlation was found between mothers' and cord blood levels of aflatoxin (unlike most reports). Also a strong negative correlation was determined between aflatoxin levels and birth-weight but no association was seen between AFM1 levels in maternal or cord blood, and rates of jaundice or infection.

Also, Hassan *et al.* [13] firstly reported on the presence of aflatoxin in both Egyptian mothers' milk and their infants' sera, correlated with infants' kidney function. Fifty healthy breast lactating mothers and their infants were investigated in this study. In conclusion, aflatoxin contamination was detected in 24 mothers (48%) and their infants (8.9±4.2 ng/ml in mothers' serum, 1.9±0.6 ng/mL in moth-

ers' milk, and 1.8±0.9 ng/mL in infants' serum). The authors reported that mothers and their infants showed a relatively high aflatoxin contamination rate, but the contamination level was not associated with renal or hepatic dysfunction.

In this study, AFM1 levels were relatively lower than those of other studies. Also, AFM1 concentrations were found to be lower than the levels considered to be safe for infant and animal milk, consumed by humans. However, in spite of the low AFM1 concentration level, the contamination range (66%) might be considered as high. Fungi, responsible for mycotoxins, produce them when between 24-35 °C, when moisture content exceeds 7% (10% with ventilation) [4]. Due to high rain fall levels and high humidity, climatic conditions of Samsun province are similar to the above-mentioned ones, and Samsun and neighbouring cities are very suitable for aflatoxin formation. Also, consumption of foods risky for aflatoxins, such as maize, wheat, other cereals and their products, is very high in this region of Turkey [14].

CONCLUSIONS

Our findings should have been considered carefully, because infants and children are exposed to aflatoxins through breast but also animal milk, food and/or foodstuff. Children are more susceptible than adults to effects of mycotoxins, because of their lower body weight, higher metabolic rate, lower ability to detoxify and incomplete development of some organs and tissues, notably the central nervous system [15]. Furthermore, concerning these adverse effects of such an exposure on immunity, liver function, and nutrition, in controlled experiments with pigs these possible effects of aflatoxin exposure for the human foetus and infant have been examined [16]. In addition, for genotoxic carcinogenic substances, such as aflatoxins, a threshold value, below which the risk for human health is equal to zero, does not exist. Hence, toxicological importance of aflatoxins in maternal milk should not be overlooked [12].

In conclusion, the study showed that at low AFM1 levels, contamination was high in breast milk of mothers who live in Samsun province. To our knowledge, this is the first report on AFM1 levels in Turkish human breast milk samples, so there are no other data available to support our results. Although, it seems that the mother's milk is not at risk for aflatoxins, adverse effects of AFM1 exposure in infants and children should be considered. Therefore, routine monitoring of AFM1 should be performed and the study must be extended to both mothers and their infants in the other cities of Turkey. Furthermore, the people should be educated and informed about the hazards of contaminated foods.

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THE ROLE OF CITRIC ACID IN ENHANCED PHYTOEXTRACTION OF HEAVY METALS IN AN ANDOSOL BY *Crotalaria juncea*

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ABSTRACT

A pot experiment was conducted to compare natural versus citric acid-assisted phytoextraction of heavy metals and their distribution in fractionated root zone soils using *Crotalaria juncea.* Seedlings of *C. juncea* were grown in an Andosol containing total concentrations of 9.2, 156.3, and 1480 mg kg^{-1} Cd, Zn, and Mn respectively, and 20 mmol kg^{-1} of citric acid were added to the pots. After 25 days of growth, the plants were harvested, and soil samples were fractionated into rhizoplane (RP), rhizosphere (RZ), and bulk soil (BK). *C. juncea* exhibited a high bioaccumulation factor (2.04) as well as a high ability for hyperaccumulation of Cd. In comparison, the plant was less able to accumulate Zn and Mn in the shoots. The pH in the soil fractions followed the series: RP<RZ<BK. *C. juncea* decreased the concentration of 0.1 M HCl extractable heavy metals in BK but increased them in RP. Higher HCl extractable metals in RP may be associated with lower pH. Citric acid showed the greatest capacity in desorbing heavy metals in the Andosol compared to other organic acids such as oxalic, malic, and tartaric acids. The addition of citric acid did not significantly change the concentrations of Cd and Zn in roots and shoots hereas the concentration of Mn in the roots and shoots increased about 2- and 4-fold, respectively. The effect of citric acid on the phytoextraction of Mn probably corresponded to the increased mobility caused by citric-manganese complexation.

KEYWORDS: Phytoextraction, *Crotalaria juncea*, Andosol, heavy metals, citric acid, cadmium.

INTRODUCTION

Contamination of ecosystems with toxic heavy metals is a serious environmental problem. Among the heavy metals, zinc (Zn), manganese (Mn), copper (Cu) and iron (Fe) are essential nutrients for living organisms. However, the concentration of heavy metals in contaminated soil frequently elevates those required as nutrients and may cause toxicity of the plant and soil microorganisms. On the other hand, cadmium (Cd), chromium (Cr), lead (Pb) and mercury (Hg) are important heavy metals that have no known biological function [15]. Concern about Cd and its toxicity for the ecosystem and human health has grown over the recent decades. Cd is mainly emitted to the environment via mining and smelting of zinc and cadmium ores, metallurgical industries, and phosphatic fertilizers. Thus, to ensure sustainable land management, metal-contaminated soil needs to be remediated. Phytoremediation is an agronomy-based technology and categorized into five techniques in which phytoextraction (use of plants to remove target contaminants, e.g., Cd, Cr, and Zn, from the soil) has attracted attention in recent years due to the low cost of implementation and environmental benefits [18, 11]. Research regarding increasing the efficacy of the phytoextraction technique has emphasized on the use of high-biomass-producing plant species with enhanced accumulation of heavy metals. Recently, *Crotalaria juncea* has been documented as a Cd hyperaccumulator and tolerant in hydroponics culture [19]. *C. juncea* is a rapidly growing crop that is extensively cultivated for fiber production and as a green manure in many tropical and subtropical areas in the world. *C. juncea* is also used as a cover crop to suppress weed populations as well as to slow soil erosion. Growing of *C. juncea* for 9 to 12 weeks will produce 5.9 t ha^{-1} dry-matter and 126 kg N ha^{-1} [14]. The plant also adapts to a wide range of soil types. The ability of this plant to remediate Cd and other heavy metal-contaminated soil remains to be investigated. Since the plant yields considerable biomass, enhanced phytoextraction (using biodegradable chelators such as organic

acids) is a suitable alternative. Among organic acids, citric acid has been reported for its high ability to extract Cd compared to other organic acids [12, 13]. The objectives of this study were to examine the ability of *C. juncea* in phytoextraction of Cd, Zn, and Mn, and to determine changes in pH and HCl extractable heavy metals in the fractionated root zone soil (rhizosphere and rhizoplane). In addition, the authors also evaluated the effectiveness of citric acid in the enhanced accumulation of these 3 heavy metals in Andosol.

MATERIALS AND METHODS

Soil samples

The soil samples for the present experiment were collected from the surface layer (A horizon; 0 to 10 cm in depth) of the Andosol in Fuchu Field Museum, Tokyo, and sieved through 2-mm mesh. The soil was previously amended with Cd nitrate and incubated for one year. The soil samples were considered initial bulk (BKi), air-dried, and stored for subsequent physical and chemical analysis. The pH of the soil in water (1:2.5) was measured with a glass electrode (Model D-52, Horiba Ltd., Kyoto, Japan). Soil electrical conductivity (EC) was also measured using an EC meter (model CM-30 V, TOA Electronics Ltd., Japan) after extraction with distilled water (1:5, W/V). The total C and N content of the soil was measured using the dry combustion method with an NC analyzer. The general characteristics of the soil are shown in Table 1.

TABLE 1 - Selected chemical properties of the soil sample.

Properties	Value
pH (H_2O)	6.23
EC (mS m^{-1})	25.40
CEC ($cmol_c$ kg^{-1})	29.20
Total C (g kg^{-1})	66.20
Total N (g kg^{-1})	3.50
Total Cd^{2+} (mg kg^{-1})	9.20
Total Zn^{2+}(mg kg^{-1})	156.30
Total Mn^{2+} (mg kg^{-1})	1480.0

Heavy metal desorption by organic acids

To evaluate the effectiveness of organic acids in desorbing heavy metals (Cd, Zn, and Mn) from Andosol, four organic acids were applied and their desorbing ability was assessed. Approximately 100 mmol L^{-1} extracting solutions of citric, oxalic, malic and tartaric acids were prepared. The original pHs of the organic acids solution were 2.03, 1.29, 2.09, and 1.95 for the citric, oxalic, malic, and tartaric acids, respectively. One-gram soil samples were placed in centrifuge tubes, and 5 ml of the desorbing solutions were added. The tubes were capped and shaken for 1 h on a reciprocator at 200 rpm and then centrifuged. The supernatants were filtered through filter paper (pore size 0.45 μm) and stored until analysis.

Growth cabinet experiment

C. juncea Seeds were grown in vermiculite and the seedlings transferred to pots filled with 200 g of air-dried soil. Each pot was watered to field capacity using distilled water. The pots were periodically weighed, and the soil was brought to field capacity. Twelve days after transplantation, the treatment pots were amended with 20 mmol citric of acid (0.7 grams of anhydrous citric acid in 50 ml of distilled water). The control and test plants were grown for 25 days in the growth cabinet supplied with fluorescent light with day/night temperatures of 28/25 °C. The locations of the pots under the lights were rotated every two days.

Plant harvesting

At the final stage of the growth period, whole plants were separated into roots and shoots (stems and leaves) followed by fractionation of the root zone soil into "rhizoplane soil" and "rhizosphere soil" after "bulk soil" was collected. The plant tissues were dried in an oven at 70 °C for 3 days and preserved before digestion. All soil samples including initial bulk, bulk, rhizosphere, and rhizoplane were air-dried and sieved through 0.5-mm mesh to make the particle size uniform [6, 16].

Analytical method

Dried plant tissues were finely ground using an electric mill. Approximately 0.2 g of plant materials were put in 75-ml digestion tubes followed by adding 1 ml of distilled water and 4 ml of concentrated H_2SO_4, and the digestion tubes were left overnight. The following day, the tubes were heated to 60°C and the temperature was gradually raised to 150°C for 4 hours after adding 2 ml H_2O_2 in every 2 hours. The digests were cooled and adjusted to a volume of 10 ml. The concentrations of heavy metals (Cd, Mn, Zn) in the soil were determined using 0.1 M HCl solution (soil: solution ratio 1:5) for more than 1 hour shaking time on a reciprocator at 200 rpm. The digests and soil extracts were filtered through filter paper (pore size 0.45μm) before analysis. The total amounts of the heavy metals were also determined using nitric-perchloric acid digestion. The concentration of metals in the plant tissues and soil extracts were determined using a Flame Atomic Absorption Spectrophotometer (FAAS; Hitachi Z-5010; Hitachi Ltd., Tokyo, Japan).

Statistical analysis

The pot experiment was set up in a completely randomized design with three replications. The dry weight of the roots, stems and leaves, and the concentration of the heavy metals in the soil fractions and plant tissues in the control and treatment groups were subjected to Fisher's comparison test at the $P < 0.05$ confidence level.

RESULTS AND DISCUSSION

Heavy metals uptake and translocation in *Crotalaria juncea*

The results of heavy metal accumulation in roots and shoots as well as the value of the shoot-to-root ratio of *C.*

TABLE 2 - Total concentration of heavy metals in soil, plant, bioaccumulation factors and shoot to root ratio for 25 days old *Crotalaria juncea* grown in Andosol.

Soil heavy metal		Concentration of heavy metal (mg kg^{-1})				$BF_{Shoot\ (S/T)}$	$BF_{Total\ (P/T)}$	S/R
		Soil (T)	Root (R)	Shoot (S)	Plant (P)			
Cd	Control	9.2	17.2	18.78	18.38	2.04	2.00	1.09
	Treatment	9.2	18.5	18.79	18.71	2.04	2.04	1.02
Zn	Control	156.3	45.8	39.27	40.99	0.25	0.26	0.86
	Treatment	156.3	56.0	41.10	44.77	0.26	0.29	0.73
Mn	Control	1480	78	20.67	35.73	0.01	0.02	0.26
	Treatment	1480	152	80.29	97.86	0.05	0.07	0.53

juncea in the control group are shown in Table 2. The concentrations of Cd, Zn, and Mn in the shoots were calculated as 18.78, 39.27, and 20.66 mg kg^{-1}, respectively. Since successful phytoextraction requires high accumulation of heavy metals in the shoots [3], the translocation factor, defined as the ratio of the shoot concentration to the root concentration (S/R), is used to evaluate the value of heavy metals translocation into the plants' shoots. The S/R ratios of Cd, Zn, and Mn were calculated as 1.09, 0.86, and 0.26, respectively, indicating that *C. juncea* was much more efficient at translocating Cd and Zn from the roots to the shoots than Mn. The larger S/R ratio of Cd and Zn is most likely due to efficient metal transporter systems [20] and probably the sequestration of metals in leaf vacuoles and apoplasts [9].

Screening of the ability of 7 cultivars of plant species on an accumulation of Cd (4.01 mg kg^{-1}, extracted with 0.1 M HCl) grown in Andosol revealed that the sugar beet has the highest ability for Cd hyperaccumulation compared to other species such as maize, *Brassica juncea*, and rice [7]. In the current study, the concentration of Cd in the shoots was nearly the same value as the sugar beet (21 mg kg^{-1} in the sugar beet versus 18.78 for *C. juncea*). The ratio of the total heavy metal content of the shoots to the roots indicates that 75%, 71%, and 43 % of the total Cd, Zn and Mn absorption were accumulated in the shoots, respectively. It is therefore concluded that harvesting only the shoots would offer a practical way to remove most of the Cd and Zn absorbed by *C. juncea*.

Bioaccumulation factor

The plant-to-soil-heavy-metal-concentration ratio or bioaccumulation factor (BF) was calculated for shoots as well as the whole plant according to the index used for screening plants and evaluating metal uptake [5, 17]. In metal excluder plants, the BF is normally less than 1, whereas in metal accumulators the factor is often greater than 1 [1, 21]. *Crotalaria juncea* exhibited a shoot BF of 2.04, 0.26, and 0.05 for Cd, Zn, and Mn, respectively, indicating that, except for Cd, the plants exhibited excluding ability for Zn and Mn upon growing in an Andosol (as shown in Table 2). The bioaccumulation factors of Cd-accumulating plants such as *Arabidopsis halleri* in moderately Cd-contaminated substrates range from 6 to 30 [4] and for the southern French population of *Thlaspi caerulescense* ranges from 3 to 73 [21]. In the current study, over the 25-day of growth period, *C. juncea* showed a high BF of cadmium in the moderately low Cd-contaminated Andosol. This high Cd accumulation in *C. juncea* indicates that an internal metal detoxification mechanism might exist in addition to the exclusion ability found for Mn and Zn [1].

The mechanism of Cd tolerance in *C. juncea* corresponded to the production of total soluble phenolics when exposed to 5 ppm of cadmium contamination in the hydroponics culture [19]. Further investigations with soils highly contaminated with cadmium may lead to more understanding of plant tolerance and its bioaccumulation factor. Assessing the potential of *C. juncea* for hyperaccumulation of other heavy metals is also a matter for further researches.

Phytoextraction potential

The phytoextraction potential of *C. juncea* was calculated based on the following equation [21]:

(% of soil Cd, Zn and Mn removed by *C. juncea*) = {(Plant metal concentration × Plant biomass) / (Total soil metal concentration × Soil mass per pot)} × 100

Crotalaria juncea extracted 1.1 % of the total soil Cd after growing on moderately low Cd-contaminated Andosol (shown in Table 3). In soils highly contaminated with Cd, *C. juncea* is estimated to have a superior BF and thereby a phytoextraction potential of more than 1.1. This would increase if a longer growth period of 25 days were considered.

The removal of Cd in a field experiment with a soil Cd concentration of 2 ± 0.4 mg kg^{-1} (extracted with 2 *M* HNO_3) ranged from 3 ± 2 g ha^{-1}a^{-1} by *Salix viminalis* to 179 ± 79 g ha^{-1}a^{-1} by *Thlaspi caerulescens* [8]. Cd removal from different levels of Cd-contaminated soil (0.51-23.92 mg kg^{-1} extracted with concentrated HNO_3) by *Calamagrostis epigejos* was also reported as between 20 and 275 g Cd ha^{-1}a^{-1} [10]. In the current experiment, based on the surface area of the pot, i.e., 50 cm^2 at the limited soil depth of 6 cm, over 4 continuous cultivations (100 days), *C. juncea* is expected to remove 143 g ha^{-1} Cd. On the other hand, a similar ability was not observed for Zn and

TABLE 3 - Heavy metal contents in soils and plants and phytoextraction potential for 25 days old *Crotalaria juncea* grown in Andosol treated with citric acid

Plot	Content of heavy metal (μg pot $^{-1}$) Plant Root	Shoot	Whole Plant	Soil ($\times 10^3$)	Phytoextraction potential (%)
Control	4.5	13.5	18	1.6	1.1
Treatment	3.5	10.5	14	1.6	0.87
Control	12	28	40	28	0.14
Treatment	10.5	23.5	34	28	0.12
Control	20	15	35	262	0.01
Treatment	29	46	74	262	0.03

Mn, which is clearly because of the low BF value and thereby low phytoextraction potential. Only 0.14% and 0.01% of the total soil concentration of Zn and Mn were taken up by the plants respectively.

Heavy metal desorption in Andosol

Among the organic acids tested, citric acid showed the greatest capacity in desorbing heavy metals (shown in Figure 1). Citric acid removed 16%, 6%, and 11% of the total Cd, Zn, and Mn from the Andosol respectively. Other chelates desorbed much smaller amounts of heavy metals compared with citric acid. Malic and tartaric acids had higher abilities in Cd and Zn desorption after citric acid whereas oxalic acid was the most effective chelate in Mn desorption.

Effect of citric acid on plant biomass

Amendment of citric acid stunted the growth of roots, stems, and leaves of *C. juncea* however, the plants survived until harvesting (shown in Figure 2). The reason is that the 20 mmol kg^{-1} of citric acid solubilized too much of the metals that could have stunted the biomass growth, a sign of phytotoxicity. However, the statistical analysis did not show significant differences between the treatment and control groups.

pH and EC changes in soil samples

Cultivation of *C. juncea* increased the pH value in the soil fractions both in the control and treatment groups. The pH levels in the control and treatment groups after plant cultivation followed the pattern bulk soil>rhizosphere soil>rhizoplane soil, yet more than the value recorded for initial bulk soil. The pH values in the rhizosphere and rhizoplane soils decreased from those of bulk soils by zero and 0.09 units, respectively, in the control group and by 0.04 and 0.24 units in the treatment group. The citric acid amendment lowered the pH minimally, compared with the control group. The pH differences in the rhizoplane soil of the treatment and control groups were statistically significant; however, no significant differences were observed in

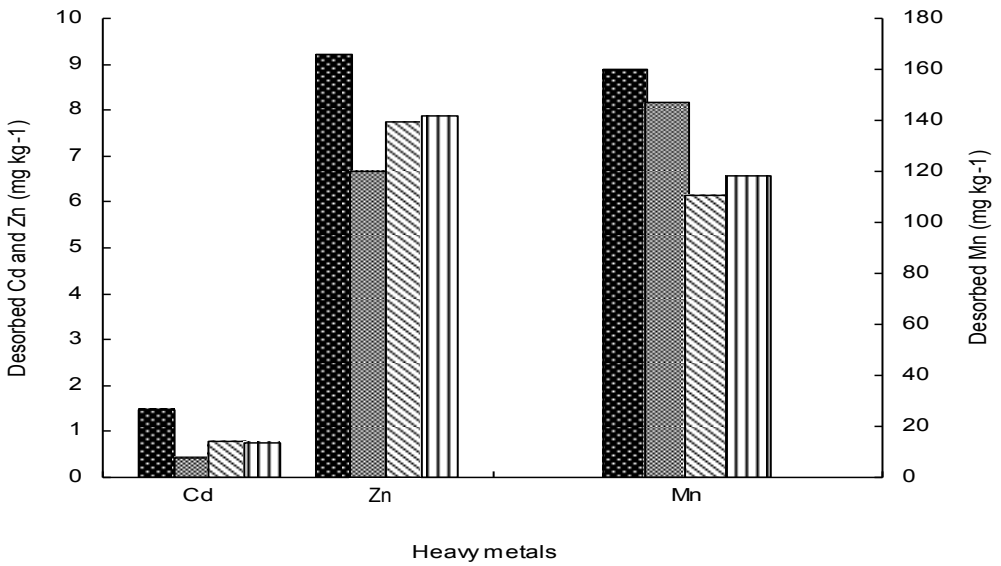

FIGURE 1 - The amount of heavy metals desorbed by organic acids (100 mmol) in Andosol

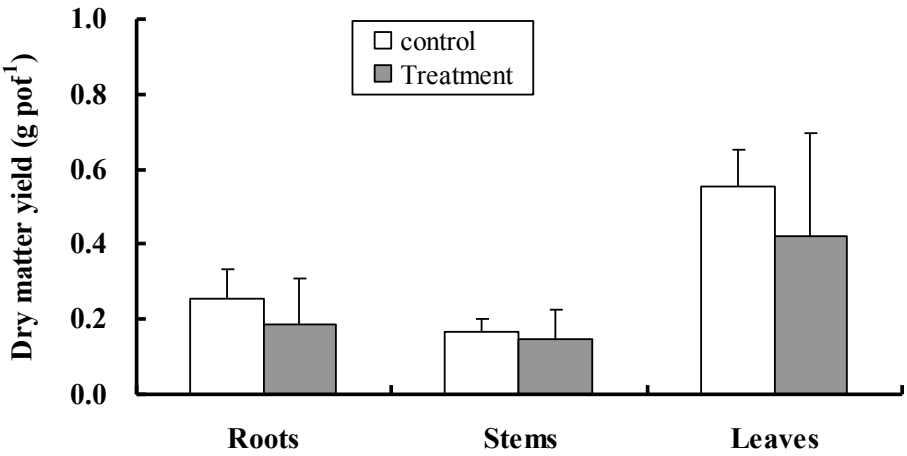

FIGURE 2 - Dry matter yield of *Crotalaria juncea* grown in Andosol amended with citric acid. Bars represent standard error.

TABLE 4 - Pre and post-growth pH, EC and concentration of water extractable nitrate in the soil fractions of *Crotalaria juncea* grown in Andosol amended with citric acid

Soil samples	pH (H_2O)		EC (mS m^{-1})		NO_3^- (mg kg^{-1})	
	Control	Treatment	Control	Treatment	Control	Treatment
Initial Bulk	6.23^a	6.23^a	25.40^a	25.40^a	455.67^a	455.67^a
Bulk	6.62^b	6.54^b	8.50^b	10.63^b	3.27^b	ND
Rhizosphere	6.62^b	6.50^{bc}	8.47^b	12.22^b	2.87^b	ND
Rhizoplane	6.53^b	6.30^{ac*}	16.39^c	28.40^a	ND	43.61^b

Values in a column followed by different letter have significant difference at $p < 0.05$ according to Fisher's test. Values denoted by * indicate significant difference between control and treatment at $p < 0.05$.
ND: Not detected

TABLE 5 - HCl (0.1 mol L^{-1}) extractable heavy metals of fractionated rhizosphere soil samples for *Crotalaria juncea* grown in Andosol amended with citric acid.

Soil samples	Heavy metals (mg kg^{-1})					
	Cd		Zn		Mn	
	Control	Treatment	Control	Treatment	Control	Treatment
Initial Bulk	6.37^a	6.37^a	21.73^a	21.73^a	62.89^a	62.89^a
Bulk	5.65^b	6.19^{a*}	21.50^a	21.51^{ab}	30.72^b	44.16^{b*}
Rhizosphere	5.82^{bc}	5.51^b	22.30^{ab}	23.98^b	35.12^{bc}	59.24^{ab*}
Rhizoplane	5.92^c	6.21^{a*}	25.51^b	30.42^{ab}	43.78^c	83.79^{ab*}

Values in a column followed by different letter have significant difference at $p < 0.05$ according to Fisher's test. Values denoted by * indicate significant difference between control and treatment at $p < 0.05$.

the bulk and rhizosphere soils following citric acid utilization. As shown in Table 4, since NO_3^- accumulated in the rhizoplane soil of the treatment group, it is likely that less effectiveness in NO_3^- uptake of the treatment group may have led to a significant decrease in the pH value of the RP soil compared with the control group. Plant cultivation also decreased the EC value in all soil fractions except the rhizoplane in the treatment group which had a greater amount than the initial bulk soil. The EC levels after plant growth followed the pattern rhizoplane soil>rhizosphere soil>bulk soil in both the treatment and control groups. Citric acid amendment induced a higher EC value in the bulk, rhizosphere and rhizoplane soils compared to the control group. The current tendency was in accordance with the solubilizing ability of citric acid, which may have brought the cations and anions into the soil samples. This was also proved by the determination of water extractable cations (Ca^{2+}, Mg^{2+}, Na^+, and K^+) and anions (SO_4^{2-} and Cl^-) having the dominant concentration in all soil fractions of the treatment group compared to the control group (data not shown).

Distribution of HCl extractable heavy metals (Cd, Zn, Mn) in root zone soil

Table 5 presents the average amount of metals dissolved by the 0.1 M HCl solution. Cultivation of *Crotalaria juncea* resulted in decreasing the concentration of 0.1 M HCl extractable of the three heavy metals. There

was an accumulation trend from the bulk to rhizoplane soils in both the control and treatment groups. For the three heavy metals, the amounts extracted by HCl solution were higher in the rhizoplane soil than in the bulk soil. Higher HCl extractable metals in rhizoplane may be associated with lower pH. The addition of citric acid caused a higher concentration of heavy metals in the treatment group compared to the control except in the concentration of Cd in rhizosphere soil, which had the reverse pattern.

Effect of citric acid on heavy metals uptake

The concentration of Cd and Zn in the roots and leaves following citric amendment did not show significant differences between the control and treatment groups, but in the stems the concentration of both heavy metals of the treatment group decreased significantly (see Figure 3a and 3b). Citric acid addition doubled the shoot-to-root ratio of Mn and increased the concentration of this metal from 78 to 152 mg kg^{-1} in the roots and from 20.67 to 80.29 mg kg^{-1} in

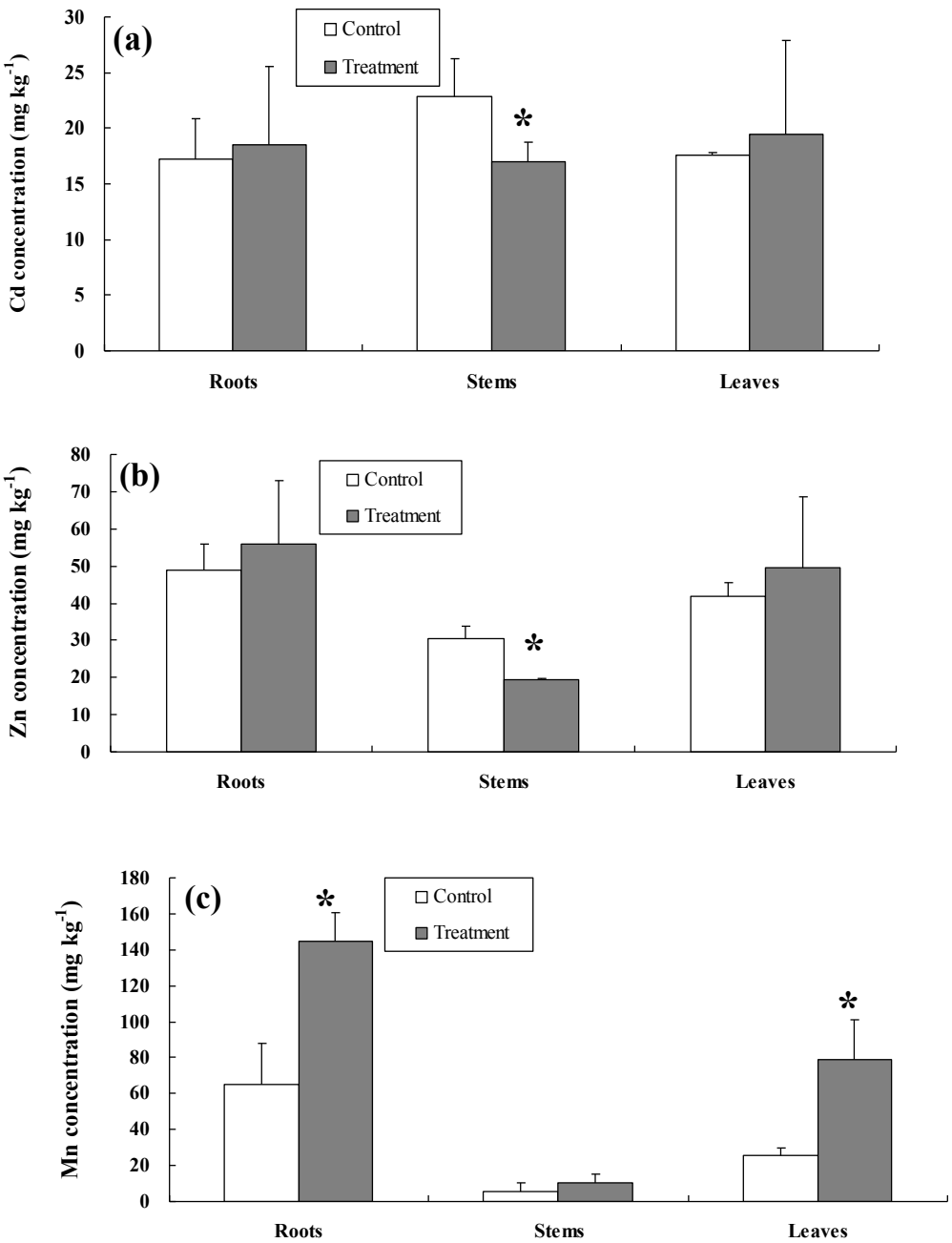

FIGURE 3 - Effect of citric acid on Cd (a), Zn (b) and Mn (c) concentration in roots, stems and leaves of *Crotalaria juncea* grown in Andosol. Bars represent standard error. Values denoted by * indicate significant difference between control and treatment at p <0.05.

the shoots or about 2- and 4-fold, respectively. As for the total absorbed heavy metals (value of content) shown in Table 3, the citric acid amendment decreased the total amount of Cd and Zn, which is the result of lower biomass produced in the treatment group. Despite the biomass stunting after the citric acid amendment, Mn content was increased due to the considerable concentration in the tissues. Chelation and acidification are chemical processes that bring sorbed metals into soil solutions and subsequently absorption by plants [17]. In the present study, although the citric acid treatment slightly decreased the pH value of the BK, RZ, and RP soils in the treatment group compared to the control group, application of this chelating agent did not decrease the pH of the initial bulk soil. The soil pH was measured after harvesting and not the day after citric acid amendment. However, high performance liquid chromatography (HPLC) analysis of the soil samples after harvesting confirmed the availability of citric acid at high concentrations in the treatment pots (data not shown). The addition of 20 mmol kg^{-1} citric acid decreased the pH from 6.8 to 3.8, resulting in U accumulation in plant tissues [17]. In contrast, soil acidification was not responsible for Cd accumulation in *Brassica juncea* [13]. In the current experiment, no effect was observed for Cd and Zn accumulation in the plants after the addition of 20 mmol of citric acid. The citric acid enhanced only the accumulation of Mn in the roots and shoots. This result indicates that acidification of soils does not seem to contribute to enhanced Mn uptake by *C. juncea.* Therefore, the effect of citric acid on the phytoextraction of Mn-contaminated Andosol is probably due to the increased mobility caused by citric-manganese complexation. Such a phenomenon did not seem to contribute to Cd and Zn phytoextraction. The results of heavy metal desorption by organic acids also confirmed that the metal-organic acid complexation is the main process to solubilize and bring cadmium, zinc and especially manganese into the soil solution.

CONCLUSIONS

The present study confirmed that *C. juncea* can hyperaccumulate Cd from Andosol containing moderately low amounts of Cd. In comparison, the plant was less able to accumulate Zn and Mn in the shoots. *C. juncea* decreased the concentrations of 0.1 M HCl extractable heavy metals in bulk soil but increased them in rhizoplane soil. Citric acid showed the greatest capacity in desorbing heavy metals compared to other organic acids in Andosol. Amendment of citric acid did not significantly change the concentration of Cd and Zn in roots and shoots whereas the concentration of Mn in the roots and shoots enhanced about 2- and 4-fold, respectively. The effect of citric acid on the phytoextraction of Mn is probably due to the increased mobility caused by citric-manganese complexation.

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IDENTIFICATION SOURCES OF POLYCYCLIC AROMATIC HYDROCARBONS (PAHs) IN SEDIMENTS FROM THE STRAITS OF MALACCA

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are a group of organic pollutants normally found in the marine environment. In the long-term, the PAHs may cause adverse effects to the aquatic life and humans through the food chain. In this study, marine sediment samples were collected from 10 locations along the Straits of Malacca. The PAHs in sediment samples were Soxhlet-extracted into a 1:1 (v/v) mixture of hexane/dichloromethane (DCM). The extract was then passed through a silica/alumina column and eluted with a mixture of hexane-DCM (20:80), and the fraction was reduced in volume under nitrogen gas flow. For quantification of PAH concentrations, the concentrated extract was injected into a GC-MS system. The ΣPAHs in sediment from the Straits coastal area ranged between 209.8-611.0 ng/g dry weight. The origin sources of PAHs were identified by comparing the various PAH ratios, such as anthracene to anthracene plus phenanthrene, fluoranthene to fluoranthene plus pyrene, benz[a]anthracene to benz[a]anthracene plus chrysene, and Σ(Low Molecular Weight) to Σ(High Molecular Weight). Generally, it was implied that the major PAH pollution sources in the Straits of Malacca are that of fossil fuel combustion (pyrolytic).

KEYWORDS: Sediment, pollution, polycyclic aromatic hydrocarbons (PAHs), Straits of Malacca.

INTRODUCTION

PAHs are common organic pollutants and they can be introduced to the environment by various ways, such as oil spill, municipal and industrial wastewater discharges, forest fires, coal burning, oil drilling and vehicle exhausts. The largest concentrations of PAHs were generally detected in urbanized and industrialized areas [1]. PAH compounds are of special concern due to their toxicity and carcinogenic properties, and also widely distribution in the environment [2-9]. PAH pollution was selected due to its potential accumulation in the living marine organisms. Some of the PAHs, especially those having four or more aromatic rings, are classified as priority pollutants by both the US Environment Protection Agency (US EPA) and European Community [8].

Many researches on PAHs have been done in order to understand their source origin, and some criteria have been developed to distinguish between different sources of PAHs, either natural or anthropogenic ones [7]. The natural PAH sources are mainly from oil seeps, bitumen, coal, plant debris, forest and prairie fires, whereas major anthropogenic sources are from fossil fuel and combustion activities [10]. Most of the criteria to distinguish the sources of PAH pollutions are made by referring to PAH-parent compounds, such as anthracene, phenanthrene, fluoranthene, pyrene, benz[a]anthracene and chrysene. Identification of possible sources of PAH pollution can be made by determining the PAH-parent ratios.

The Straits of Malacca is representing the West Coast of Peninsular Malaysia with a length of about 800 km. The major activities along the Straits of Malacca are fisheries, agriculture, industries and tourism. The major environmental problems in the Straits of Malacca are mainly due to agricultural, industrial [11] and sea-based activities. The goal of this work was to determine the concentration level of PAH compounds in the sediment, and also to identify the possible origin sources of PAH pollution in the Straits of Malacca.

MATERIALS AND METHODS

Sample collection

Ten sampling locations were selected during Kuala Lumpur (KL) Paus 12-days cruise along the Straits of Malacca's coastal area, on February 2004. The sediment samples were collected using a Van Veen grab sampler, and

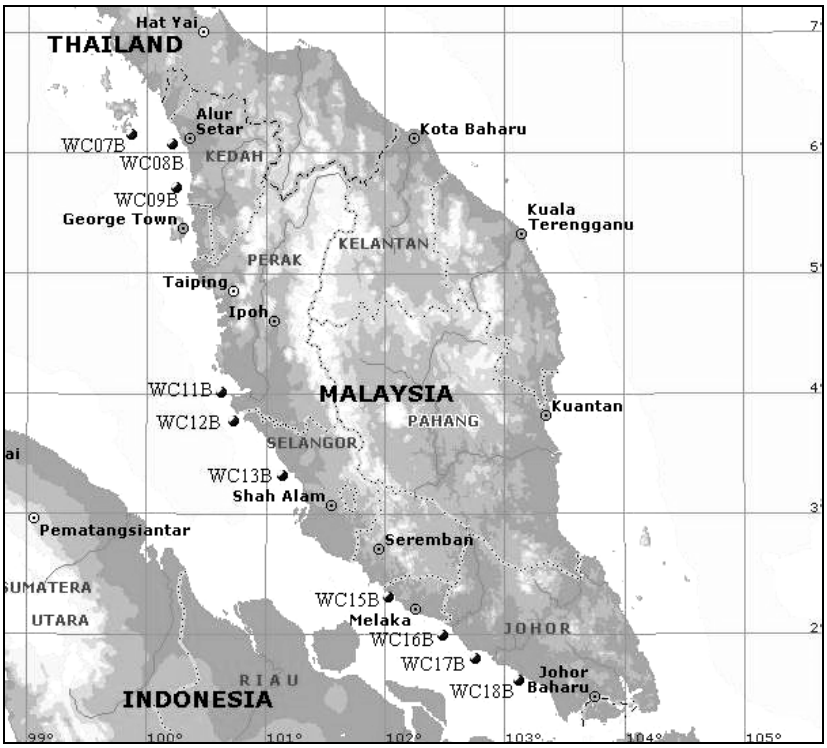

FIGURE 1 - Sampling locations of sediment from the Straits of Malacca.

TABLE 1 - Sampling stations, coordinates and other information.

Stations	Latitude	Longitude	Water depth (m)	Distance from onshore (nautical miles)	Surface temperature (°C)
WC07B	06° 09.90' N	099° 51.40' E	9.8	2.20	28.1
WC08B	06° 04.94' N	100° 12.91' E	11.0	4.25	28.8
WC09B	05° 42.43' N	100° 14.98' E	7.1	6.00	29.7
WC11B	04° 00.25' N	100° 36.99' E	12.6	5.50	30.2
WC12B	03° 46.98' N	100° 43.94' E	20.2	4.60	29.9
WC13B	03° 19.43' N	101° 08.97' E	13.2	4.90	30.2
WC15B	02° 19.56' N	102° 01.98' E	10.4	1.30	28.1
WC16B	01° 59.89' N	102° 28.07' E	8.6	5.00	28.3
WC17B	01° 48.84' N	102° 44.85' E	14.6	3.32	27.9
WC18B	01° 37.27' N	103° 06.90' E	8.9	2.30	28.2

they were transferred into glass bottles with aluminium caps using a stainless steel spatula. The sampling locations and coordinates are shown in Fig. 1 and Table 1, respectively.

Soxhlet extraction

Approximately 20 g wet sediment sample was thoroughly mixed with sodium sulfate anhydrous ($NaSO_4$). The sediment sample was then Soxhlet-extracted with 250 ml of hexane and DCM (1:1, v/v) for 18 h. The extract was dried by rotary evaporation and rinsed with 5 ml DCM. Copper granules were used to remove sulphur from the extract, which was then passed through a silica/alumina chromatographic column. About 40 ml of hexane-DCM (20:80) mixture was used to elute the aromatic hydrocarbon compounds. The effluent was collected and concentrated to a few ml in a rotary evaporator. The residue was collected, transferred into a vial, and reduced to 1 ml under a gentle nitrogen gas stream. The final solution was analyzed by GC-MS to determine the aromatic hydrocarbon compounds quantitatively.

Certified Reference Material (CRM), that is SRM 1944, was analyzed in order to examine the applicability of the adopted analytical procedure. The CRM was also used as quality control material of analysis. All 17 of SRM 1944 aromatic hydrocarbons were determined, and their recovery rates varied between 51.2-128%. However, the recovery of indeno[1,2,3-cd]pyrene was low, (43.4%), dibenz-[a,h]anthracene was more than 300%, whilst benzo[b]-fluoranthene was not detected. The internal standard (o-

terphenyl) was spiked into respective sediment samples to be analyzed in order to evaluate the recovery of the analytical process.

Gas Chromatography–Mass Spectrometry (GC-MS) analysis

The aromatic hydrocarbons were analyzed by GC-MS (Shimadzu QP5050A model) using selected ion monitoring (SIM) mode. A 30 m x 0.25 mm x 0.25 µm ZB1 fused silica capillary column (Phenomenex) was used to identify them. Helium was used as carrier gas with a flow-rate of 3 ml/min. The column temperature was started at 70 ^{o}C, held for 2 min, then increased at 15 ^{o}C/ min up to 300°C. The aromatic hydrocarbon analysis took about 1 h to be complete. The 17 aromatic hydrocarbons analyzed, were, namely; naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, chrysene, perylene, benz[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, indeno[1,2,3-cd)-pyrene, dibenz[a,h]anthracene, and benzo[g,h,i]perylene.

RESULTS AND DISCUSSION

Most of the 17 PAHs (Table 2) were detected in marine sediment samples from the Straits of Malacca. The Table 2 also showed that the ΣPAHs in sediment samples ranged from 209.8 to 611.0 ng/g, with a mean of 353.4 ng/g dry weight (Table 2). The PAH concentrations could be characterized as low, moderate, high and very high ones when the ΣPAHs were 0–100, 100–1000, 1000–5000, and more than 5000 ng/g, respectively [12]. Generally, based on PAH levels, the Straits of Malacca can be characterized as moderately polluted.

Anthropogenic PAHs can be classified as pyrolytic and petrogenic. Pyrolytic PAHs are formed as a consequence of incomplete combustion, whereas petrogenic PAHs are derived from crude oil or unburnt fuel, and their refined products [1, 9]. Special PAH ratios have been used to distinguish both anthropogenic PAH pollution sources, such as anthracene to anthracene + phenanthrene (An/An+Phen), fluoranthene to fluoranthene + pyrene (Fl/ Fl+Py), benz[a]anthracene to benz[a]anthracene + chrysene (B[a]A/ B[a]A+Chry) [1, 10, 13], as well as Σ low-molecular to Σ high-molecular weight (ΣLMW/ΣHLW) [1]. Two common parent PAH ratios of anthracene and phenanthrene were used to distinguish the PAH pollution sources (combustion and petroleum). A ratio of An/An + Phen less than 0.1 was usually taken as an indication of petroleum (petrogenic) origin while ratios more than 0.1 indicated combustion process (pyrolytic) [10, 13]. Table 2 shows that An/An+Phen ranged from 0.30 to 0.60, thus indicating that the source of PAH pollution on the Straits of Malacca coastal area mainly originated from combustion processes (pyrolytic).

Another parent PAH pair, used to identify sources of PAH pollution, was fluoranthene to fluoranthene + pyrene (Fl/Fl+Py), and its parent PAH ratio can be used to differentiate origin sources of PAH pollution, such as petroleum,

TABLE 2 - PAH concentration (ng/g d.w) in marine sediment sample collected from the Straits of Malacca.

PAH Compounds	Stations									
	WC07B	WC08B	WC09B	WC11B	WC12B	WC13B	WC15B	WC16B	WC17B	WC18B
Naphthalene	29.0	35.5	25.6	25.7	23.2	22.0	30.6	23.2	24.1	27.7
Acenaphthylene	21.0	n.d	21.0	21.0	20.0	21.0	20.0	30.0	20.0	20.0
Acenaphthene	15.0	24.0	15.0	16.0	15.0	n.d	15.0	n.d	12.0	14.0
Fluorene	24.0	36.0	24.0	23.0	23.0	24.0	22.0	35.0	23.0	22.0
Phenanthrene	17.4	21.2	15.0	15.0	13.5	13.0	18.0	57.0	14.2	16.0
Anthracene	21.0	32.0	21.0	21.0	20.0	24.0	20.0	24.6	20.0	19.0
Fluoranthene	19.4	23.7	17.0	17.1	15.4	15.0	20.8	15.8	16.1	18.8
Pyrene	28.6	34.7	19.9	25.0	22.7	21.0	30.6	23.4	24.0	28.0
Chrysene	24.0	33.7	47.0	52.4	23.0	24.0	26.9	20.8	20.7	25.2
Perylene	n.d	n.d	10.0	11.0	10.0	21.0	13.0	16.0	13.0	11.0
Benz[a]anthracene	n.d	n.d	47.7	38.5	n.d	n.d	n.d	n.d	n.d	51.1
Benzo[b]fluoranthene	48.6	53.2	81.2	92.2	n.d	n.d	24.1	39.9	18.7	34.7
Benzo[k]fluoranthene	25.1	29.9	40.7	45.9	19.7	n.d	n.d	35.8	21.1	27.0
Benzo[a]pyrene	31.3	38.3	51.4	72.0	27.7	24.8	n.d	25.6	26.1	30.1
Indeno [1,2,3-cd)pyrene	n.d	41.2	n.d	71.0	30.3	n.d	n.d	28.2	n.d	37.8
Dibenz[a,h]anthracene	n.d	n.d	n.d	64.1	n.d	n.d	n.d	n.d	n.d	n.d
Benzo[g,h,i]perylene	n.d	n.d	n.d	n.d	n.d	n.d	n.d	22.3	n.d	31.6
Σ PAHs (ng/g)	304.4	403.4	436.5	611.0	263.5	209.8	241.0	397.6	253.0	414.0
Ratio An/(An+Phen)	0.55	0.60	0.58	0.58	0.60	0.65	0.53	0.30	0.59	0.54
Ratio Fl/(Fl+Py)	0.40	0.41	0.46	0.41	0.40	0.42	0.40	0.40	0.40	0.40
Ratio B[a]A/(B[a]A+Chry	n.c	n.c	0.50	0.42	n.c	n.c	n.c	n.c	n.c	0.67
Σ LMW (2-3 rings)	0.13	0.15	0.12	0.12	0.11	0.10	0.13	0.17	0.11	0.12
Σ HLW (4-6 rings)	0.18	0.25	0.31	0.49	0.15	0.11	0.12	0.23	0.14	0.30
Ratio Σ LMW/Σ HLW	0.72	0.58	0.39	0.25	0.77	0.98	1.09	0.75	0.81	0.40

n.d – below detection limit (10 ppb); n.c – not calculated. An = anthracene; Phen = phenanthrene; Fl = fluoranthene; Py = pyrene; B[a]A = benz[a]anthracene; Cry = chrysene; LMW = Low Molecular Weight; HMW = High Molecular Weight.

grass, wood and coal combustion. The Fl/Fl+Py ratio of 0.5 is usually defined as the petroleum and combustion transition point [10], and Fl/Fl+Py ratios below 0.50 stand for most petroleum products, such as gasoline, diesel, fuel oil and crude oil combustion, as well as emissions from cars and diesel trucks. Fl/Fl+Py ratios above 0.50 imply kerosene, grass, most coal, creosote and wood combustions [10]. Fl/Fl+Py ratios determined ranged from 0.40 to 0.46 (Table 2), indicating that PAH pollution is mainly derived from the combustion of petroleum. According to Yunker *et al.* [17], Fl/Fl+Py values between 0.40 and 0.50 are more characteristic of liquid fossil fuel (vehicle and crude oil) combustion, whereas those higher than 0.50 are characteristic of grass, wood or coal combustion.

Benz[a]anthracene to benz[a]anthracene + chrysene (B[a]A/B[a]A+Chry) ratio may also be used to identify the source origin of PAH pollution in the Straits of Malacca. This ratio is less frequently used as parent PAH indicator, and few guidelines have been established for its interpretation [14-17]. In this study, only at three stations (WC09B, WC11B and WC18B) benz[a]anthracene was detected. However, at all stations, chrysene was recorded. The B[a]A/B[a]A+Chry ratio for stations WC09B, WC11B and WC18B were 0.50, 0.42 and 0.67, respectively. B[a]A/B[a]A+Chry ratios less than 0.2 indicated a petroleum (petrogenic) source, from 0.2 to 0.35 either petroleum or combustion, and more than 0.35, a combustion (pyrolytic) source of PAH pollution [10]. These three stations implied that the origin sources of PAH pollution could mainly originated from the combustion process.

Generally, the sources of PAH pollutions can also be determined by comparing Σ LMW/Σ HMW of PAH species. The LMW group consists of 2-3 aromatic ring-PAHs and HMW group is represented by those of 6 aromatic rings. Ratios of Σ LMW/Σ HMW lower than 1 indicate pyrolytic origin pollution [1]. Ratios determined in this study were 0.25-1.09 with an average of Σ LMW/Σ HMW of 0.67, implying that PAH pollution source of the Straits of Malacca was derived from pyrolytic processes, except at WC15B, in which the source of PAH pollution was uncertain (ratio 1.09).

CONCLUSIONS

Based on the determination of some aromatic hydrocarbons obtained in this work, the PAH pollution in the sediment of the Straits of Malacca was characterized as moderate, with an average of about 353.4 ng/g dry weight in the samples. However, the status of PAH pollution in the Straits of Malacca is still inconclusive as not all PAHs, especially the alkyl-substituted ones, were not measured.

From the identification of the PAH pollution sources based on parent PAH ratios of anthracene, phenanthrene, fluoranthene, pyrene, benz[a]anthracene, chrysene, and that of Σ Low Molecular / Σ High Molecular Weight can be generally concluded that the PAH pollution in the sediment of Straits of Malacca is dominated by a single source, i.e. fossil fuel combustion.

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EFFECTS OF LAND-USE MANAGEMENT ON SURFACE SOIL PROPERTIES, EROSION INDICES AND GREEN TEA YIELD IN HUMID BLACKSEA REGION

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ABSTRACT

Many basins with forest eco-systems in the north-east of Turkey have been converted to agricultural catchments, to which varying quantities and types of manure have been applied. The objectives of this study were to investigate the effects of land-use on surface soil properties in four adjacent management types: Alder coppice (control), and three types of tea cultivation management (at the first: compound fertilizer and stable manure, besides mulching; at the second, compound fertilizer and stable manure; and at the last one, only compound fertilizer) were applied to assess the impact of long-term cultivation and mulching on green tea yield.

A total of 80 soil samples (4 replications x 4 treatments x 5 soil sample points) were collected from 0-10 cm soil depth for laboratory analyses. Results according to land conversion and land-use types showed that the plant-available water (PAW), saturated hydraulic conductivity (Ksat), wet stable aggregates (WSA), soil organic matter (SOM), soil organic carbon (SOC) and green tea yield decreased significantly, while clay, permanent wilting point (PWP), bulk density (Db) and soil penetration resistance increased significantly. The highest soil degradation was determined in TC-III (only compound fertilizer). The results indicate that an accurate planning and land management model is required. This would consider the characteristics of existing land areas, help to conserve soils in tea plantations, and ensure that tea cultivation is carried out sustainably.

KEYWORDS: Land-cover change; alder coppice; tea cultivation; soil properties; green tea yield

INTRODUCTION

Land-use change is one of the most important environmental problems in the north-eastern part of Turkey, as in other parts of the world. In this region, forestry lands between 0-600 m elevation were converted to agricultural uses (particularly tea and nuts), resulting in subsequent environmental damage. The causes of land conversion are broadly categorized as related to population, personal preferences, policies and economic considerations, which result in impacts, such as environmental degradation. Much of the pressure to convert forests to agricultural uses comes from increasing population growth and demands for development.

The eastern part of Black Sea region is mountainous, with land divided into both large and small parcels. Therefore, the land which is suitable for agriculture is very limited and, within the east Black Sea region, only 2 % of the total land area is suitable for this purpose. Much of the productive lands, which are located on coastal area, have been converted to urban settlements, industry and an airport [1]. Tea is an important cash crop in the northern Black Sea region and, because of its economic value, many farmers have replaced their traditional annual as well as food crops with tea. Moreover, since 1950, many of the farmers have converted their private coppice forest to tea cultivation. While there was 2,800 ha of tea plantation in Rize in 1951, the area under tea cultivation is presently 49,968 ha, which represents 12.74 % of land area in Rize [2]. Of this, 9,255 ha of tea plantations are on land suitable for land-use classes between I-IV [3], while the other parts of the tea cultivation area (40,713 ha) are maintained on unsuitable land.

The available experience shows that human activity is the main cause of the degradation of soil, and the soil cover as a whole. Increased anthropogenic influence on soil leads, at first, to its degradation (i.e. deformation and destruction of micro-macro aggregates). By soil tillage, structural aggregates are exposed to fragmentation during rapid and fast wetting due to the impact of rain drops and the direct influence of agricultural machines [4]. Soil structure varies in time and between locations as a function of soil proper-

ties, climatic conditions and land management practices [5]. Forest vegetation generally improves the soil structure while long-term cultivation often results in structural degradation, mainly as a result of a loss of ground cover, the loss of organic inputs from forest vegetation, and due to soil disturbance from tillage [6]. It has been reported by some researchers that, as a result of the damaging of the forest vegetation and conversion to agricultural land, the texture [7] and structure, especially of topsoil, is degraded, due to the dense cultivation; while the volume weight [8-11] of the topsoil increases, pore volume (St) [10, 11], wet stable aggregate (WSA), saturated hydraulic conductivity (Ksat) and cumulative infiltration (Ic) were significantly changed [12, 13]. In addition, as a result of the conversion of forest land to agricultural uses, rain drops directly contact the soil surface and degrade the aggregate structure of the topsoil. Many researchers have reported that this increases the tendency of erosion and excessive water in the soil surface and may cause surface flow [14-17].

Tea (*Camellia sinensis* L.) is a globally important crop, and is unusual because it both requires an acid soil and also acidifies soil. Tea stands tend to be extremely heavily fertilized in order to improve yield and quality, resulting in a great potential for diffuse pollution [18]. Assessing land use-induced changes in soil systems is, therefore, essential for addressing the problem of agro-ecosystem transformation and sustainable land productivity [19]. Soil structure, organic matter content and nutrient concentrations are related to root distribution [20]. Surface application of the mulched plant material can provide protection from erosion, conserve moisture [21] and moderate soil temperature. How-ever, little of the mulched material is initially incorporated into the soil and potential for soil compaction exists. Incorporating the mulched material by tilling the soil can en-hance soil structure and loosen the soil, permitting improved air and water infiltration. However, the soil will be exposed, and oxidation of soil organic matter (SOM) will be more rapid than on sites where the mulch remains on the surface. Another consideration is that many mulched plant materials have a high C:N ratio and will tie up large amounts of nitrogen during decomposition, thus reducing nutrient availability for the new vegetation. Incorporating fertilizer application in the site preparation may provide sufficient nutrients to replace those tied up in the mulch [22]. Mulching drastically reduces the rate of evaporation. The protective effect of mulch in reducing runoff is generally related to the quantity of mulch. The infiltration rate normally increases with increasing levels of mulch applications. Lal [23] reported that mulching improves water conservation in two ways. It reduces water runoff, and decreases water loss due to evaporation from soil surface. Improvement in soil structure caused by mulching is partly due to prevention of surface crusting by checking raindrop impact, and partly due to enhancement of soil fauna activity, notably that of earthworms [24].

The aim of the present study is to determine the long-term effects of different land management strategies on a range of physical, hydrological and chemical soil properties, and the impact of mulching on topsoil properties and green tea yield. The trends in the changes observed were used to evaluate the impacts of the tea cultivation management on soil attributes, and as indicators of the sustainability of the tea cultivation management. For this purpose, a small agricultural catchment located at Pazar watershed in Rize, Turkey was chosen as study area.

MATERIALS AND METHODS

Site description and history

The study area is located in the Pazar river basin, northeastern part of Turkey (lat. 41° 08' 31"-41° 08' 31 " N and long. 40°53'49"-40°53'52" E). The area has a humid Black Sea climate: average annual precipitation is 2,019 mm, with a minimum in spring (260 mm) and maximum in autumn (758 mm), and the mean annual temperature is 14 °C (data from the Pazar Meteorological station, located 3 km distant at a similar altitude) [25]. The altitude of the study sites ranges between 70-180 m, and the mean slope of the study area is moderate (18±2 %). The soil types of the area were classified as yellow-red podsol according to the International Soil Classification System (ISCS) [26]. The rock mass is extensively volcanically disrupted, and the parent material is andesite [15]. The sand and silt content of soils varies between 40-74 % and 15-40 %, respectively. The clay content of soils varies between 8-34 %. All soil profiles in the study area had higher sand than silt and clay content. The pH values of the soils vary from 4 to 6 [16]. The organic carbon and total nitrogen values vary from 0.20 to 5.51 % and 0.04 to 0.32 %, respectively. C:N values vary between 4.4-21.8. These soils do not have any $CaCO_3$ content due to the non-calcareous parent material and high precipitation in the region [26]. The area adjacent to that of tea plantations mainly comprises stands of alder (*Alnus glutinosa* L. Gaertner subsp. *barbata)* with lesser proportions of *Rubus platyphyllos*, *Urtica* sp., *Frangula alnus* Miller., and annual forage plants [15, 16]. Between 1950-1980, 4-5 ha of alder coppice forest was clear-cut annually and, in total, 120-150 ha of forestland has been converted to tea plantation in Pazar river basin. The terraces on which the tea is cultivated range between 40-60 m in length and 0.4-0.6 m width, and the soils of the terraces were tilled to a depth of 0.6 m.

Experimental design and soil sampling

A preliminary study was carried out in May 2006 with the purpose of determining the tea plantations in which various procedures (mulching, organic or artificial manure, trimming) are applied. For this purpose, a public survey and visual observation was carried out in 2006. In the present study, research sites were categorized according to these visual observation and public survey. Four management types were identified (Table 1).

Four sample plots (20 m × 30 m) were established per management area for the purpose of data collection. Soil

TABLE 1 - The application on tea plantations (mulching, organic or artificial manure).

Experimental sites	Tea Plantation Date	Mulching	Mulching + organic manure	Artificial manure
TC-I (Site-I) (Tea cultivation)	1974	Between 1974-1980, mulch was applied in tea terraces using the leaves collected from beneath alders. With this method, approximately 200 kg leaves were spread over 0.1 hectares of tea terrace.	Between 1980-1990, low quantities of mulch and organic animal manure were used. Approx. 50 kg mulching and 250 kg animal manure, kept for 1 year, were applied to 0.1 hectares of land.	Until the years 1990-2000, 50 kg ammonium sulfate manure was used in 0.1 hectares. Between the years 2000-2008, an average of 50 kg N-P-K (2:1:1) manure mixturess were used in 0.1 hectares. Manure application was made in March each year.
TC-II (Site-II) (Tea cultivation)	1978	Using the leaves collected under alders between 1978-1980 mulching was applied in tea terraces. With this method, approximately 200 kg leaves were spread over 0.1 hectares of tea terrace.	Between 1980-1985 approximately 250 kg animal manure which was kept for one year was spread over 0.1 hectares of land.	Until the years 1990-2000, 50 kg ammonium sulfate manure was used in 0.1 hectares. Between the years 2000-2008, an average of 50 kg N-P-K (2:1:1) manure mixtures were used in 0.1 hectares. Manure application was made in March each year.
TC-III (Site-III) (Tea cultivation)	1979	Mulching is not applied	Mulching or organic manure is not applied	Until the years 1980-2000, 50 kg ammonium sulfate manure was used in 0.1 hectares. Between the years 2000-2008, an average of 50 kg N-P-K (2:1:1) manure mixtures were used in 0.1 hectares. Manure application was made in March each year.

samples were collected during spring from the experimental sites having the same physiographic unit, soil conditions and slope aspects. Soil samples were taken randomly at a soil depth of 0–10 cm in each plot of the study area, comprising five samples where the soil had been disturbed, and five undisturbed samples. The undisturbed soil samples were obtained using a steel core sampler of a 98.16 cm^3 volume (5 cm in diameter and 5 cm in height). Prior to analysis of physical and chemical soil characteristics, all samples were air-dried at room temperature and passed through a 2 mm soil sieve.

Laboratory analysis

The particle size distribution was determined by the Bouyoucos hydrometer method using disturbed soil samples sieved through a 2-mm mesh-sized sieve [27]. Dispersion ratio (DR) was determined according to the methods described by Middleton [28]. The clay and silt fractions obtained by chemical dispersion were taken as Tc and Ts, while water-dispersible clay and silt (WDCS) was obtained as described above, except that no chemical dispersant was used. Colloid moisture equivalent ratio (C-MER) and erosion ratio (ER) were determined according to the methods described by Balcı [29]. The field capacity (FC) was measured by subjecting saturated soil samples <2 mm to tensions of 1/3 bars. The permanent wilting point (PWP) was measured at 15 bar until equilibrated in pressure membrane and pressure plate extractors. The plant-available water (PAW) content was calculated from the difference between the field capacity and the permanent wilting point [30]. The bulk density, total porosity, and saturated hydraulic conductivity were determined with the undisturbed soil samples. The dry bulk density (Db) was determined by the core method [31]. Particle density (Dp) was determined by the pycnometer method. Total porosity (St) was calculated from the following equation: [St (%) = $(1-D_b/D_p)$ x 100], where St is total pore space, D_b is bulk density and D_p is soil particle density [32]. A wet sieving method was used to determine the water stable aggregates (WSA) [33]. The saturated hydraulic conductivity (Ksat) was measured by the falling-head method according to Klute and Dirksen [34]. The samples were used for saturation and consecutively oven-dried at 105± 2 °C to determine bulk density. The soil penetration resistance (SPR) [35] was measured as 0-40 cm depth. 20 measurements were recorded for each plot, at depth intervals of 5 cm, using a manual (hand-pushed) 13-mm diameter cone (30°) penetrometer. Cumulative infiltration (Ic) in the field was determined using a single ring infiltrometer [36] having a cylinder of 20 cm diameter and height, and 4 measurements were done on a leveled surface at each plot. The site was prepared by removing all residues and any large clods (in tilled soils) that would interfere with achieving a levelled surface. The cylinder was inserted into the surface to a depth of approximately 5 cm. The change in water depth of the cylinder was measured at time intervals of 5, 10, 15, 20, 30, 40, 60, 75, 90, 105 and 120 min. Soil pH was determined in a 1:2.5 soil/water mixture [37]. Electrical conductivity (EC) (of the saturation) was determined according to the method described by Rhoades [38]. Lime ($CaCO_3$) was determined according to the method described by Richard and Donald [39]. The concentrations of soil organic matter (SOM) and soil organic carbon (SOC) were determined using the Walkley-Black method [40]. Total Nitrogen (TN) was determined using the Kjeldahl method [41]. The C:N ratio was calculated from the following equation:

$$[C:N = (SOC/ \text{Total } N)] \quad (1)$$

Where C is carbon, N is total nitrogen, SOC is soil organic carbon, and Total N is total nitrogen.

Estimation of green tea yield

Green tea yield in the tea plantations, between the years 1975-2005, was calculated based on the sales of the

producers from the tea gardens in which related test sites were created and the values which were registered to the tea account book. By adding the value of all green tea sold from the related test sites, the total annual green tea yield was calculated.

Statistical analysis

Statistical analysis was performed using of variance (ANOVA), and the means were subjected to Duncan test (P <0.05) to identify the main differences between the treatments (sites). The data were also examined for correlation using SPSS software package. Mean values found for all properties are shown in relevant tables.

RESULTS AND DISCUSSION

Soil texture and water characteristics

The soil types of the study area were sandy loam (in control site), sandy clay loam (in TC-I and TC-II sites), and light clay (in TC-III site) (Table 2).

The mean value for sand content was the highest in control site, followed by the TC-I, TC-II and the TC-III sites. Compared to control site, sand ratio was significantly lower (p<0.012), while the clay ratio was significantly higher (p<0.008) in TC-I, TC-II and TC-III sites (Table 2). The FC decreased from control site to TC-I, TC-II, and then TC-III site. The PAW was significantly lower (p<0.021), while PWP was significantly higher (p<0.038) in the TC-I, TC-II and the TC-III than in control site. Under normal conditions, a change of land usage would not be expected to cause a large change in the sand and clay ratios. However, with field transformation in the research area, terraces at an approximate width of 60-90 cm were formed, stones in the terraces larger than 10 cm were removed and tea seedlings were set after discombobulating the soil. Also, beans and other crops were seeded in the newly set tea terraces and the soil was hoed. It can be said that these operations caused displacement of the existing topsoil and, as a result of this, the texture and the structure of the soil changed. Numerous previous studies have reported that discombobulating changes the soil's structure, especially in the upper part of the soil profile [7]. Brye *et al.* [42] reported that land leveling significantly altered soil particle-size fractions, although the soil textural class remained a silt loam. In the forest areas transformed to tea cultivation, there is less protection of the soil and, as a result of this, in terraces were tea cultivations are present, some of the topsoil had been removed by surface flow. This can be shown as another effect causing the soil's texture to change.

In the tea cultivation sites, due to heavy field traffic and hoeing, the volume weights and penetration values of

TABLE 2 - Investigated soil properties in 0–10 cm soil depth (mean± Std Dev).

Soil Characteristics	Land-use type				F ratio	P
	Control Site	Tea cultivation (TC)				
	(Alder coppice)	TC- I	TC- II	TC- III		
Sand (%)	70.70±(6.75)^{a}	61.50±(5.88)^{b}	58.90±(5.61)^{b}	54.10±(5.13)^{c}	4.35	0.012
Clay (%)	12.25±(1.30)^{c}	20.55±(2.60)^{b}	21.88±(2.80)^{b}	25.67±(3.12)^{a}	8.24	0.008
Silt (%)	17.05±(2.21)^{a}	17.95±(2.23)^{a}	19.22±(2.54)^{a}	20.23±(2.88)^{a}	0.56	0.755
FC (% vol.)	33.16±(3.11)^{a}	32.54±(3.38)^{a}	31.78±(3.51)^{a}	30.34±(3.77)^{a}	0.44	0.815
PWP (% vol.)	14.18±(1.52)^{d}	16.34±(2.11)^{c}	17.90±(2.29)^{b}	20.40±(2.58)^{a}	3.77	0.038
PAW (% vol.)	18.98±(1.22)^{a}	16.20±(2.06)^{b}	13.88±(1.33)^{c}	9.94±(1.04)^{d}	4.15	0.021
Dp(gcm^{-3})	2.32±(0.25)^{a}	2.29±(0.33)^{a}	2.30±(0.34)^{a}	2.14±(0.25)^{b}	2.79	0.042
Db (gcm^{-3})	0.98±(0.12)^{c}	1.09±(0.17)^{b}	1.11±(0.17)^{b}	1.18±(0.22)^{a}	4.67	0.004
St (%)	57.75±(5.88)^{a}	52.40±(4.45)^{b}	51.74±(4.40)^{b}	44.86±(4.12)^{c}	10.92	0.001
WSA (%)	72.50±(6.22)^{a}	66.50±(5.77)^{b}	64.80±(5.71)^{b}	59.20±(5.24)^{c}	3.88	0.023
Ksat (mmh^{-1})	44.56±(4.88)^{a}	28.55±(2.50)^{b}	21.70±(1.98)^{c}	12.87±(1.12)^{d}	10.65	0.002
Ic (mm h^{-1})	452±(12.98)^{a}	320±(12.10)^{b}	283±(11.57)^{c}	118±(10.79)^{d}	14.99	0.000
SPR (MPa)	0.315±(0.11)^{c}	0.361±(0.13)^{b}	0.374±(0.13)^{b}	0.576±(0.17)^{a}	5.12	0.008
DR (%)	10.46± (1.09)^{d}	18.77± (1.55)^{c}	28.25± (3.75)^{b}	55.79± (5.75)^{a}	10.96	0.001
C/MER	0.37±(0.10)^{a}	0.656±(0.14)^{b}	0.688±(0.19)^{c}	0.789±(0.24)^{d}	7.92	0.003
ER (%)	28.27±(2.16)^{c}	28.63±(2.80)^{c}	41.06±(4.15)^{b}	70.71±(8.89)^{a}	16.78	0.000
pH (1/2.5 H_2O)	4.55±(0.45)^{a}	4.13±(0.20)^{b}	3.99±(0.19)^{b}	3.53±(0.17)^{c}	2.92	0.039
E.C ($dS.m^{-1}$)	0.27±(0.06)^{c}	0.31±(0.06)^{b}	0.33±(0.06)^{b}	0.41±(0.09)^{a}	3.52	0.034
$CaCO_3$ (%)	0.43±(0.11)^{b}	0.54±(0.12)^{a}	0.55±(0.12)^{a}	0.57±(0.15)^{a}	4.56	0.025
SOM (%)	5.88±(0.96)^{a}	4.50±(0.89)^{b}	4.01±(0.89)^{c}	2.65±(0.71)^{d}	4.90	0.019
SOC (%)	3.57±(0.65)^{a}	2.54±(0.45)^{b}	2.41±(0.40)^{c}	2.05±(0.35)^{d}	7.35	0.007
Total N (%)	0.25±(0.05)^{a}	0.22±(0.03)^{b}	0.21±(0.03)^{b}	0.16±(0.03)^{c}	2.91	0.040
C: N Ratio (%)	14.28±(1.95)^{a}	11.54±(1.86)^{c}	11.48±(1.86)^{c}	12.81±(1.98)^{b}	2.88	0.041
STC^{x}	SL	SCL	SCL	LC	--	--

FC: Field Capacity, PWP: Permanent wilting point, PAW: Plant-available water, Dp: Particle density, Db: Bulk density, St: Total porosity, WSA: Water-stable aggregate, Ksat: Saturated hydraulic conductivity, Ic: Cumulative infiltration (mm h^{-1}), E.C: Electrical conductivity, SOM: Soil organic matter, SOC: Soil organic carbon, STC: Soil texture class, DR: Dispersion ratio, ER: Erosion ratio,: Colloid-moisture equivalent ratio, P: Significance Level. Values are means, and Significance levels are: NS represents non-significant, * 0.05–0.01, ** 0.01–0.001 and *** 0.001; values within columns followed by the same letter are not statistically different at 0.05 significance level.. ^{X}: According to international soil texture class, SCL: Sandy Clay Loam; SL: Sandy Loam; LC: Light clay. SPR: Soil penetration resistance.

the soil increased (TC-III> TC-II> TC-I) by a significant amount, and the total pore volume and the net structure of the pores were destroyed. Resulting from this, the penetration of water into the soil was slower and the FC and PAW values of the soil decreased. The decrease in the amount of organic matter can be seen as one reason for the decrease of the field capacity and plant-available water content in the tea and forest soils, with increasing depth. As the amount of organic matter decreases, the water holding and available water capacities of the soil decrease [7, 43]. Terracing with human power and, applying high levels of organic fertilizers (in the first six years) and mulching (in the first 10 years) following the tea setting help to increase the plant-available water capacity of the soil in the TC-I area. The use of organic inputs in the terraces formed by human power increases the water-resistant aggregate stability of the soil. In these terraces, the volume weight of the soil decreases when compared to terraces in which no organic input is used, whereas the water holding capacity, plant-available water and hydraulic conductivity increase [7]. Under normal conditions, the water holding capacity and PAW content in the forest soils can be expected to be higher than the cultivated tea soils. However, in the research region, the dry leaves of the alders are collected and used in the tea terraces for compost or other purposes (such as animal bedding in stables). Also, the herbaceous plants growing under the alders are being harvested and used as animal food or animal bedding in stables. These operations cause a loss of minerals in the forest ecosystem and have negative effects on the soil properties. Le Bissonnais [44] mentioned that the aggregate stability of the soil increases with the increase of organic materials in the soil. Yüksek [15] found that the water holding capacity of the soils decreases, and the surface run-off increases in heavy rain conditions, as a result of using the organic wastes that are presently collected from the forest for different purposes.

Bulk density (Db), total porosity (St), water-stable aggregates (WSA), and soil penetration resistance (SPR)

The highest Db (1.18 g.cm^{-3}) and the lowest St (44.86%) were determined in TC-III, while the lowest Db (0.98 g.cm^{-3}) and highest St (57.75%) were determined for the control site (Table 2). According to mean values, Db content followed the sequence TC-III>TC-II>TC-I> Control; and mean St followed the sequence Control>TC-I> TC-II>TC-III. The mean WSA content was determined to be 59.20-66.50 %. The highest WSA (72.50%) was found in the control site, while the lowest WSA (59.20%) was determined in the TC-III (Table 2).

It can be stated that the dense cultivation and trampling of soil in the tea plantations decreased the pore volume, especially in the topsoil, and caused bulk weight increase. Under normal conditions, as a result of the dense plantation traffic and cultivation, total porosity can be expected to be lower and bulk weight is expected to be higher. Organic wastes applied to the tea terraces improve the structure of the topsoil to a certain degree. This prevents the further decrease of pore volume and a further increase in bulk weight. Many researchers have reported that compost and animal manure applied to the topsoil decreased bulk weight [10, 45, 46], but increased WSA and total porosity value [5, 47-49].

The effect of soil moisture content is quite important in the increase of soil penetration resistance. However, the humidity levels held at critical tensions at the tea and forest soils are close to each other. Statistically, the penetration values in both of the land-use types are significantly different. It can be said that this is due to the field traffic present in the tea soils. Many researchers reported that the SPR increases as a result of the increase in the topsoil traffic density [43].

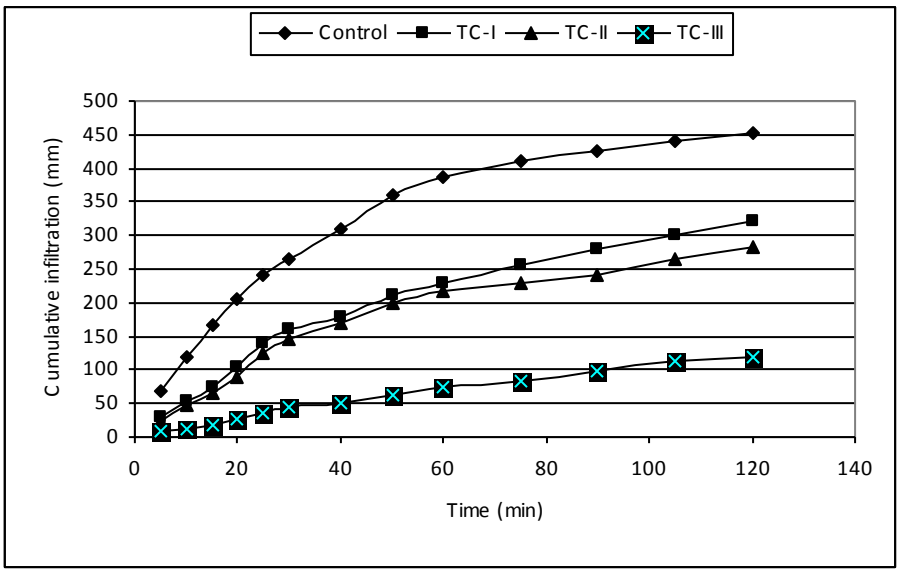

FIGURE 1 - Cumulative infiltration on control, TC-I, TC-II and TC-III sites.

Saturated hydraulic conductivity (Ksat) and cumulative infiltration (Ic)

The highest Ksat and Ic values were determined in control sites, and the lowest Ksat and Ic were determined in TC-III (Table 2, Fig. 1). Average permeability values were 44.56 mmh^{-1} in control site and 12.87-28.55 mmh^{-1} in tea plantations.

As seen in Fig. 1, the mean Ic contents in TC-I and TC-II were lower than the control site but was higher than that of TC-III.

Soil hydraulic properties, including soil hydraulic conductivity function and water retention characteristics, are affected by soil texture, bulk density, soil structure, and organic carbon content, many of which are strongly influenced by land-use and management, even though the soil classification may be the same. Due to the procedures applied to soils in tea plantations, with the degradation of texture and soil structure, the macro-pores and pore structure of the soil were damaged. Surface pressure from trampling and the increase in soil bulk weight caused Ksat and Ic values to decrease. The fact that root structure and the total root density were lower when compared to the forest soils had an effect on the low Ksat and Ic values. The temporal change of land-use and management, or natural disturbances and cycles, such as diurnal and seasonal changes, can affect soil hydraulic properties. Soil compaction, caused by human trampling, wheeled traffic or animal grazing, can immensely destroy the large-pore structure and, therefore, reduce saturated or near-saturated hydraulic conductivity [50]. Tilling can create more large pores for surface soil but may also disrupt pore network connectivity, especially for subsurface flow [51]. Other factors, such as soil organism activity, root dynamics, and formation of cracks at the surface during dry periods, all contribute to the dynamic nature of soil hydraulic properties in different soils [8].

Dispersion ratio (DR), Colloid/Moisture equivalent ratio (C-MER), and Erosion ratio (ER)

The highest DR, C-MER and ER values were determined in TC-III, while the lowest ones were found in the control site. According to mean values, DR and C-MER were significantly higher in tea cultivation sites, with regard to control site, while mean ER was significantly higher in TC-II and TC-III compared to control and TC-I (see Table 2). The vegetation in the control site enriched the soil in terms of organic matter, and prevented rainfall from impacting the soil surface and eroding the soil by dislodging soil particles. In addition, mulching and the application of organic manure to the soil similarly improved soil properties and protected the soil against the direct effect of rain drops. For this reason, DR, C-MER and ER values of the soils in control sites and long-term mulching/organic manure sites were found to be significantly lower than those of the other sites (TC-II and TC-III). Many researchers reported that mulching protects the topsoil from the direct effect of rain drops and improves soil properties [21, 23, 24]. Another reason for higher DR, C-MER and ER contents in tea plantations is probably that the aggregate structure of topsoil in these sites is degraded due to the cultivation of these soils along the terrace in certain intervals. Many previous studies based in different regions [14, 16, 17] have reported the DR, C-MER and ER contents in cultivated soils to be higher than those in forest soils.

Values of pH and EC

The highest pH and the lowest EC values were determined in control site, while lowest pH and highest EC were determined in TC-III (Table 2). Different types of mulching and manure use were applied in tea plantations. With the mulching applied in the earlier period, the acidification of tea soils was prevented to some degree. However, as result of excessive use of chemical fertilizers and washing out, the acidity values of tea soils have been increased. It was reported that generally there is a strong positive relationship between the clay content in the soils and EC [52]. In addition, it was reported that the application of mulch on the soil surface has an effect on the increase in EC values [46]. In the present study, clay content and EC values in test sites followed the sequence: TC-III> TC-II>TC-I>Control. In other words, there is a positive correlation between clay content and EC value.

The movement of electrons through bulk soil is complex. Electrons may travel through soil water in macropores, along the surfaces of soil minerals (i.e. exchangeable ions), and through alternating layers of particles and solutions [52]. Therefore, multiple factors contribute to the variability of soil EC, including factors that affect the amount and connectivity of soil water (e.g. bulk density, structure, water potential, precipitation, timing of measurement), soil aggregation (e.g. cementing agents, such as clay and organic matter, soil structure), electrolytes in soil water (e.g. salinity, exchangeable ions, soil water content, soil temperature), and the conductivity of the mineral phase (e.g. types and quantity of minerals, degree of isomorphic substitution, exchangeable ions) [53].

Soil organic matter (SOM) and soil organic carbon (SOC), Total N, C:N ratio and lime

While the average value for organic matter content varied between 2.65-4.50% in tea soils, this value reached 5.88% in the control site. The highest SOM, SOC, total N and C:N contents were determined in the control site, while the lowest SOM, SOC and total N were found in TC-III. According to the test sites, the change in SOM and SOC values are statistically significant (Table 2).

It can be stated that the land-use change and the cultivation in tea terraces caused a decrease in the organic matter content of the soils. However, it can also be stated that the organic matter contents in tea topsoils are not particularly low. Mulching and organic manure were applied to tea topsoil during a certain period of time, preventing a decrease of organic matter in tea topsoil. Land-use change and long-term cultivation may lead to changes in SOM quantity and quality [54]. Soil carbon loss first occurs pre-

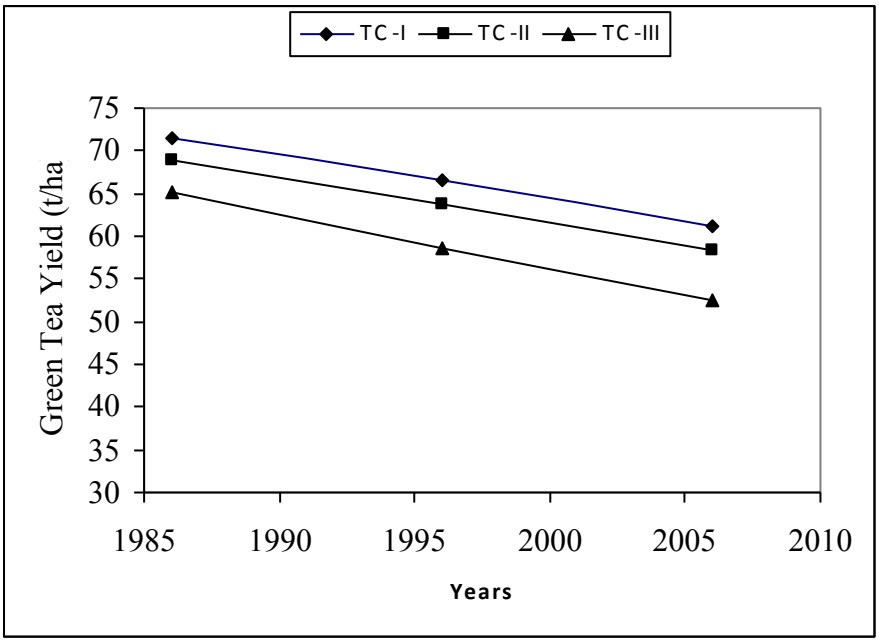

FIGURE 2 - Green tea yield in experimental sites (TC-I, TC-II, and TC-III).

dominately by mineralization after conversion of virgin land into cultivated land, followed by soil erosion as the dominating process of soil carbon loss in later years [55]. It can be suggested that the application of organic wastes, collected from the forest and stable manure to tea terraces, caused the total nitrogen amount in the tea topsoil to be higher than tea soils that did not have organic inputs. In addition, thanks to the organic wastes and the spread dry tea leaves, the carriage of N and P by surface flow was prevented to some extent. This may have caused the relatively high N value. However, due to the fact that the study area is located in a humid region (annual average precipitation approximately 2,000 mm) and, in addition, due to the topsoil cultivation in the terraces, a certain amount of N may have been leached from the soil. Leaching losses are influenced by soil texture, N management, and irrigation practices [56].

Green tea yield (Kg/ha)

Total annual yield (1976-2006) of tea plantation in hectares is shown in Fig. 2. Generally, tea yield decreased in TC-I (7.14-14.42%), TC-II (7.31-15.32%) and TC-III (10.28-19.49%) in given ratios. The values indicate that the greatest decrease in tea yield occurred at TC-III site, in which mulching + organic manure was not applied (Fig. 2).

As Fig. 2 shows, although mulching and organic manure was applied for a certain period, tea yield still decreased. However, this decrease was lower than the one observed in the test site in which only chemical fertilizer was applied. The application of mulch + organic manure in tea terraces increases the crop yield when compared with the test site, in which only chemical fertilizer was applied. Forest soils, in general, have higher organic matter content than agricultural soils. This probably has an effect on the relatively high yield in the new tea plantations. Degradation of soil properties in the following years (for example, decrease in organic matter, pressure, decrease in permeability etc) has probably caused loss of yield in tea plantations [9]. The aging of tea cuttings causes tea yield to decrease. In addition, while, in the first years after establishing the plantation, tea cuttings were young having a positive effect on the yield; aging may have a negative effect on the yield of the tea cuttings. In addition, the change in climate (10% increase in total precipitation amount, especially in the last 10 years) may have caused more plant nutrients to be leached with surface flow or the displacement of the topsoil with erosion, which may have contributed to the reduced yield of the soils in which tea plantations were established.

CONCLUSIONS

As a result of the degradation of alder coppice and their conversion to tea plantations, their 10 physical (sand, clay, PWP, D_b, Dp, St, WSA, DR, C-MER, and ER), 2 hydro-physical (Ksat and Ic) and 6 chemical (pH, EC, $CaCO_3$, SOM, SOC, and total N) properties changed significantly. As a result of forest degradation, the erodibility values of the soils increased and green tea yields decreased. With the mulching application, soil properties were protected to some extent, and further reduction of yield was prevented. There is insufficient availability of land in the Pazar area that is suitable for agriculture. For this reason, the introduction of agriculture on ill-suited land involves a high level of risk of declining crop yields

and environmental damage. For the protection of soils in these areas and to enable more sustainable forms of agriculture, an accurate planning and land management model should be prepared by considering the properties of the existing soils. In tea plantations, in order to protect soils and increase yields, mulching applications should continue, and the effects of mulching applications on soil protection and tea yield should be investigated. This strategy can help land managers to utilize the soils more effectively and productively.

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TRACE ELEMENT CONTENTS IN DIFFERENT SOILS OF A SEMIARID MEDITERRANEAN ENVIRONMENT: CASTILLA-LA MANCHA, SPAIN

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ABSTRACT

Pedo-geochemical baseline concentration levels of trace elements were assessed in 72 soil horizon samples of 24 soil profiles of a semiarid Mediterranean environment region (Castilla-La Mancha. Spain). The concentrations of the elements (Sc, V, Cr, Ni, Cu, Zn, Ga, Ge, As, Se, Br, Rb, Sr, Y, Zr, Nb, Mo, Ag, Cd, Sn, Sb, I, Cs, Ba, La, Ce, Nd, Sm, Yb, Hf, Ta, W, Tl, Pb, Bi, Th and U) were found to be affected mainly by the nature of the bedrock. These values are mainly of natural origin and only to a lesser extent of anthropogenic origin. Enrichments in some soils do not necessarily suggest a direct influence of pollution from recent point sources.

The microelement vertical concentrations showed no significant differences between the arithmetical means of the "amount of an element" found for the three different horizons (A. B and C). Only zirconium and lead (and to a lesser extent arsenic showed a similar pattern with relative depletion within the bottom soil. These vertical distribution variations can be associated mainly with natural sources. Those seem to depend mainly on the bedrock and secondly on soil processes, such as accumulation of calcium carbonate, leaching of elements due to dissolution of host minerals, etc. Other factors could be the underlying young, loose sediments (material mobilized by water, wind, or gravitation).

KEYWORDS:
Trace elements; baseline concentrations, soils, Mediterranean environment, spatial variability, Castilla-La Mancha, Spain.

INTRODUCTION

Trace elements are natural constituents of the Earth's crust. Geochemical background concentrations should represent natural concentrations, which ideally exclude human influence. In contrast, geochemical baselines represent specific concentrations to one area and time, and are not always backgrounds [1, 2]. Free of human influence, their contents in soils depend on the mineralogical composition of the bedrocks and on the pedogenetic processes to which the soil-forming materials have been subjected [1-4]. In fact, these elements would be derived from the weathering of parent materials and redistribution in the soil profile due to soil-forming processes. In addition, in some zones, the contents of these elements can be increased by anthropogenic input [5]. According to these authors [5], as a general rule, the "natural background" concentrations of trace elements in soils can be seen as a function of their concentrations in the parent material. An understanding of the parent material and the typical concentrations of elements therein provide a good indication of the concentrations of these elements in soils.

Physical and chemical weathering of rocks and minerals leads to soil formation. To establish the content and distribution of a given element in soils, it is necessary to determine the accumulation and/or release rates of rock-forming elements during weathering. But human activities can alter the balance and the biochemical and geochemical cycles of trace elements, as their concentrations in topsoils can increase with greater industrial and agricultural activity. Thus, these activities must be taken into account.

The objective of this study was to characterize, in quantitative terms, the total contents (concentration levels) of a large group of trace elements in soils of a semiarid Mediterranean environmental region. For this purpose, soil profiles were selected as a function of the various pedogenetic processes developed on different parent materials and land-uses in Castilla-La Mancha (Central Spain). The provenance of trace metals in soil, whether from the parent material or from pollution, is rarely known with certainty, and the metals' history must usually be pieced together from fragmentary statistical information [6]. In addition, to investigate vertical variability also measured out a statistical analysis to test whether there were differences in the

mean values of the "amount of an element" between horizons.

MATERIALS AND METHODS

Study area and sampling procedure

The study area, located in the central of Iberian Peninsula, Castilla-La Mancha, is part of the so-called "*Meseta Sur* (Southern Plateau)" (Fig. 1a). This area, including Toledo, Ciudad Real, Cuenca, Guadalajara and Albacete province, presents several environmental conditions. It is an area with a large diversity of geologically parent material composition. The geological formations extend from the Precambrian and Paleozoic (which lithologically are rich in slates, schists, gneisse, granites and migmatites. etc) to the Mesozoic (mainly limestones, dolomites, marls, more or less clayey sands, clays and conglomerates), the Tertiary (more represented, with limestones, clays, sands, gypsums, marls and, in much smaller proportion, volcanic rocks) and the Quaternary (silts, clays, sandy soils and gravels, mainly). The climate is temperate Mediterranean, somewhat modified by continental conditions and by altitude. This is a typical continental Mediterranean climate, with 300 to 800 mm average annual precipitations and 10-15 ºC average mean annual temperature, with hot summers and cold winters. The potential vegetation is a *Quercus ilex* forest, but in the region over half of the soils are occupied by crops (wheat, vineyards, olive groves etc.). Agriculture is important in the region; agricultural practices extend over 4.2 million hectares [7]. The present day vegetation is the result of a complex interactive network of a large number of different factors (floristic, climatic, pedologic and anthropic impacts).

a

b

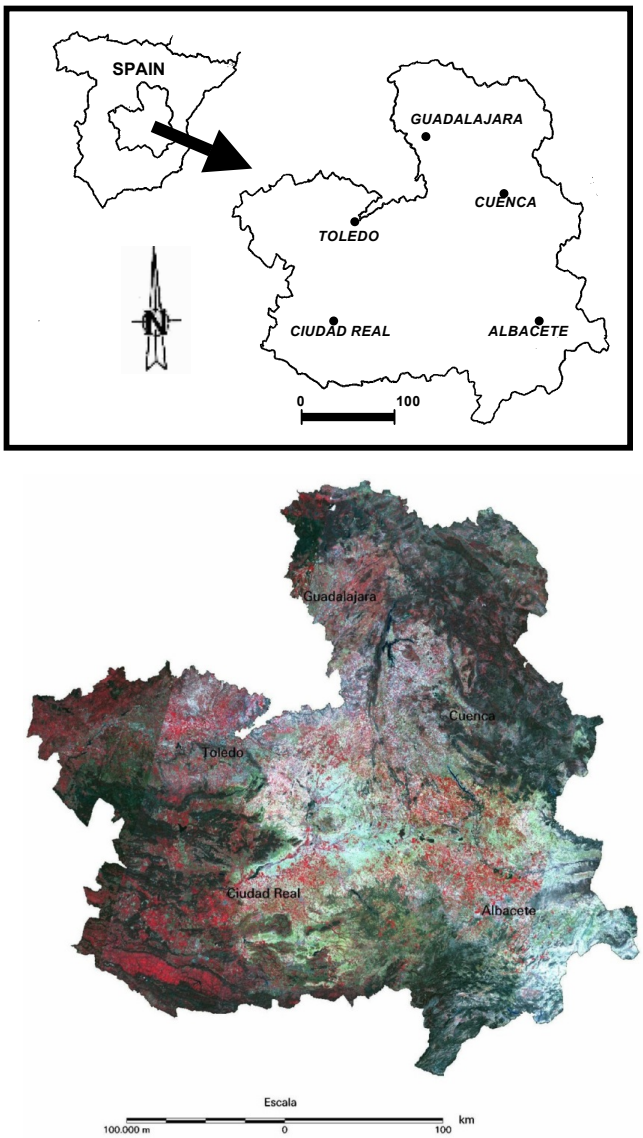


FIGURE 1 - Location map of the studied area (Castilla La Mancha. Spain).

TABLE 1 - General and pedological characteristics of investigated soils.

Profile number	Hor. Type/ Depth (cm)	Soil Type (FAO. 2006)	Location	Parent Material	Land Use	Coordinates
1	Ap 0-28 C 28-92 R> 92	Leptic Regosol (Gypsiric. Siltic)	Ontigola (Toledo)	Gypsums	Uncultivated	0450766 (X) 4425763 (Y)
2	Ap 0-38 Bt 38-72 C> 72	Haplic Luvisol (Chromic. Profondic)	Madridejos (Toledo)	Marls	Dry farming	304534 (X) 4370675 (Y)
3	Ap 0-48 Bt 48-154 C> 154	Vertic Luvisol (Profondic. Rhodic)	La Solana (C. Real)	Argillites	Dry farming	0479715 (X) 4306634 (Y)
4	Ah 0-42 Ck 42-107 2R> 107	Haplic Calcisol (Ruptic.Chromic)	Villahermosa (C. Real)	Sandstones/ quarcites	Forest/ dry farming	519091 (X) 4288625 (Y)
5	Ap 0-8 C 8-25 R> 25	Haplic Leptosol (Skeletic. Novic)	Alcaraz (Albacete)	Quarcites	Pasture	544057 (X) 4211625 (Y)
6	Ap 0-32 Bt 32-84 C> 84	Cutanic Luvisol (Clayic. Rhodic)	Povedilla (Albacete)	Argillites	Dry farming	0540151 (X) 4282000 (Y)
7	Ap 0-4 Bt 4-29 R> 29	Leptic Luvisol (Humic. Chromic)	El Bonillo (Albacete)	Limestones	Forest/ Reforestation	0537754 (X) 4310260 (Y)
8	Ah 0-12 Bwg 12-58 C> 58	Endogleyic Cambisol (Humic. Dystric)	Velada (Toledo)	Granites	Pasture	331058 (X) 4431848 (Y)
9	Ah 0-36 Bt 36-79 2Ck 79-163	Calcic Luvisol (Chromic. Abruptic)	Mirabueno (Guadalajara)	Limestones/ marls	Forest	0521246 (X) 4531563 (Y)
10	Ap 0-12 C 12-23 R > 23	Haplic Leptosol (Calcaric. Skeletic)	Torremocha (Guadalajara)	Limestones/ calcarenites	Scrub/ pasture	0591915 (X) 4540195 (Y)
11	Ap 0-14 Bw 14-82 R> 82	Leptic Cambisol (Calcaric. Chromic)	Alcolea del Pinar (Guadalajara)	Poligenic/ calcarenites	Dry farming/ pasture	543521 (X) 4543529 (Y)
12	Akp 0-34 cm Ck 34-79 2C 79-131	Haplic Regosol (Gypsirc. Calcaric)	Tarancón (Toledo)	Marls/ gypsums	Uncultivated	0504409 (X) 4430745 (Y)
13	Ap 0-21 Bt 21-94 Ckm >94	Cutanic Luvisol (Chromic . Clayic)	Belmonte (Cuenca)	Limestones/ calcarenites	Dry farming	0519693 (X) 4380744 (Y)
14	Ap 0-18 Bw 18-83 2Ckm >83	Haplic Cambisol (Calcaric. Yermic)	Olivares del Jucar (Cuenca)	Marls	Forest	0554157 (X) 4406257 (Y)
15	Ap 0-12 Bw 12-45 R > 45	Leptic Cambisol (Dystric. Skeletic)	S. Cruz De Mudela (C. Real)	Slates/ quarcites	Uncultivated/ Dry farming	459873 (X) 4278354 (Y)
16	Ap 0-32 Bt 32-73 C >73	Calcic Luvisol (Rhodic. Profondic)	Bazán (C. Real)	Quarcites/ slates	Dry farming	0457194 (X) 4270927 (Y)
17	Ap 0-35 Bw 35-82 C >82	Calcic Vertisol (Humic. Pellic)	Valverde (C. Real)	Basalts	Dry farming	409417 (X) 4316228 (Y)
18	Ap 0-15 Bt 15-74 Ckm >74	Calcic Luvisol (Skeletic. Rhodic)	Ocaña (Toledo)	Limestones	Uncultivated	455084 (X) 4423063 (Y)
19	Ap 0-15 C1 15-73 C2m > 73	Haplic Fluvisol (Calcaric. Skeletic)	Balazote (Albacete)	Fluvial sediments	Dry farming	0580442 (X) 4307137 (Y)
20	Ap 0-12 Bw 12-55 R > 55	Leptic Cambisol (Humic. Dystric)	Almadén (C. Real)	Slates	Uncultivated/ Pasture	340446 (X) 4290941 (Y)
21	Ah 0-18 Bw 18-78 C>78	Cambic Umbrisol (Humic. Arenic)	Almorox (Toledo)	Granites	Forest/ Reforestation	0383535 (X) 4460106 (Y)
22	Ah 0-14 2Bt 48-113 3Cg >113	Haplic Acrisol (Ruptic. Profondic)	Viñuelas (Guadalajara)	Rañas	Forest	0470537 (X) 4515645 (Y)
23	Ah 0-5 C 5-25 R > 25	Umbric Leptosol (Humic. Dystric)	Valdepeñas de la S. (Guadalajara)	Quarcites/ slates	Forest	0466594(X) 4529098 (Y)
24	Ah1 0-12 Ah2 12-47 R> 47	Rendzic Leptosol (Humic. Brunic)	El Bonete (Albacete)	Limestones	Scrub/ Forest	0642565 (X) 4306691 (Y)

The selected sites (Figs. 1b, Table 1) are located across the Castilla-La Mancha Community and represent varied typical soils of the vast area (79226 km^2). The undertaking could not have been carried out without knowledge of the soils, their formation, their morphology and their functioning, as Baize and Sterckeman [8] take into account. Most of the territory has a rural character with a great variety of landscapes.

Three horizons (principal) of 24 soil profiles, the A horizon (SUP), B or C-horizon (MED) and the C or R-horizon (SUB) were collected covering environments ranging mainly from arable land zones, forest and pasture, scarcely polluted and some almost pristine. The 72 samples corresponding to different horizon types (Ap, Ah, Bw, Bt and C or R, with or without calcium carbonate accumulation) were sampled between the surface and 150 cm maximum depth, and stored in polyethylene bags. Some details on sampling are given in Table 1 and Fig. 1. The A horizon is usually restricted to a layer with small to moderate amounts of organic matter at the top (typical of soils of Mediterranean environment). The B horizons (which may reach several percentages in calcium carbonate contents), consist of a cambic or argillic horizon [9], sometimes reds. The C horizon is commonly made up of a mixture of clay and partially weathered parent material with concretions (petrocalcic).

Samples were air-dried at room temperature and the <2 mm fraction separated by sieving. The dried samples were ground with agate mortar to below 74 mesh size, and were utilized for subsequent analysis. Aliquots of this fraction were taken randomly for chemical and physico-chemical analysis.

Analytical methods

Total trace element contents of the <2 mm soil samples were determined by XRF (X-ray fluorescence) using the Panalytical Pro Trace Program. Quality control is assured by reference material analyses.

All statistical analyses were performed with the SPSS software (14.1). Thus, the variable analyzed is the quantitative variable "amount of an element", where the elements we are considering in the study are Sc, V, Cr, Ni, Cu, Zn, Ga, Ge, As, Se, Br, Rb, Sr, Y, Zr, Nb, Mo, Ag, Cd, Sn, Sb, I, Cs, Ba, La, Ce, Nd, Sm, Yb, Hf, Ta, W, Tl, Pb, Bi, Th and U. The factor we consider in the study is the horizon: the layer in which the "amount of an element" is measured.

RESULTS AND DISCUSSIONS

Total concentration of trace elements

The median concentrations and ranges of some trace elements are shown in Table 2. Out of the 14 elements that constitute the REE (Rare Earth Elements), only 5 could be detected, Ge, Se, Nb, Mo, Ag, Cd, Sb, Sm, Yb, Hf, Ta, W, Tl, Bi, Th and U are present under detection limits in some samples. The data indicated variations in the amount of trace element contents in soils; the median values were within the range of normal abundance reported from various sources for world soils [3, 10-15]. The local background values can be interpreted as the presence of soil profiles with abundant soil mineral components coupled with scarce anthropogenic incorporation from the associated development activities nearby. This is the case of the Almadén mining district [16-18].

The overall abundance of trace elements in superficial materials depends initially on their concentrations in the bedrocks. Only small differences were found between median data of the surface and the deeper horizons. Only in some profiles, there are clear differences between horizons.

TABLE 2 - Trace element contents and concentration ranges in soils.

Trace element	Concentration mg/Kg	Concentration Range (variation)	Trace element	Concentration mg/Kg	Concentration Range (variation)
Sc	23.0	55.0	**Cd**	4.0	1.8
V	55	196.0	**Sn**	5.1	75.2
Cr	67.6	346.0	**Sb**	2.9	9.9
Co	8.12	64.4	**I**	7.3	68.0
Ni	24.0	188.7	**Cs**	7.6	17.6
Cu	11.9	49.5	**Ba**	408.9	1394.9
Zn	39.5	102.3	**La**	25.2	51.3
Ga	11.8	28.7	**Ce**	60.0	93.5
Ge	1.0	1.5	**Nd**	23.1	52.5
As	7.7	26.4	**Sm**	7.0	11.2
Se	0.8	0.9	**Yb**	2.8	4.2
Br	6.8	33.0	**Hf**	5.0	11.3
Rb	86.0	432.7	**Ta**	2.2	6.3
Sr	369.2	3372.6	**W**	3.2	5.2
Y	18.26	39.8	**Tl**	1.5	2.1
Zr	177.9	523.2	**Pb**	20.7	96.4
Nb	9.9	59.7	**Bi**	1.2	3.0
Mo	1.0	3.9	**Th**	9.7	24.4

Ag	4.3	10.1	**U**	3.8	16.4

Thus, the variety of parent materials could be the main reason causing the difference in the content of soil trace elements. Trace element backgrounds depend firstly on the composition of parent material; therefore, the main proportion of trace elements in the studied soil is inherited from the parent material. Variations were significant, interacting with local lithological variables. The natural concentrations of heavy metals in arable soils depend primarily on the lithological composition [19]. These results showed that traditional agricultural land-use practices do not have important effect on soil quality, and, only at local level, can be sensitive to a change in trace element concentrations. We considered that, in general, trace element contents in common semiarid Mediterranean environments of a region as Castilla-La Mancha are not affected by human activity, except in local places: high levels correlate with the presence of unusual rocks or mineralization-related events, as Reimann and Melezhik [20] found in the Finnish project area. Besides, the nature of weathering processes and migration/accumulation of substances (calcium carbonate, clay minerals. etc.) of these soils can play an important role in the immobilization.

Discussion on some trace elements

Arsenic. There is little difference in its concentration in different rocks. Its concentrations in soils are generally ranging from 0.8 to 27.2 mg/kg (Fig. 2e).

Cadmium. Appears under the detection limit. In general, most soils can be expected to contain <1 mg/kg Cd.

Chromium. Generally, chromium content increases with soil depth, and clay-rich soils have higher Cr concentrations than coarse loamy, sandy and peaty soils. Chromium is known for its low mobility and is found only in soils where the main pedogenetic process has been leaching in clay accompanied by Fe. Its concentrations in soils range from 2 to 348 mg/kg (Fig. 2a).

Copper. Copper is an element that is distributed in distinct quantities in the rock of the terrestrial crust. Its abundance in basaltic rocks is greater than in granitic rocks, whereas the concentration in carbonate rocks is very low. Melting of Cu is the largest single anthropogenic input. Concentrations in rocks range between 0.2-50 mg/kg [21]. Concentrations in soils generally range from 2 to 100 mg/kg [3], agreeing with our results reported in Fig. 2b. In a survey of 4179 Scottish subsoil samples [22], it gave a geometric mean Cu level of 10 mg/kg [5].

Lead. Soils located near industries are a sink for anthropogenic Pb, and there are several well-recognized major sources [5]. The high concentrations of lead are usually attributable to the pollution from combustible fossil fuels [21]. Lead and zinc represent together with cadmium a coherent group of metals, both from the metallogenic viewpoint and as contaminants of the environment [23, 24]. Concentrations in rocks range from 10 to 20 mg/kg [24]. Concentrations in investigated soils ranged from 1.12 to 97.5 mg/kg (Fig. 2g). On the contrary, no high Pb concentrations were observed in the deeper horizons. Lead tends to show the highest concentrations in the surface horizon and decreases with depth.

Nickel. The content of Ni in a soil depends very much on the nature of the parent material In general, clay-rich soils contain higher Ni concentrations than coarse loamy, sandy and peaty soils. Nickel does not show any marked increase with soil depth. Disposal of fly-ash is the largest single input of Ni to soils. as indicated by [25].

Zinc. The total Zn concentration in soils is largely dependent on the composition of the parent materials [2, 26]. Concentrations in soils range from 10 to 300 mg/kg [27]. Zn concentrations in most soils of Western Europe, with the exception of those in mining areas, are consistent with the levels considered as background values [5, 28]. Zn is often considered to be very mobile, particularly in slightly acid soils [29].

Strontium. Appears in high quantity in some soils; is mainly associated with carbonate rocks or with gypsum rocks.

Rare Earths Elements. Severe REE depletion in the upper horizons of an acid soil in France has been shown [25]. We did not find such loss, probably associated to the bedrock and some soil processes.

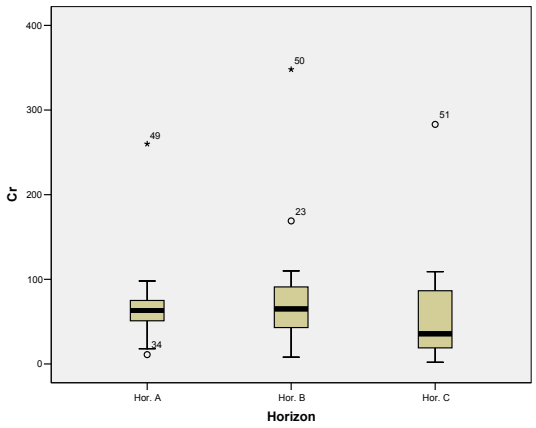
Certain soils in Castilla-La Mancha are still in a relatively primitive state, affected or controlled mainly by natural factors. The physical cracks, weathering, calcium carbonate migration and accumulation, rubefaction and clay illuviation are the dominating soil forming processes. Some of the elements measured show increasing concentration with depth. In the case of Sr, there is an increase with depth (profile 1).

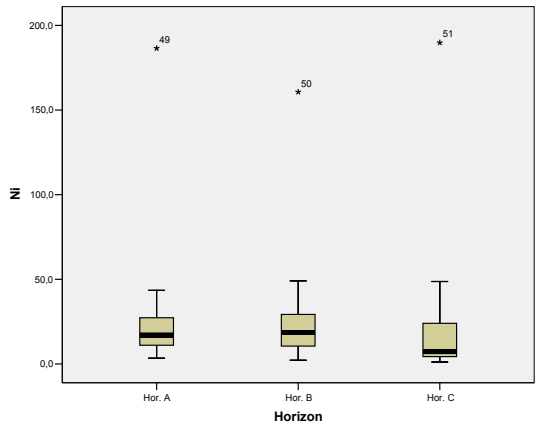
A SUP/SUB-ratio as an indication of relative enrichment/depletion of elements in the uppermost soil layer, which can, to a certain extent, make sense on agricultural land. Here both horizons, the Ap- (SUP) and the deeper B- and/or C (SUB), are predominantly mineral. However, even for agricultural soil, a high value in the SUP/SUB-ratio is no proof of contamination or other human interferences because the SUP-layer is not 1:1 comparable to the SUB-layer. It contains more organic material.

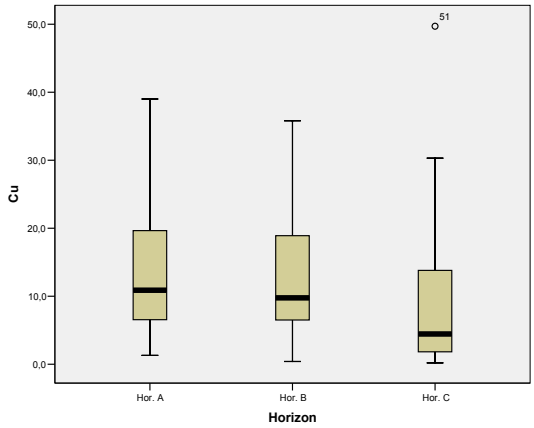
Vertical variation in soil profiles

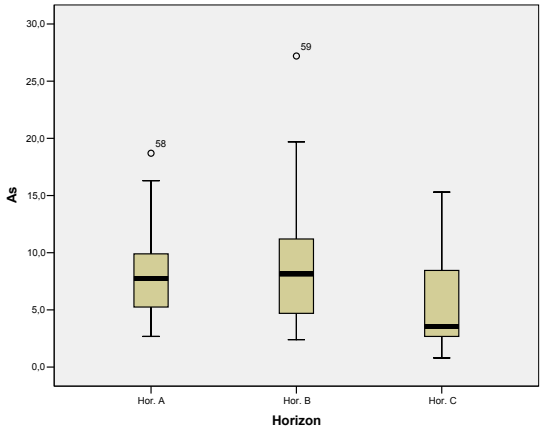
Microelement vertical concentrations showed no significant differences between the means of the "amount of an element" found for the three different horizons (A, B and C). Only zirconium and lead (and to a lesser extent arsenic) show a similar pattern with relative depletion within the bottom soil. These vertical distribution variations can be associated mainly with natural sources that seem to depend mainly on the bedrock and secondly on soil processes,

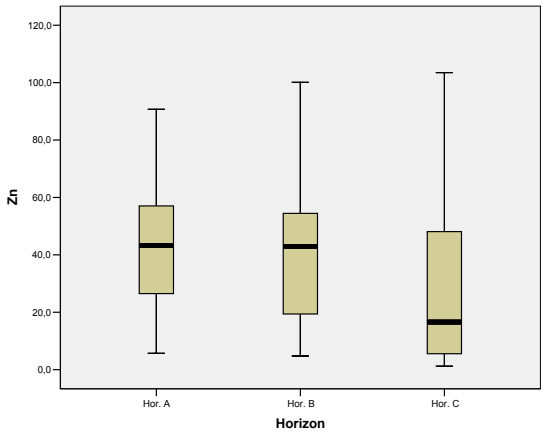
such as accumulation of calcium carbonate, leaching of elements due to dissolution of host minerals, etc. The fact that the concentration of the trace elements diminished in the calcic horizons could be the result either of dilution or

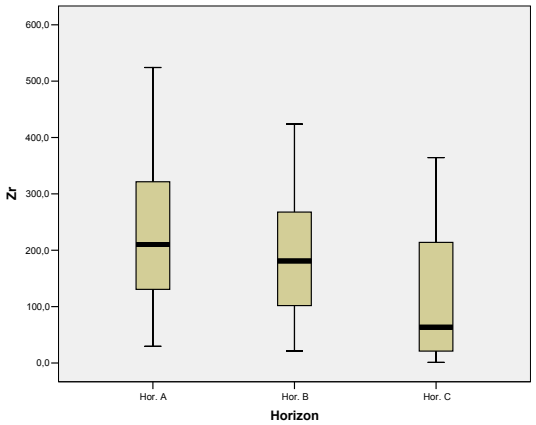












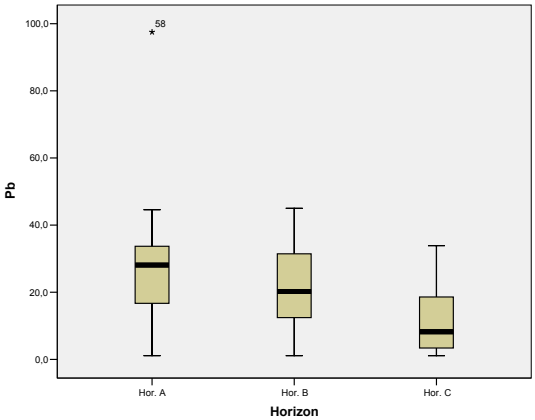


FIGURE 2 - Box-plots showing graphically the differences between the values for trace elements (in mg/kg): a) Cromium, b) Copper, c) Zinc, d) Nickel, e) Arsenic, f) Zirconium and g) Lead. The outliers for the studied area were also indicated by stars and circles.

of the carbonates having accumulated at a different depth from that normally accumulating these elements. Other factors could be the underlying young, loose sediments (material mobilized by water, wind or gravitation).

Concentrations and vertical distribution of trace elements are controlled by two factors: the mineralogical composition of the starting material and the geochemical environment (weathering and pedogenesis). Among the latter, the most important factors governing their behavior in the soil are pH, redox potential and the amount and type of colloids: clay minerals and organic matter [1, 11]. The small precipitation to evaporation ratio does not allow migration of elements down the profile. The result is scarce leaching of trace metals. The SUP-layer is often relatively depleted in the fine fraction (clay-size particles and oxyhydroxides), a process known as lessivation. Clay size particles commonly contain much higher metal concentrations than the coarser soil size fractions. The leaching of carbonates and lessivation appear to indicate that the current climate is drier than the wetter climate under which the soil must have formed. This wetter climate must have also determined the distribution of the trace elements in the soil. This process can lead to their relative depletion in the SUP-layer. Most of the trace elements are highly enriched in the fine fraction of soils as a result of the processes of weathering [31, 32]. Evidence from the data demonstrates that only 3 elements (out of 38 analyzed) show a general enrichment in the SUP or MED-layer. These are As, Zr and Pb. This combination of elements indicates that contamination is not the most likely explanation of the enrichment.

Statistical analysis

The second aim of this paper is to carry out a statistical analysis to test whether there are differences between the means of the "amount of an element" to three different levels in the soils. An Analysis of Variance (ANOVA) was carried out (Table 3) for each variable "amount of an element" (where the elements are Sc, V, Cr, and so on), with respect to the factor "horizon". The main conclusions in the statistical analysis are as follow:

1. There are no significant differences between the means for the following elements, at the three different horizons (A, B and C): Sc, V, Cr, Ni, Cu, Zn, Ga, Ge, Se, Br, Rb, Sr, Y, Nb, Mo, Ag, Cd, Sn, Sb, I, Cs, Ba, La, Ce, Nd, Sm, Yb, Hf, Ta, W, Tl, Bi, Th and U. The reason is that, in all these cases, p-values in the ANOVA are greater than 0.05 (Table 3). Figures 2 (a,b,c,d, box-plots) graphically show the situation for some of the most important

TABLE 3 - Statistical analysis: ANOVA of some trace elements.

		SS	df	MS	F	p-value
Sc	Between groups	790.083	2	395.042	2..271	0.111
	Within groups	12000.792	69	173.925		
	Total	12790.875	71			
V	Between groups	2539.669	2	1269..835	0.701	0.5
	Within groups	125032.993	69	1812.072		
	Total	127572.662	71			
Cr	Between groups	5808.111	2	2904..056	0.852	0.431
	Within groups	235311.542	69	3410..312		
	Total	241119.653	71			
Co	Between groups	10.914	2	5.457	0..032	0.969
	Within groups	11822.144	69	171.335		
	Total	11833.059	71			
Ni	Between groups	372.991	2	186.496	0..148	0.862
	Within groups	86687.572	69	1256..342		

	Total	87060.563	71			
Cu	Between groups	151.809	2	75.905	0.691	0.505
	Within groups	7584.055	69	109.914		
	Total	7735.864	71			
Zn	Between groups	2255.948	2	1127.974	1.469	0.237
	Within groups	52971.111	69	767.697		
	Total	55227.059	71			
Ga	Between groups	69.688	2	34.844	0.566	0.57
	Within groups	4245.056	69	61.523		
	Total	4314.744	71			
Ge	Between groups	0.023	2	0.011	0.445	0.643
	Within groups	1.746	69	0.025		
	Total	1.769	71			
As	Between groups	163.855	2	81.928	3.445	0.037
	Within groups	1640.941	69	23.782		
	Total	1804.796	71			
Zr	Between groups	142098.170	2	71049.085	5.102	0.009
	Within groups	960783.343	69	13924.396		
	Total	1102881.513	71			
Pb	Between groups	2927.702	2	1463.851	7.019	0.002
	Within groups	14390.494	69	208.558		
	Total	17318.196	71			

elements. In particular, Cr, Cu, Zn and Ni have been chosen. Of course, the mean values we obtain for each element for the three different horizons (A, B and C) are not exactly the same, but the differences found are not significant from the viewpoint of the ANOVA (at significance level 0.05).

2. There are significant differences between the means for As found for the three different horizons (A, B and C) because, in this case, the p-value in the ANOVA is 0.037 (Table 3). We then carried out multiple comparisons between the three pairs of horizons (by applying the Bonferroni correction) revealing significant differences only between horizons B and C (Table 4a). It is important to verify that the hypotheses of the statistical model (Normality and Equal Variances) are fulfilled. The hypothesis of Normality is acceptable in this case because the p-value of the Kolmogorov-Smirnov test for the Normality of Residuals is 0.228. The hypothesis of Equal Variances is also acceptable in this case because the p-value for Levene's statistic is 0.258. Figure 2 (e, box-plot) graphically shows the differences between the values for As found for the three different horizons (A, B and C).

TABLE 4a - Multiple comparisons between the three pairs of horizons (by applying Bonferroni correction): case As

Horizon	**Horizon**	**p-value**
Hor. A	Hor. B	1
Hor. A	Hor. C	0.277
Hor. B	Hor. C	0.036

3. There are very significant differences between the means for Zr found for the three different horizons (A, B and C) because, in this case, the p-value in the ANOVA is 0.009 (Table 3). We then carried out multiple comparisons between the three pairs of horizons (by applying the Bonferroni correction), and found significant differences only between horizons A and C (Table 4b). It is important to verify that the hypotheses of the statistical model (Normality and Equal Variances) are fulfilled. The hypothesis of Normality is acceptable in this case because the p-value of the Kolmogorov-Smirnov test for the Normality of Residuals is 0.193. The hypothesis of Equal Variances is also acceptable in this case because the p-value for Levene's statistic is 0.868. Figure 2 (f box-plot) graphically shows the differences between the values for Zr found for the three different horizons (A, B and C).

TABLE 4b - Multiple comparisons between the three pairs of horizons (by applying Bonferroni correction): case Zr

Horizon	**Horizon**	**p-value**
Hor. A	Hor. B	0.980
Hor. A	Hor. C	0.008
Hor. B	Hor. C	0.108

4. There are very significant differences between the means for Pb found for the three different horizons (A, B and C) because, in this case, the p-value in the ANOVA is 0.002 (Table 3). We then carried out multiple comparisons between the three pairs of horizons (by applying Bonferroni correction), and found significant differences only between horizons A and C (Table 4c). It is important to verify that the hypotheses of the statistical model (Normality and Equal Variances) are fulfilled. The hypothesis of Normality is acceptable in this case because the p-value of the Kolmogorov-Smirnov test for the Normality of Residuals is 0.619. The hypothesis of Equal Variances is also acceptable in this case because the p-value for Levene's statistic is 0.610. Figure 2 (g, box-plot) graphically shows the differences between the values for Pb found for the three different horizons (A, B and C).

TABLE 4c - Multiple comparisons between the three pairs of horizons (by applying Bonferroni correction): case Pb

Horizon	**Horizon**	**p-value**
Hor. A	Hor. B	0.620
Hor. A	Hor. C	0.001
Hor. B	Hor. C	0.055

All the tools we have used for the statistical analysis can be found, for instance, in Townend [33].

As remarked, some authors, such as Blaser et al. [34], have suggested that element concentrations measured in a

deeper soil horizon can be taken as local background for the upper soil horizons that are more likely to be affected by anthropogenic contamination. Considerably higher metal values in overlying soil horizons are often interpreted as evidence of anthropogenic contamination [35]. A large proportion of toxic concentrations of trace elements is introduced from cultivation methods, such as pesticides addition, fertilization etc. [36]. Therefore, since there was no significant difference between the superficial and sub-superficial horizons, calculation of the "generic reference levels" can be searched indistinctly through any of them. Finally, distinguishing between the baseline and background levels becomes less relevant in soils of Castilla La Mancha.

CONCLUSIONS

The trace element concentrations were mostly within normal ranges in Castilla La Mancha (Mediterranean semi-arid environment) and are controlled by the geochemical characteristics of the underlying rock and the nature of weathering processes and migration/accumulation of substances (such as calcium carbonate). Natural variations of element concentrations in different pedological and environmental sample materials will often be very small. This study has confirmed the fact that the knowledge of the trace elements content of soils recognizes the effect of parent materials and, to a lesser extent, of pedogenic processes. Therefore, the background depends on location and the scale of the area investigated. The result is scarce leaching of trace metals, because the small ratio of precipitation to evaporation does not allow migration of elements down the profile. Only zirconium and lead (and to a lesser extent arsenic) show a similar pattern with relative depletion within the bottom soil.

Results also indicated that baseline concentrations in common soils in semiarid Mediterranean environments, such as Castilla La Mancha, are not significantly affected by human impact. No concentration value was considered to be pollutant. Therefore, the present study indicates that the analyzed trace elements do not pose environmental risks under the existing physicochemical environment. Finally, since there was no significant difference between the superficial and sub-superficial horizons, we propose to search the "generic reference levels" indistinctly through either one.

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STUDY ON HIGH EFFICIENT POLLUTANT-DEGRADING BACTERIA FOR THE TREATMENT OF WASTEWATER

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ABSTRACT

Four types of high efficient pollutant-degrading bacteria: DWT-2, DWT-4, DWT-6 and DWT-7, which could be inoculated for enhancing biological treatment of domestic wastewater, were isolated from sludge samples. Based on their morphological, physiological and biochemical characteristics, these bacteria were identified as *Alcaligenes sp.*, *Pseudomonas sp.*, *Klebsiella sp.* and *Pseudomonas sp.*. DWT-2 and DWT-4 were identified as highly efficient COD-degrading bacteria, and DWT-6 and DWT-7 were identified as highly efficient phosphorus-degrading bacteria on the basis of bench scale experiments. Experiment was conducted to assess the performance of wastewater treatment with different bacterial combinations. The performances of three combinations of DWT-2 and DWT-7 with proportions of 2:1, 1:1 and 1:2 were relatively good. The COD and phosphorus removal efficiencies of those combinations, which could be used for inoculation for enhancing biological degradation of organic matter and phosphorus from domestic wastewater, were 69.5-74.3% and 72.6- 80.6%, respectively. Influences of inoculum percentages, rotation speeds, incubation temperatures and initial pH values on COD and phosphorus removal were also studied in flask-level optimization experiments.

KEYWORDS: Domestic wastewater; wastewater treatment; biological degradation; isolation; inoculation.

INTRODUCTION

Sullage and black water discharges has become a significant water pollution source as they seriously affect both surface and ground water qualities [1-3]. State Environmental Protection Administration of China (SEPAC) [3] reported that in 2006, 54.40% of wastewater came from domestic wastewater sources, overall Chinese domestic discharges increased by 29.18% between 2001 and 2006. A large proportion of households throughout the world use onsite wastewater treatment systems, because there are no service connections providing discharge to central wastewater collection and treatment systems [1, 4].

Some of designs are constructed and utilized as an alternative to onsite wastewater treatment systems, such as constructed wetlands, waste stabilization ponds, and biofilters [1, 5-8]. It was also reported that the removal efficiencies of organic matter and phosphorus were less efficient in some systems [4, 9]. Biological organic matter and phosphorus removal can be enhanced by inoculating highly efficient pollutant-degrading bacteria [10, 11].

Isolation and screening of bacteria and optimization of process parameters, inoculum percentage, shaker rotation speed, incubation temperature and initial pH are significant for the success of inoculation for enhancing biological treatment of domestic wastewater. In this study, high effective pollutants-degrading bacteria which could be used for enhancing biological pollutants removal from domestic wastewater were isolated and identified from sludge samples, and the effects of process parameters on the performance of treatment were also studied based on the bench scale experiments.

MATERIALS AND METHODS

Synthetic Wastewater

In order to avoid the interference of bacteria from domestic wastewater, disinfected synthetic wastewater of a similar composition with domestic water was used in this study. Synthetic wastewater comprising distilled water, glucose (0.469 g/L), urea (0.086 g/L) and potassium dihydrogen phosphate (0.035 g/L) was selected as feed for the experiment. The main constituents of the synthetic wastewater were as follows: chemical oxygen demand (COD), 500 mg/L; total phosphorus (TP), 8.0 mg/L; total nitrogen (TN), 40.0 mg/L; pH, 7.1.

Isolation and Screening of Bacteria

The bacteria used in this study were isolated from sludge samples and collected from domestic wastewater receiving tank near Lake East in Wuhan, China. First, sludge samples (5 g) were added to 100 ml sterile water, the suspend liquid was then mixed on an orbital shaker at a temperature of 30ºC at 150 rpm for 48 hours. Second, 10 ml of standing supernatant liquid was collected and added to a medium which consisted of KH_2PO_4, 0.5g; K_2HPO_4, 0.5 g; $MgSO_4$, 0.2 g; $CaCl_2$, 0.1 g; NaCl, 0.2 g; NH_4NO_3, 1 g; $MnSO_4$, 0.05g; 10 % $FeCl_2$, 0.05 ml; and synthetic wastewater, 100 ml. The initial pH of the medium was adjusted to 7.2-8.0. Then, the liquid was mixed on an orbital shaker at 30ºC at 150 rpm for 48 hours. The second step was repeated for three times to prepare inoculation liquid. After that the dilutions (0.1 ml) were seeded on Luria broth (LB) (containing 5 g/L yeast, 10 g/L protein and 5 g/L NaCl; pH adjusted to 7.2-7.4) agar plates and incubated at 30ºC. Well-proportioned colonies on LB agar plates were selected for analysis according to the size, color and the configuration of the surface and the edge of the colony [12]. All bacterial isolates (10 ml of each) were added into conical flasks containing 70 ml of synthetic wastewater and incubated on an orbital shaker at 150 rpm at 30ºC, for 24 hours. Screening of bacteria was made according to the pollutants degradation efficiency.

Identification of Bacterial Isolates

Morphological and physiological characterizations were defined according to Bergey's Manual of Determinative Bacteriology [13]. Morphological observations included shape, colour and size of the colony. Physiological identification included: gram staining, oxidase, catalase, aerobic experiments, hydrogen sulfide production, arginine dihydrolase, L-arginine, nitrate reduction, hydrolysis of gelatin, voges-proskauer test, methyl red test, fructan formation, indole production, pyocyanine, fluorescent pigment, glucose utilization, phenylalanine dehydrogenation, D-xylose fermentation and hydrolysis of starch.

Wastewater Treatment Efficiencies with Different Bacterial Combinations

According to the result of experiment 2.2, high efficient COD-degrading bacteria (DWT-2 and DWT-4) and high efficient phosphorus-degrading bacteria (DWT-6 and DWT-7) were mixed in different combinations (Table 1). Each type of bacterial combination was labeled by their species and proportion number (e.g., A-1 represents that DWT-2 bacterium and DWT-6 are mixed in a proportion of 2:1, see Table 1). All inoculations were made at 5% i.e. the equivalent of 5 ml inoculum to 100ml synthetic wastewater and incubated on an orbital shaker at 30ºC at 150 rpm for 24 hours.

TABLE 1 - Proportion of added bacterium.

Number	Combination	1	2	3
A	C1+P1	2:1	1:1	1:2
B	C1+P2	2:1	1:1	1:2
C	C2+P1	2:1	1:1	1:2
D	C2+P2	2:1	1:1	1:2

Optimization of Process Parameters

Process parameters, such as inoculum percentage, shaker rotation speed, incubation temperature and initial pH, were optimized at flask-level. According to the result of experiment 2.4, the bacterial combinations of DWT-2 + DWT-7 with three different proportions (B-1, B-2 and B-3) were selected to assess the effects of process parameters on the pollutants degradation.

Three bacterial combinations (B-1, B-2 and B-3) were inoculated into synthetic wastewater in conical flasks containing 100ml of synthetic wastewater. The initial pH of each inoculation was adjusted to 8.0. All of three groups' inoculations were made at 1%, 5%, 10%, 15% and 20%. The conical flasks were kept on the orbital shaker at 30ºC at 150 rpm for 24 hours. Samples were withdrawn from the conical flasks, centrifuged and analyzed for residual pollutants concentrations.

Three bacterial combinations (B-1, B-2 and B-3) were inoculated to synthetic wastewater in conical flasks containing 100ml of synthetic wastewater, initial pH of each inoculation was adjusted to 8.0. Inoculations of B-1, B-2 and B-3 were made at optimum inoculum percentages (5%, 5% and 10%, respectively.). Every inoculation was incubated at 30ºC for 24 hours at different rotation speed: 100 rpm, 120 rpm, 150 rpm and 180 rpm, respectively. Samples were withdrawn from the conical flasks, centrifuged and analyzed for residual pollutants concentrations.

Three bacterial combinations (B-1, B-2 and B-3) were inoculated to synthetic wastewater in conical flasks containing 100ml of synthetic wastewater, initial pH of each inoculation was adjusted to 8.0. Inoculations of B-1, B-2 and B-3 were made at optimum inoculum percentages (5%, 5% and 10%, respectively.) and incubated at optimum rotation speeds (150 rpm, 150 rpm and 120 rpm, respectively.) for 24 hours at different temperatures: 20ºC, 30ºC, 37ºC, 40ºC and 45ºC, respectively. Samples were withdrawn from the conical flasks, centrifuged and analyzed for residual pollutants concentrations.

Three bacterial combinations (B-1, B-2 and B-3) were inoculated to synthetic wastewater in conical flasks containing 100ml of synthetic wastewater, initial pH values were adjusted to 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 and 11.0, respectively. For each pH value, inoculations of B-1, B-2 and B-3 were made at optimum inoculum percentages (5%, 5% and 10%, respectively.) and incubated at optimum temperatures (30 ºC) at optimum rotation speeds (150 rpm, 150 rpm and 120 rpm, respectively.) at different pH values in the range of 5.0-11.0 for 24 hours. Samples were withdrawn from the conical flasks, centrifuged and analyzed for residual pollutants concentrations.

Water Quality Analysis

Water samples were analyzed for COD, TP, TN, temperature and pH according to standard methods for the Examination of Water and Wastewater [14].

Statistical Analyses

All statistical analyses were performed using the software Origin 7.0 and Microsoft Excel. Differences with $P < 0.05$ were considered as statistically significant.

RESULTS AND DISCUSSION

Isolation and Screening of Bacteria

Primarily, about 7 types of domestic wastewater-treating bacteria designated as DWT-1 to DWT-7 were isolated and screened form sludge samples. Those bacteria were used for further screening on the basis of their performances of synthetic wastewater treatment. The synthetic wastewater treatment efficiencies with those 7 types of bacteria are summarized in Table 2. The average removal efficiencies for COD, phosphorus and nitrogen by 7 types of bacteria were 41.3%, 45.8% and 16.7%, respectively. It can be seen that DWT-2 and DWT-4 with COD removal rates of 58.9% and 60.4% were higher than other bacteria in terms of COD removal, and the COD removal efficiency of DWT-3 was the lowest. DWT-6 and DWT-7 performed very well for phosphorus removal, the corresponding removal efficiencies were 67.9% and 70.5%, respectively. This result indicated that DWT-6 and DWT-7 were heterotrophic bacteria. The nitrogen removal efficiencies which were all less than 30% were relatively low comparing to those of COD and phosphorus. The linear regression analysis was used to assess the relationship between the degraded COD and phosphorus concentrations by 7 types of domestic wastewater-treating bacteria (Equation 1). The result showed that the COD and phosphorus were up consumed by bacteria with a proportion of 500:9 approximately.

$$\text{Phosphorus (mg/L)} = 0.018 \times \text{COD (mg/L)} + 0.393 \quad R=0.738 \quad P=0.038 \qquad (1)$$

TABLE 2 - Synthetic wastewater treatment performances of 7 types of bacteria.

Bacteria	Removal rates (%)		
	Chemical oxygen demand	Total phosphorus	Total nitrogen
DWT-1	29.0	21.1	10.8
DWT-2	58.9	56.1	12.9
DWT-3	18.0	30.7	28.8
DWT-4	60.4	54.7	15.8
DWT-5	30.0	19.8	14.1
DWT-6	46.8	67.9	20.5
DWT-7	45.9	70.5	13.7

Finally, DWT-2 and DWT-4 were selected as highly efficient COD-degrading bacteria, and DWT-6 and DWT-7 were selected as highly efficient phosphorus-degrading bacteria for the subsequent experiments. The growth curves of those four bacteria types are shown in Fig. 1. It can be seen that the logarithmic phases of bacterial growth of 4 types of bacteria were reached after 4 hours, and stationary phases were reached after 20 hours approximately.

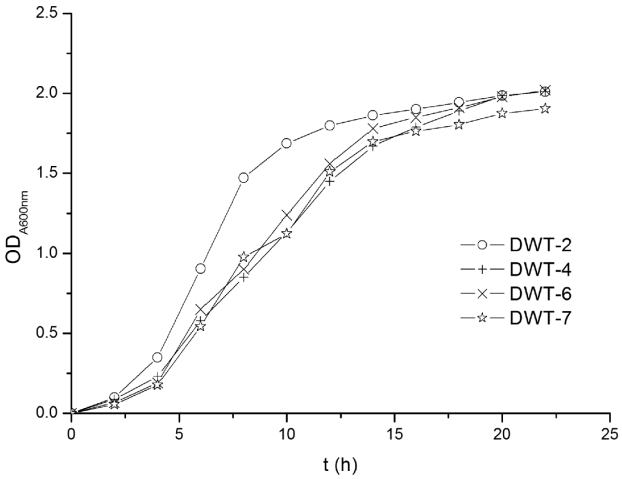

FIGURE 1 - Growth curves of DWT-2, DWT-4, DWT-6 and DWT-7.

Identification of Bacteria

On the basis of morphological, physiological and biochemical characteristics (Table 3), DWT-2 belonged to *Alcaligenes sp.*, DWT-4 and DWT-7 were identified as *Pseudo-monas sp.*, DWT-6 was identified as *Klebsiella sp.* [13].

Wastewater Treatment Efficiencies with Different Bacterial Combinations

After 24 hours incubation, water samples were collected and analyzed to evaluate COD and phosphorus removal efficiencies. Fig 2 shows the performances of COD and phosphorus removal with different bacterial combinations. It can be seen that the COD removal rate of B-3 was the highest (74.3%), and D-2 had the lowest COD removal rate (41.2%). The combinations of B-1, B-2 and B-3 (DWT-2 + DWT-7) performed very well for COD removal. On the contrary, the COD removal rates of D-1, D-2 and D-3 (DWT-4 + DWT-7) combinations were lower than the other combinations. As shown in Fig. 2 (b), the COD removal rate of B-2 (80.6%) was the highest while that of A-2 was the lowest (50.3%). The efficiencies of B-1, B-2 and B-3 (DWT-2 + DWT-7) combinations were the highest in terms of phosphorus removal which is equal to COD removal rates for the above combinations. The phosphorus removal efficiencies of the A-1, A-2 and A-3 (DWT-2 + DWT-6) combinations were the lowest. It was reported that *Acinetobacter calcoaceticus* accumulated 80.5% of phosphate in a medium containing 174 mg PO_4/L [15]. Qin *et al.* [16] showed that the species isolated from activated sludge removed 30% of phosphate from the media containing 315 mg PO_4/L. Moreover, the phosphate-accumulating bacteria studied by Merzouki *et al.* [17] accumulated phosphate with rates of 30-70 mg P/L/h in the A/O sequencing batch reactor and 15-32 mg P/L/h in the A_2 sequencing batch reactor.

The result indicates that the bacterial combinations with DWT-2 and DWT-7 were better than other bacterial

combinations for both COD and phosphorus removal.

TABLE 3 - Proportion of added bacterium.

Characteristics	DWT-2	DWT-4	DWT-6	DWT-7
Color of colonies	Milk white	Green	White	Milk white
Colony morphology	Rod-shaped, flagella	Short rod-shaped, polar flagella	Globular, no flagella	Rod-shaped, flagella
Gram staining	-	-	-	-
Oxidase	+	+	-	+
Catalase	+	+	+	+
Aerobic experiments	Strictly aerobic	Aerobic	Facultative anaerobic	Aerobic
Hydrogen sulfide production	-	-	-	-
Arginine dihydrolase	-	+	-	+
L-arginine	-	+	-	+
Nitrate reduction	+	+	+	+
Hydrolysis of gelatin	+	+	+	+
Voges-proskauer test	+	+	+	-
Methyl red test	+	+	+	-
Fructan formation	-	-	+	-
Indole production	+	+	-	+
Pyocyanine	-	+	-	+
Fluorescent pigment	-	+	-	+
Glucose utilization	-	+	-	+
Phenylalanine dehydrogenation	-	-	-	-
D-xylose fermentation	+	+	+	+
Hydrolysis of starch	+	-	+	+
Nitrate reduction	+	+	+	+
Hydrolysis of gelatin	+	+	+	+
Voges-proskauer test	+	+	+	-

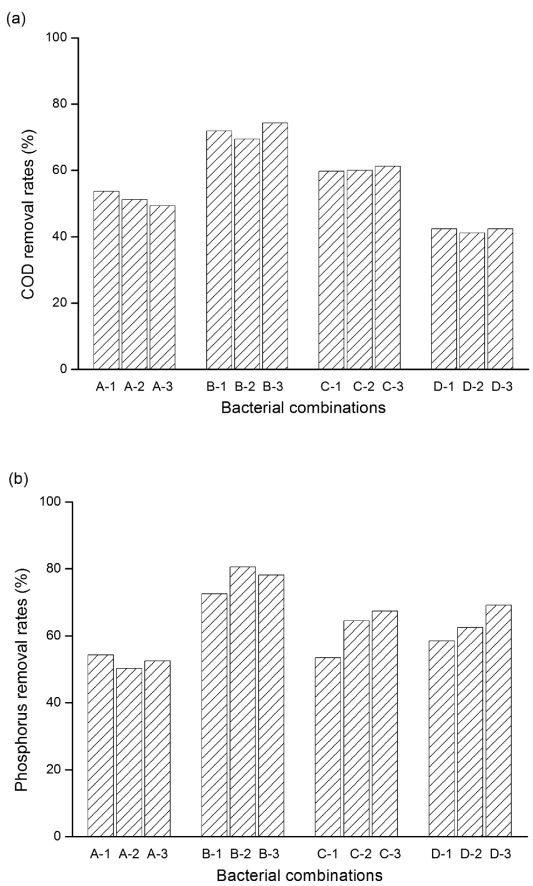

FIGURE 2 - The (a) chemical oxygen demand (COD) and (b) phosphorus removal rates with different bacterial combinations.

Effect of inoculum percentage

Fig. 3 shows the performances of COD and phosphorus degradation at different inoculum percentages. It indicates that the optimum inoculum percentage of B-1, B-2 and B-3 for COD and phosphorus removal reached the values form 5% to 10%, respectively. At the optimum inoculum percentages, inoculations with B-1 and B-3 were effective, with 60-65% COD removal efficiencies. On the contrary, the COD removal efficiencies in terms of inoculations with B-2 were relatively low; B-2 had the best COD removal efficiency of 45.6% at the inoculum percentage of 10%. Inoculations with B-3 had best phosphorus removal efficiency (78.3%) at the optimum inoculum percentage (10%). In terms of B-1 and B-2, the phosphorus removal efficiencies decreased by 3.9% and 1.6% respectively while the inoculum percentage increased from 5% to 10%.

Effect of Rotation Speed

The effect of shaker rotation speed on the COD and phosphorus removal is shown in Fig. 4. The increase in rotation speed from 100 rpm to 150 rpm resulted in gradual increase in COD removal efficiency of B-1 and B-2. It indicated that as the rotation speed increased, more surface area was made available. The oxygen mass and nutrient mass transfer were high, as gas-liquid oxygen transfer and solid-liquid nutrient mass transfer were critical in determining the activity of bacteria [18]. Further increase in rotation speed to 180 rpm resulted in the reduction in COD removal efficiencies of B-1 and B-2. The variations of the phosphorus removal efficiency of B-2 were similar

to the COD removal of B-2, but the phosphorus removal efficiency of B-1 increased gradually as the rotation speed

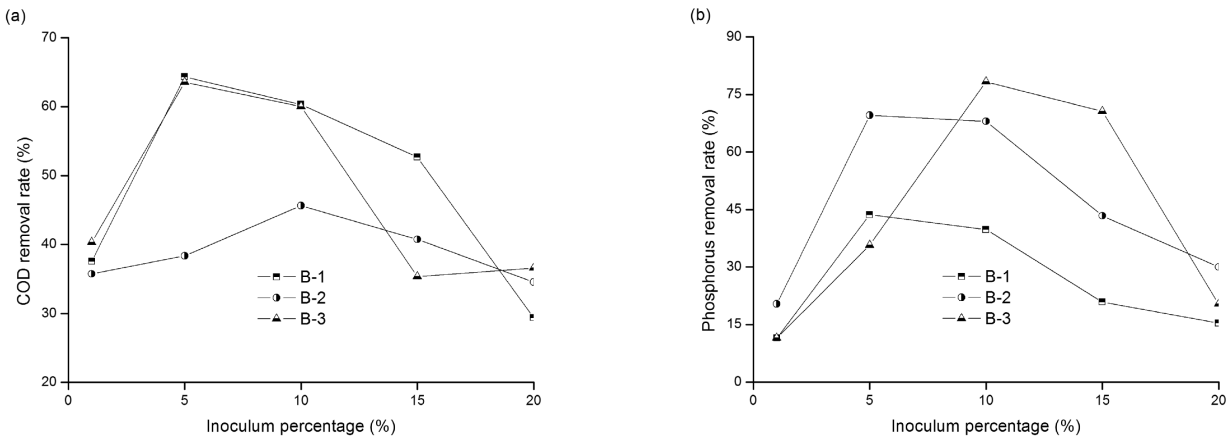

FIGURE 3 - Effect of inoculum percentage on the (a) chemical oxygen demand (COD) and (b) phosphorus removal

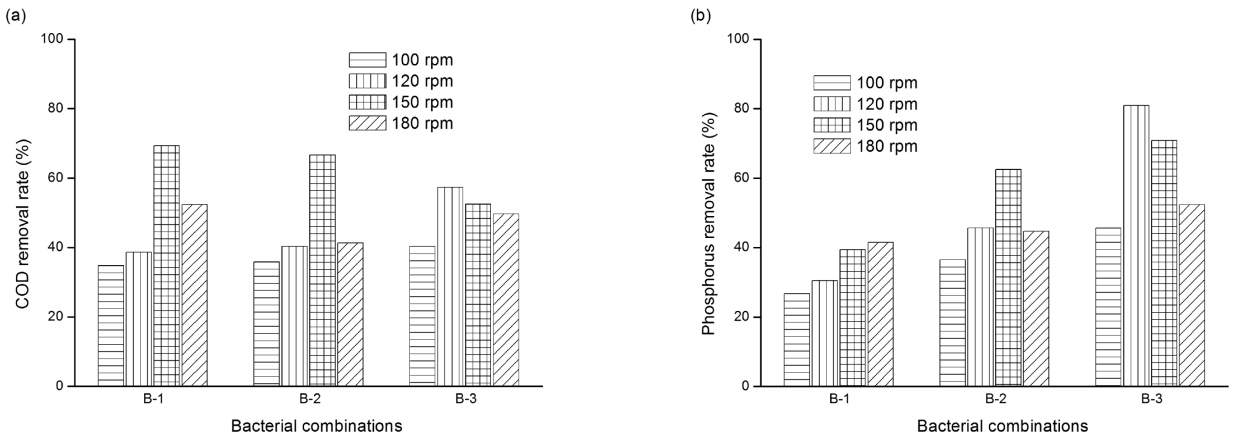

FIGURE 4 - Effect of rotation speed on the (a) chemical oxygen demand (COD) and (b) phosphorus removal

increased. The result indicates that the optimum rotation speeds for B-1 and B-2 were 150 rpm to 180 rpm. The COD and phosphorus removal rates of B-1 and B-2 were 69.4% and 41.5%, 66.7% and 62.5%, at the optimum speed.

In terms of B-3, the COD and phosphorus removal rates were highest at the optimum rotation speed of 120 rpm: 57.4% and 81.0%, respectively.

Effect of Incubation Temperature

Temperature is an important environmental parameter and has been known to effect the biomass composition, growth, nutrient requirement, nature of metabolism and the metabolic reaction rate for decades [19, 20]. The effect of the temperature on pollutants degradation was studied in batch culture (Fig. 5). The degradation of COD and phosphorus was less effective when incubation temperatures were above 40ºC or below 30ºC, at which the activities of three bacterial combinations would be relatively low. The optimum B-1, B-2 and B-3 temperatures for COD degradation were 30ºC, 30ºC and 37ºC, respectively. B-1 showed higher capacity for COD degradation than others. The COD removal rate of B-1 at incubation temperature of 37ºC (69.5%) was slightly lower than that at 30ºC (73.4%). The optimum temperatures of B-1, B-2 and B-3 for phosphorus degradation were also in the range of 30ºC - 40ºC. At those temperatures, the mean phosphorus removal rate of B-3 was 72.7±5.1%, which was relatively higher than those of B-1 and B-2 with efficiencies of 36.8±0.6% and 34.3±11.2%, respectively. B-3 had the highest phosphorus removal efficiency of 78.7% at 30ºC, and incubation temperature of 37ºC, B-1 and B-2 had the highest phosphorus removal efficiencies of 37.5% and 41.0%, respectively.

Effect of pH

Fig. 6 shows the performances of COD and phosphorus removal at different initial pH values in the range of

5.0-11.0. This range is typical in wastewater treatment plants. The bacteria studied were acid sensitive and less

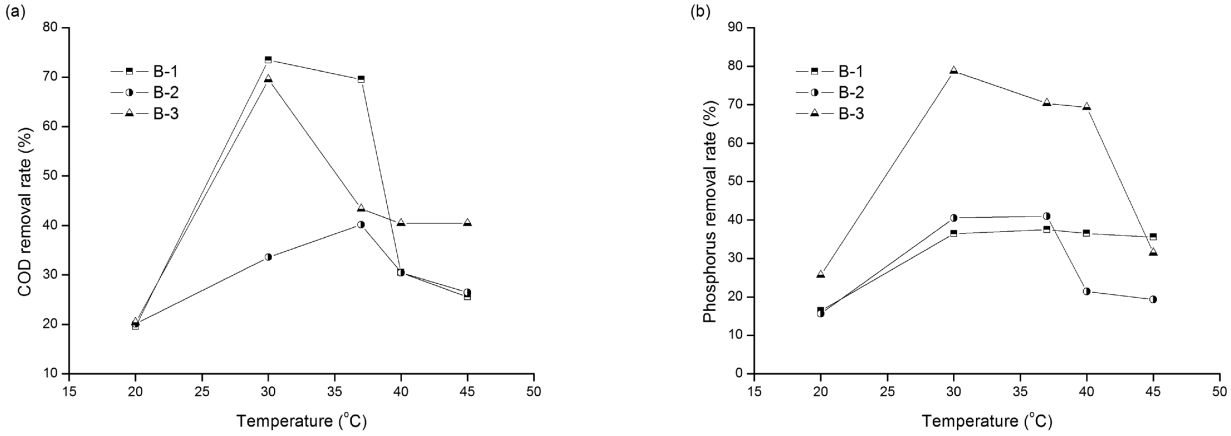

FIGURE 5 - Effect of temperature on the (a) chemical oxygen demand (COD) and (b) phosphorus removal

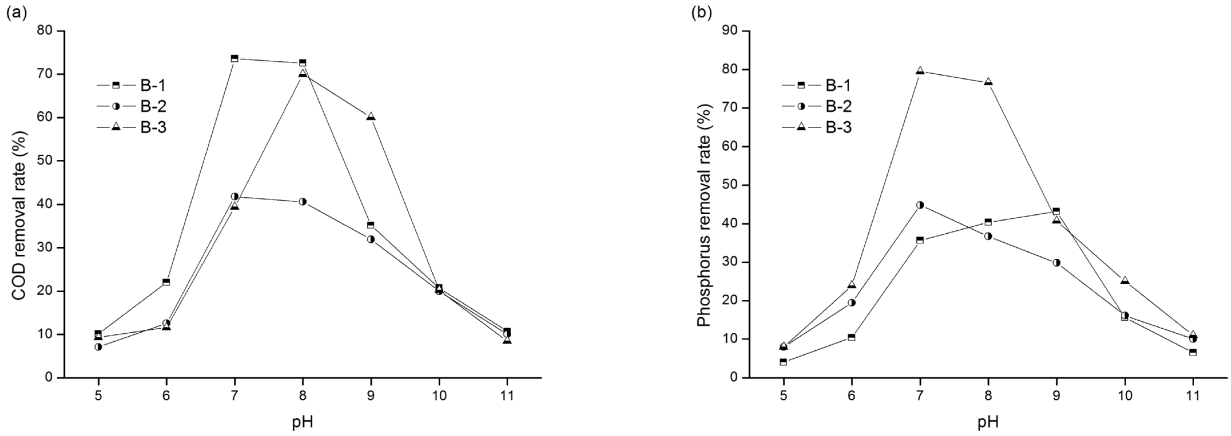

FIGURE 6 - Effect of pH on the (a) chemical oxygen demand (COD) and (b) phosphorus removal

alkaline sensitive. The activities and quantities of bacteria isolates would be lower when initial pH values were below 6.0 or above 10.0. Neutral pH values (7.0-8.0) were the optimum pH values for both COD and phosphorus degradation. At neutral pH, the COD removal efficiency of B-1 and the phosphorus removal efficiency of B-3 were the highest. The highest COD removal efficiencies of B-1, B-2 and B-3 were 73.6%, 41.8% and 70.0% at pH 7.0, 7.0 and 8.0, respectively. In terms of phosphorus removal, those were 43.2%, 44.8% and 79.4% at pH 9.0, 7.0 and 7.0, respectively. The effect of pH in the range of 7.0-8.0 on phosphorus degradation was not obvious.

CONCLUSION

Based on the bench scale experiments conducted in this study, the conclusions are as follows:

- Based on the morphological, physiological and biochemical characteristics, two types of high efficient COD-degrading bacteria (DWT-2 and DWT-4) and another two types of high efficient phosphorus- degrading bacteria (DWT-6 and DWT-7) isolated from sludge samples were identified as *Alcaligenes* sp., *Pseudomonas* sp., *Klebsiella* sp. and *Pseudomonas* sp., respectively.
- Three combinations of DWT-2 + DWT-7 (with proportions of 2:1, 1:1 and 1:2, respectively) provided the best results for pollutants degradation, with COD and phosphorus removal efficiencies of 69.5-74.3% and 72.6-80.6%, respectively. These bacteria could be used for inoculation for enhancing biological degradation of organic matter and phosphorus from domestic wastewater.
- The optimum inoculum percentages, rotation speeds, incubation temperatures and initial pH values for pol-

lutants degradation were 5-10%, 120-150 rpm, 30-37ºC and 7.0-8.0, respectively.

ACKNOWLEDGEMENT

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SPRING PELAGIC COMMUNITIES OF PHYTOPLANKTON, CYANOBACTERIA, ASSOCIATED HETEROTROPHIC BACTERIA AND VIRUSES IN AN EUTROPHIC SHALLOW TEMPERATE LAKE

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ABSTRACT

The high viral counts reported in aquatic systems suggest that viral infection may be an important factor controlling bacterial and phytoplankton populations in natural waters. In order to test the importance of viruses on bacterial and phytoplankton dynamics, we have taken advantage of the spring phytoplankton bloom, which occurs regularly in the shallow freshwater Vela Lake. Viral particles were counted by epifluorescence microscopy after staining with SYBR gold. Phytoplankton composition, viral and bacterial densities were determined every second day between April and June of 2006 in surface water samples (32 samples). The concentration of viruses through the spring bloom decrease from 1,27x109 viruses mL^{-1} after the Chlorophyta bloom to $5,40x10^7$ viruses mL^{-1} during the peak of Cyanobacteria, with a mean of $2,70x10^8$ viruses mL^{-1}. Bacterial numbers followed the same pattern of variation, decreasing during the bloom from $3.33x10^8$ cells mL^{-1} to $1.01x10^8$ cells L^{-1} with a mean of $1.64x10^8$ cells L^{-1}. Although viruses counts were, on average, higher than bacterial counts, yielding mean virus-to-bacterium ratio of 1.6 (range 0.3 to 7.2), the virus-to-bacterium ratio was frequently low, showing values below 1 in 43 % of the cases. The same pattern of variation of viruses and bacteria during the phytoplankton bloom as well as the significant correlation between viruses and bacteria ($p<0.05$) suggest that a significant fraction of viruses are bacteriophages. By considering not only the environmental parameters, bacteria and phytoplankton but also viruses as explanatory variable in a canonical correspondence analysis, the variance explained during the study period was increased in about 3% achieving a total of 67.3%, indicating a correlation that may be due to the known regulation of the ecosystem by viruses.

KEYWORDS: shallow lake, eutrophication, phytoplankton dynamics, environmental parameters, virioplankton, bacterioplankton.

INTRODUCTION

Cyanobacterial-microbial associations occur during both active growth and senescent phases of cyanobacteria blooms. These interactions may lead to parallel optimization of growth and bloom potentials among microbial epiphytes and cyanobacterial hosts acting as consortia, and may also reflect mutually beneficial physiological and ecological adaptations and exchange processes allowing cyanobacteria to periodically dominate planktonic communities [1, 2]. Cyanobacteria optimal growth in the presence of bacteria is related to the exchange of mutually required metabolites and growth factors e.g., vitamins, and with a detoxifying role that phycosphere-associated bacteria may play. Diverse freshwater (and marine studies) have noted relatively high densities of strain-specific planktonic and phycosphere-associated bacteria during peak cyanobacterial bloom conditions [1, 3, 4].

There is a lack of recently published information concerning planktonic dynamics in Mediterranean lakes, especially in Portuguese territory where there is a strong climatic influence from the Atlantic Ocean. Vela Lake is a polymictic shallow lake exhibiting some characteristics typical of an advanced trophic state, namely, the permanently turbid water, the reduction in biodiversity, and the recurrent occurrence of Cyanobacteria blooms [2, 5]. For Vela Lake the works of de Figueiredo *et al*. [2], Abrantes *et al*. [5], and Antunes *et al*. [6], establish that the phytoplankton assemblages are highly correlated with temperature and nutrient concentrations (particularly phosphorus); Chlorophytes dominate in early spring and Cyanobacteria from early spring until the beginning of autumn (low nutrient levels, along with high temperatures).

New techniques of enumeration have shown that viruses are abundant in marine and freshwaters. As summarized by Wommack & Colwell [7], the abundance of viruses ranged from $<10^4$ to $>10^8$ mL^{-1} in (the studied) aquatic

systems. Recent estimates reveal ca 10^{10} viruses per litre which are far higher than previous estimates [7-12]. They can regulate biomass production, species diversity, population dynamics and community structure in the aquatic microbial communities, influence carbon and nutrient recycling in food chain and water environment, and even have implications on the global climate [13, 14]. The high viral counts reported in aquatic systems [12] suggest that viral infection may be an important factor controlling bacterial populations in natural waters, and can match grazing by protists as a source of mortality for bacteria [15].

Since the ecological significance of virioplankton in aquatic ecosystems must be realized by their interaction with the hosts (e.g. bacteria, cyanobacteria and algae), enumeration of viruses and their hosts in water samples is an important first step elucidating the virus-host interaction. In order to test the importance of viruses on bacterial dynamics, we have taken advantage of the spring phytoplankton bloom, which occurs regularly in the shallow freshwater Vela Lake.

MATERIALS AND METHODS

Studied area and sampling

Vela Lake is a shallow eutrophied freshwater body (mean depth = 0.9 m, maximum depth = 2.4 m, with 70 ha of floodable surface area and a total volume of 70×10^4 m^3) [2, 5] located in Quiaios (Figueira da Foz, Central Portugal). The water volume is predominantly influenced by the variation of groundwater levels and rainfall, which makes the lake very susceptible to drought during summer months. This lake is classified as eutrophic since 1960 [16], showing high N and P levels typical of eutrophic systems [6]. The increase of phytoplankton biomass, with the presence of algal blooms, the depletion of submerged macrophytes, the increase in turbidity, and a subsequent reduction in biodiversity are some of the characteristics associated with the eutrophication process in Vela Lake [2]. The organic matter and nutrient inputs come mainly from human activities (such as agriculture and modification of land) in surrounding areas. Antunes *et al*. [6] has reported an increase of the nutrient levels during the last decade.

The study started at April 18th and ended at June 19th of 2006. Samples were collected always at the same place (44º58'N, 5º18'W) every two days (32 samples). Dissolved oxygen (O_2 mg L^{-1} and O_2%), conductivity, water and air temperature and pH were determined *in situ* (WTW multiliner P4).

The samples were collected 0.45 m bellow water surface. A 1.5 L plastic bottle and a 1L glass bottle with Lugol (1% v/v) were used: the content of the first was filtered and used for the determination of chemical parameters (phosphates, nitrates and ammonia), organic mater (total and volatile solids) and chlorophyll *a* in the laboratory; and, the second, was used for the phytoplankton analyses. A sterile analyses flask was also filled with water for bacterial analyses. Glutaraldehyde-fixed samples for virus and bacteria enumeration were also collected. Sampling occurred always in the same location and during the morning period (10±2h).

The samples were maintained in the obscurity and processed after an hour, in the laboratory. The processing consisted in the sample filtration using Whatman GF/C filters, the filters used for the evaluation of organic material were previously dried at 103ºC until they obtain a constant weight [17]. After the processing, the filtered water was used for determination of chemical parameters and the filters were used for determination of chlorophyll *a* and organic matter (which were conserved a -20ºC).

Environmental parameters and chlorophyll a

Phosphate, nitrate and ammonia concentrations were determined spectrophotometrically (Genesys 6 Thermo spectronic): phosphate was assayed using the stannous chloride method [17]; nitrate was determined applying the sodium salicilate method [18] and ammonia was quantified as described by Hall & Lucas [19].

Quantification of organic matter (total and volatile solids) was made as described in APHA [17]. Chlorophyll *a* was determined after maceration and extraction with acetone 90%, overnight at 4ºC, centrifugation at 3000rpm during 10min, as described by Lorenzen [20].

Phytoplankton diversity and abundance

For the phytoplankton quantification, samples were fixed with (1% v/v) Lugol and the enumeration was done in agreement with Lund *et al*. [21]. During enumeration, were considered as unit colony for colonial organisms, filament for filamentous organisms and cell for unicellular organisms.

The more important groups in this study were Chlorophyta, Cryptophyta, Cyanoprokariota and Euglenophyta. The identification of phytoplankton species, belonging to these groups, was made by observation through a light microscope using different references for Chlorophyta (Chlorococcales) [22], Cryptophyta [23], Cyanoprokaryota [24-26] and Euglenophyta [27].

Bacteria

Culturable bacteria were enumerated by the pour-plate method of the convenient dilution, using TSA (Tryptic Soy Agar) as culture medium and Ringer solution as dilution liquid, and incubation at 25ºC for 96h [18].

Total cell counts were accessed by epifluorescence counts after DAPI staining [28, 29].

Viruses

Viral particles were counted by epifluorescence microscopy after staining with SYBR gold [30].

Statistical analysis

A canonical correspondence analysis or CCA [31] was used to ascertain the relationships between phytoplankton, bacteria, virus and environmental variables along the study period. CCA extracts synthetic gradients from the biotic and environmental matrices and the explanatory variables are quantitatively represented by arrows in graphical biplots. The arrow direction indicates positive or negative correlations and their length is relative to the importance of the explanatory variable in the ordination. A Monte Carlo permutation test was used to assess the significance of the relationships between the biotic data and the explanatory variables.

Potential relationships between biotic and environmental data sets were tested by Pearson correlation analysis [32].

RESULTS

Environmental parameters

During the sampling period water temperature showed an average value of 22.5±2.4 ºC, and registered a higher value for sample 21 and a lower value in sample 3 (Table 1, Figure 1a). Water conductivity showed an increase over the analyzed period, with a maximum of 641 µS cm^{-1} in sample 29 (Table 1, Figure 1a). In general, the higher pH values were recorded in the last sampling period (sample 21-32). Dissolved O_2 showed an average value of 9.53 mg L^{-1}, the higher value was registered in sample 25, and lower value was observed in sample 32. The higher pH values were observed between sample 17 and 28, the highest pH value measured was 9.82 (Table 1, Figure 1b).

NH_4^+ concentration reached its highest value in sample 29 (0.131 mgN L^{-1}), this value was about 6 times greater than the other registrations, of this parameter. NO_3^- values ranged between 0.004 and 0.017 mgN L^{-1}, with an average value of 0.008±0.003 mgN L^{-1}. Phosphate concentration (detection level: 0.003 mg L^{-1}) reached a maximum of 0.009 mg L^{-1}, during a very rainy period (≤ 0.003 mg L^{-1}) (Table 1, Figure 1c).

Total and volatile solids values decreased during the first sampling days (sample 1- 13), than they increased to values higher than 33.47 mg L^{-1}, reaching a maximum in sample 26, 52.900 mg L^{-1} and 47.900 mg L^{-1}, respectively. Chlorophyll *a* concentration also registered an increase in the last sampling period (sample 20-32), the maximum value for this parameter was 0.098 µg L^{-1} (sample 25) (Table 1, Figure 1d).

Phytoplankton

The more representative *taxa* were Chlorophyta and Cyanoprokariota, while the Cryptophyta and Euglenophyta were less representative, i.e., had a lower frequency and number of effectives (Figure 2 a, b).

Euglenophyta had the lowest abundance during the studied period. Nevertheless, between May 26th and June 19th of 2006, due to *Euglena viridis* (1.56x10^3 cells mL^{-1}) in May 28th and to the genus *Trachelomonas* (*T. nigra*; *T. stokesii*; *T. volvocina*) (3.37x10^2 cells mL^{-1}) in June 5th (1.35x 10^3 cells mL^{-1}), this group abundance increased.

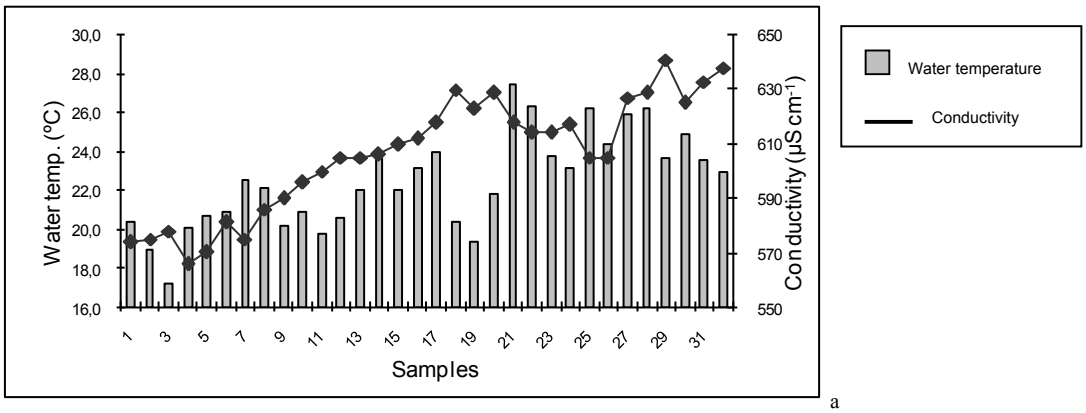
Cryptophyta division abundance was lower than the Chlorophyta and Cyanobacteria. Nevertheless it was always represented during the sampling period; the exception occurred between May 26th and June 7th. The major abundance due to *Cryptomonas* sp. was observed during April 20th (1.07x10^3 cells mL^{-1}) and June 11th and 17th (1.68x10^3 and 1.54x10^3 cells mL^{-1}, respectively) (Figure 2b).

Chlorophyta division was more abundant in the sampling beginning. Afterwards a shift in the dominant division occurred and the Cyanoprokariota division became dominant. This shift occurred as the water temperature, conductivity, pH and dissolved oxygen were getting higher (Figure 1). During the sampling period, a vast diversity in Chlorophyta species and abundance was observed. The species that during this period contributed for this group abundance belong to the genera *Phacotus* (*Phacotus lenticularis*), *Coelastrum*, (*Coelastrum pseudomicroporum* and *Coelastrum reticulatum*), *Pediastrum* (*Pediastrum boryanum*, *Pediastrum duplex*, *Pediastrum simplex* and *Pediastrum tetras*), *Scenedesmus* (*Scenedesmus acuminatus*, *Scenedesmus acutus* and *Scenedesmus oahuensis*), and *Tetraedron* (*Tetraedron caudatum* and *Tetraedron minimum*). The higher values observed between the April 18th and 24th of 2006 occurred due to a bloom of *Scenedesmus* spp., with a maximum observed in April 20th (2.71x10^4 cells mL^{-1}). A new increase was observed in May 22nd (1.03x10^4 cells mL^{-1}), with *Pediastrum* spp., *Tetraedron* spp. and still higher number of *Scenedesmus* spp. In June 3rd

TABLE 1 - Maximum (max), minimum (min), average and standard errors (σ) values for each measured parameter. Sampling was performed every second day between April and June of 2006 (32 samples of Vela Lake).

Air temp. ºC		Water temp. ºC		Cond. µS cm^{-1}		pH		Dissolved O_2 mg L^{-1}		Dissolved O_2 %sat	
min	**max**	**min**	**max**	**min**	**max**	**min**	**max**	**min**	**max**	**min**	**max**
14.7	28.2	17.2	27.5	566	641	7.75	9.82	4.75	13.7	58.4	173.3
average ± σ		**average ± σ**		**average ± σ**		**average ± σ**		**average ± σ**		**average ± σ**	
20.7±3.4		22.5±2.4		606±21		8.63±0.55		9.53±1.80		110.9±22.9	
Chl *a* µg L^{-1}		**TS mg L^{-1}**		**VS mg L^{-1}**		**C P µg L^{-1}**		**C N(NH_4^+) mg L^{-1}**		**C N(NO_3^-) mg L^{-1}**	
Min	**max**	**min**	**max**	**min**	**max**	**min**	**max**	**min**	**max**	**min**	**max**

0.005	0.098	12.700	52.900	12.700	47.900	0.000	0.009	0.002	0.131	0.004	0.017
average ± σ		**average ± σ**		**average ± σ**						**average ± σ**	
0.035±0.028		28.624±12.453		27.384±11.220						0.008±0.003	



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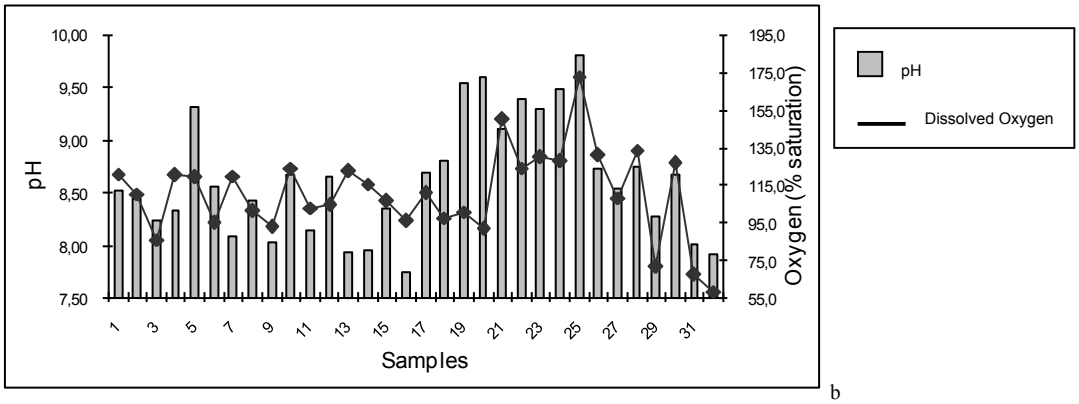

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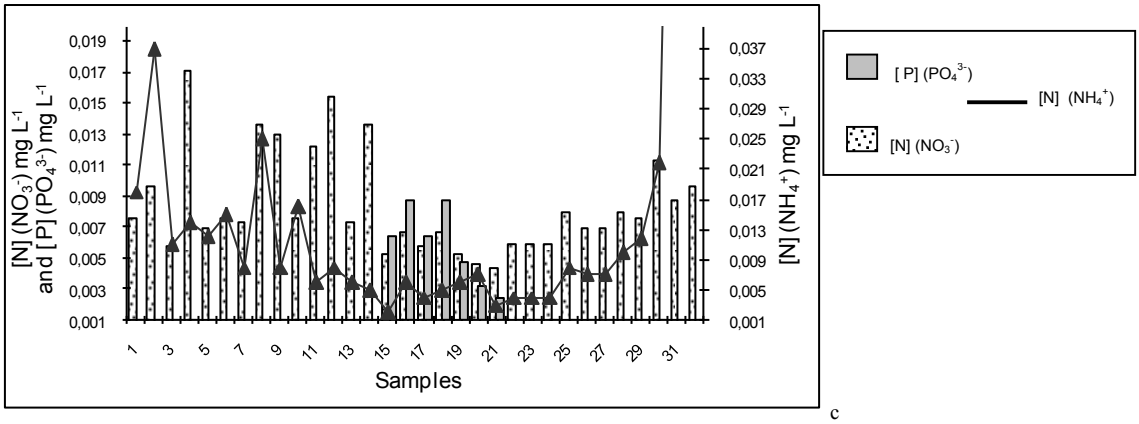

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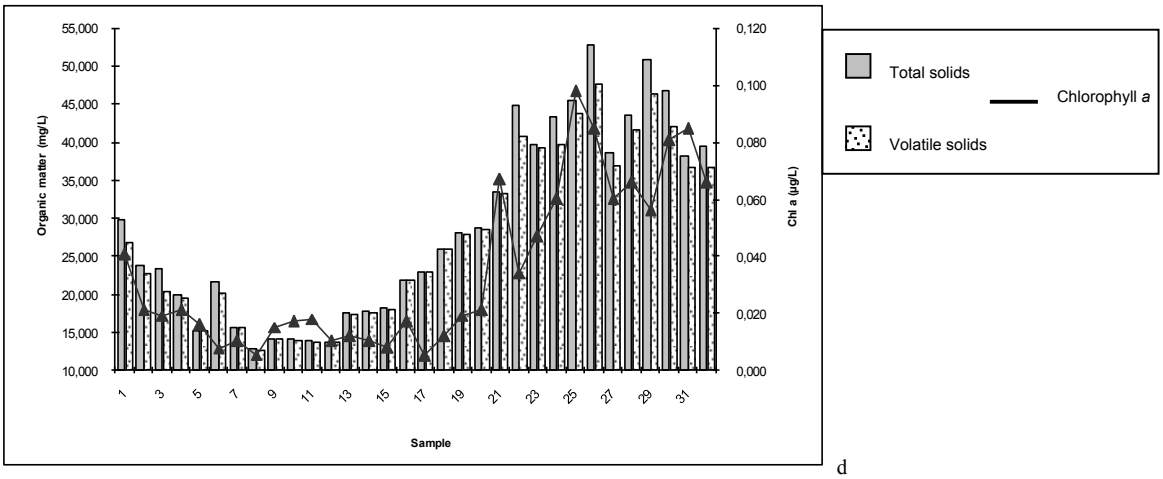


FIGURE 1 – Variation of a) water temperature, conductivity, b) pH, oxygen concentration (saturation %), c) nitrate, phosphate and ammonia, d) total and volatile solids and chlorophyll a in Lagoa da Vela, during the sampling period.

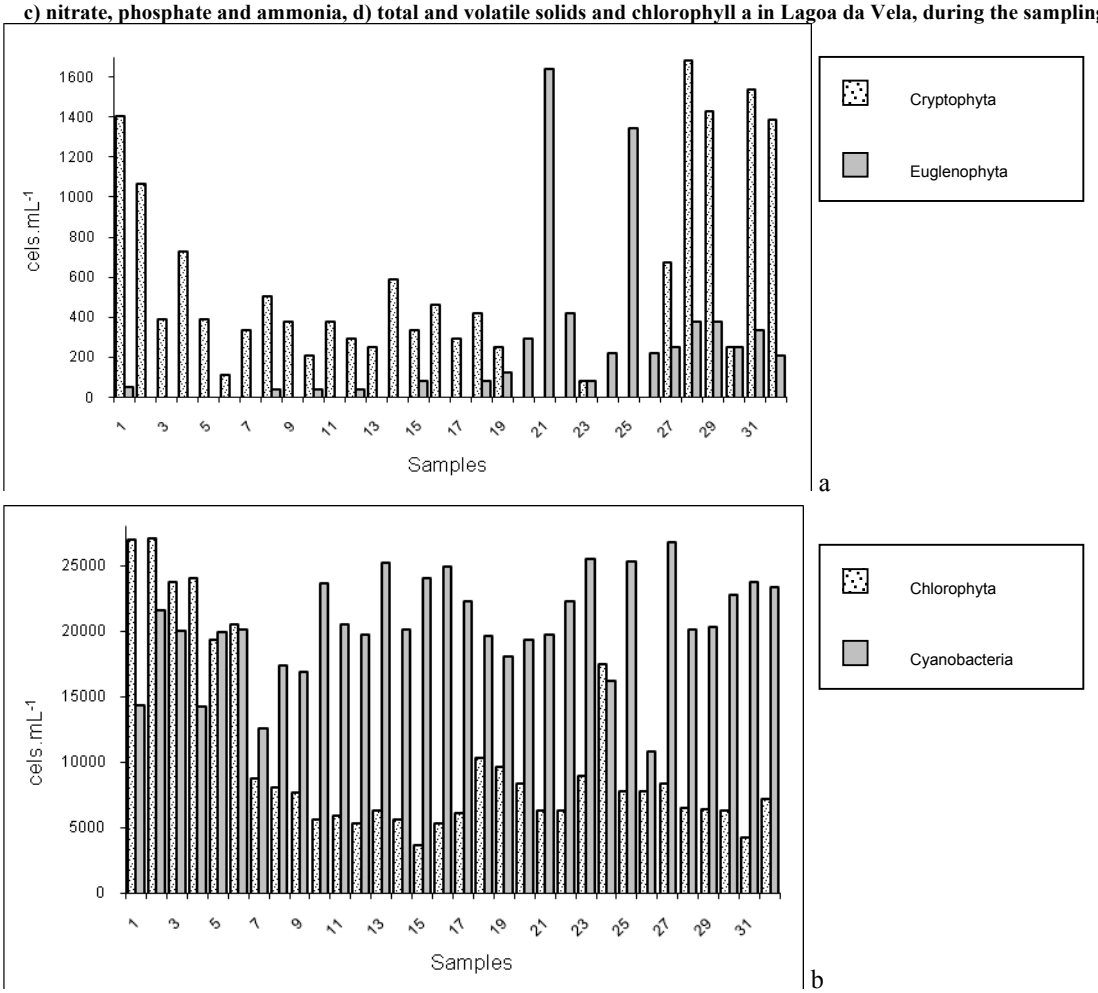


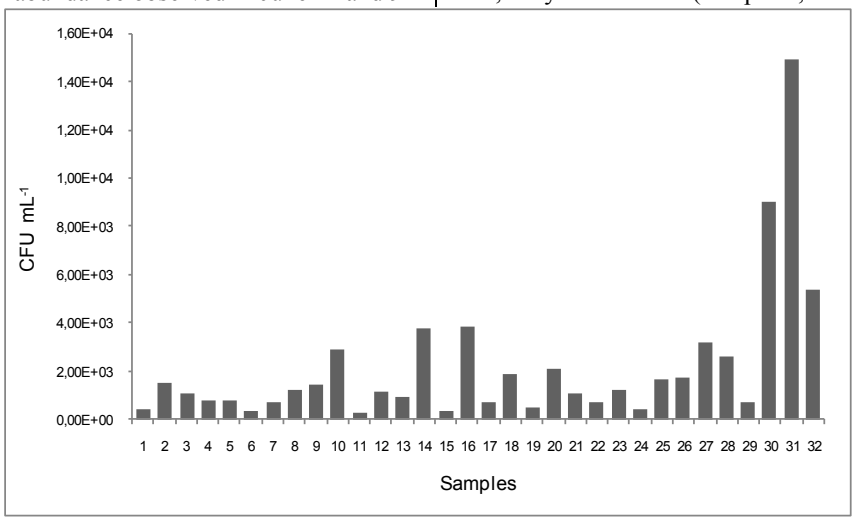
FIGURE 2 – Seasonal dynamics of a) Chlorophyta and, Cyanobacteria, b) Cryptophyta and Euglenophyta, during the sampling period and variation of the absolute abundance of Chlorophyta and Cyanobacteria, during the sampling period.

was observed a new peak ($1.75x10^4$ cells mL^{-1}) due to a *Tetraedron* spp. bloom ($8.20x10^3$ cells mL^{-1}). Species from the *Coelastrum* genera have been always present during the studied period; its higher abundance occurred between April 22nd and 28th of 2006 with a maximum in April 26th ($5.06x10^2$ cells mL^{-1}), and between the June 3rd and 13th of 2006, with the highest value in June 9th ($3.37x10^2$ cells mL^{-1}). In figure 2b it is possible to observe that, although

Cyanobacteria division had always a high cellular concentration, it only became dominant after April 20th of 2006. This shift followed a decrease in total cellular concentration of Chlorophyta and an increase in cellular concentration of cyanobacteria species *Aphanizomenon flos-aquae*. During the sampling period, there were two cyanobacterial species that have always been present (*Microcystis* cf. *pulveria* and *Chroococcus limneticcus*) and were responsible for the high cellular level observed. After the 19th sample (May 24th of 2006), a similar behaviour was observed for this group and for the bloom-forming filamentous cyanobacteria (*Aphanizomenon flos-aquae*), detectable between May 22nd and June 19th of 2006, with an exception in the following days June 11th and 13th. In this way the high abundance observed in May 6th, 12th and 18th (2.36x 10^4 cells mL^{-1}, 2.52x10^4 cells mL^{-1} and 2.49x10^4 cells mL^{-1}, respectively) was caused by the species *Microcystis* sp.. In opposite, the high abundance observed in June 1st and 9th (2.55x10^4 cells mL^{-1} and 2.68x10^4 cells mL^{-1}, respectively) was mainly caused by the filamentous species. The highest cellular concentration obtained for these *taxa* occurred in June 9th, being coincident with the day in which the highest cellular concentration for the filamentous species was observed (8.34x10^3 cells mL^{-1}). The appearance of the bloom forming filamentous cyanobacteria *Aphanizomenon flos-aquae* occurred simultaneously with an increase in phosphate concentration in the water (May 30th to June 19th), after a very rainy period and with a low level of nitrogen source compounds (NO_3^- and NH_4^+) (Figure 1).

Bacteria

The abundance of culturable bacteria varied during the study period. The highest values were observed in June 15th and 17th (sample 30 and 31, respectively) (maximum of 1.5x10^4 CFU mL^{-1}) and the lowest values occurred in April 28th, May 8th and 16th (sample 6, 11 and 15, respectively).



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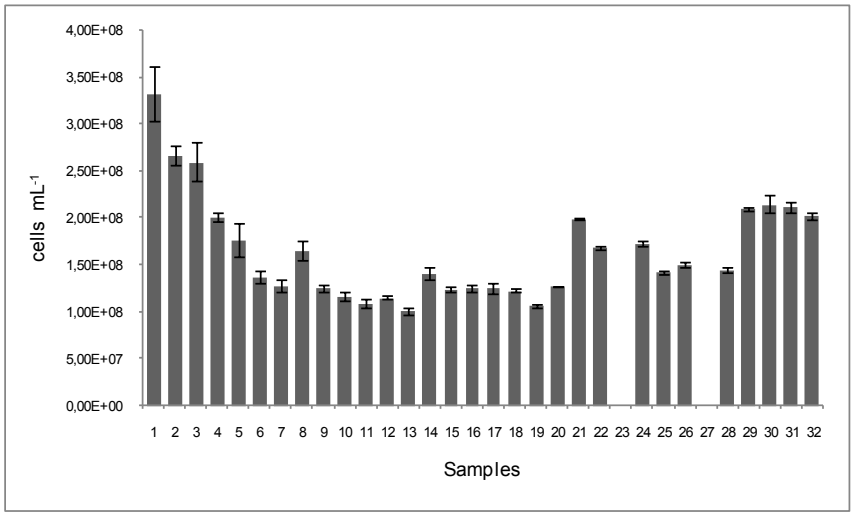
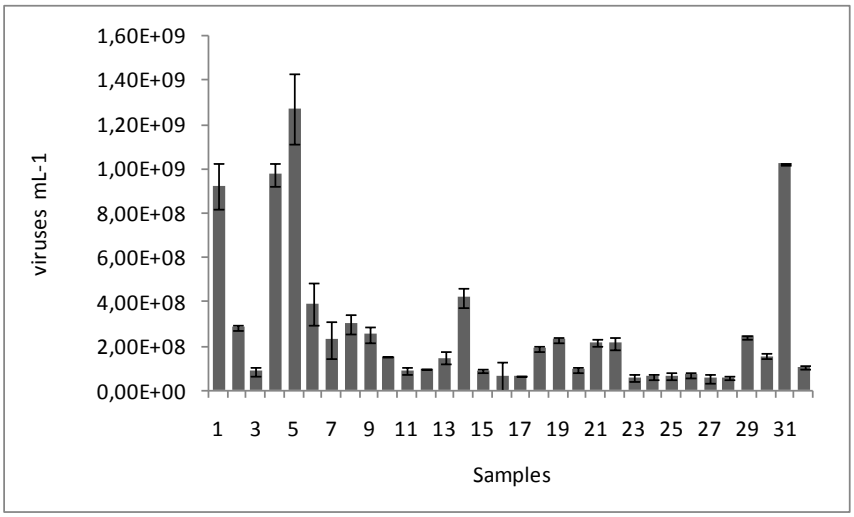


b

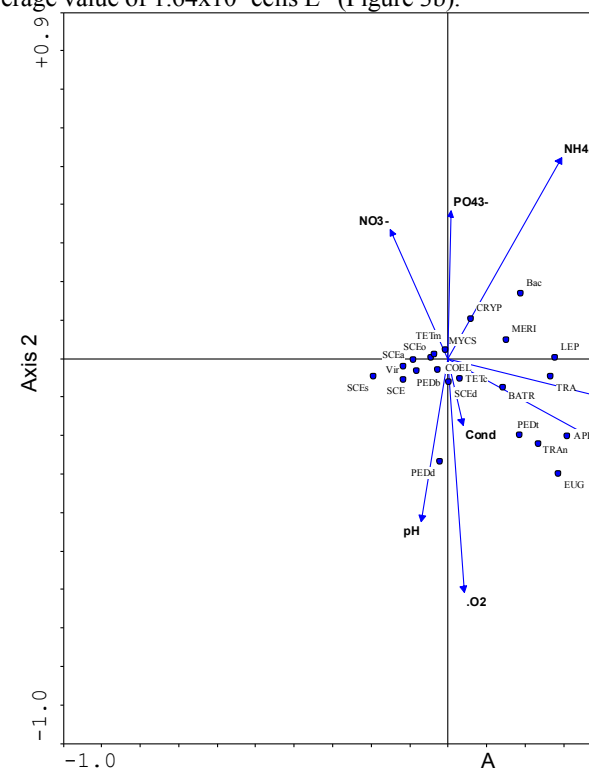


c

FIGURE 3 – a) Abundance of culturable bacteria (colony counts); Variation of b) total cells (average ± σ) accessed by DAPI staining and c) viral abundance (average ± σ) every second day between April and June of 2006 in surface water samples (32 samples) of Vela Lake.

The minimum was observed in May 8th (3.23×10^2 CFU mL^{-1}) (Figure 3a). The highest CFU abundance occurred during a peak of ammonia concentration (June 17) (Figure 1).

Total cell counts, accessed by epifluorescence counts after DAPI staining, varied from 3.33×10^8 cells mL^{-1} (sample 1, April 18th) to 1.01×10^8 cells mL^{-1} (sample 13, May 12th) with an average value of 1.64×10^8 cells L^{-1} (Figure 3b).



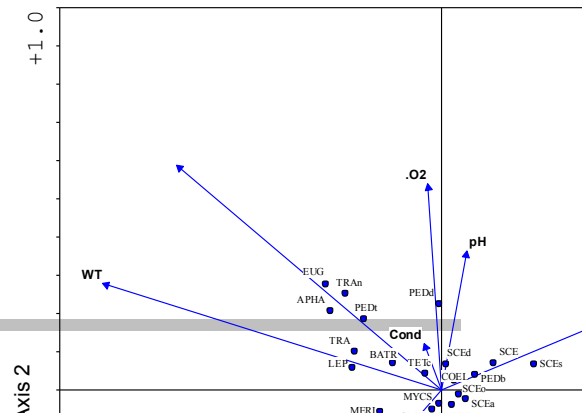


FIGURE 4 - Results from canonical correspondence analysis for Vela Lake between 18th April and 19th June of 2006: a) biplot for most abundant phytoplankton species, bacteria, virus and environmental variables; b) biplot for most abundant phytoplankton species, bacteria and environmental variables plus virus (as explanatory variable). (WT (Water temperature), VS (Volatile solids), O_2 (Dissolved oxygen),pH (pH), PO_4^{3-} (Phosphates), NO_3^- (Nitrates), APHA (*Aphanizomenon flos-aquae*), BATR (*Botryococcus braunii*), COEL (*Coelastrum pseudomicroporum*), CRYP (*Cryptomonas* sp.), EUG (*Euglena* sp.), LEP (*Lepocinclis salina*), MERI (*Merismopedia* sp.), MYCS (*Mycrocistis* sp.), PEDb (*Pediastrum boryanum*), PEDd (*Pediastrum duplex*), PEDt (*Pediastrum tetras*), SCEa (*Scenedesmus acuminatus*), SCEd (*Scenedesmus disciformes*), SCEo (*Scenedesmus oahuensis*), SCE (*Scenedesmus* sp.), SCEs (*Scenesdesmus spinosus*), TETc (*Tetraedron caudatum*), TETm (*Tetraedron minimum*), Bac (Total Bacteria), Vir (Total viruses), TRAn (*Trachelomonas nigra*) and TRA (*Trachelomonas* sp.))

TABLE 2 - a) Summary of canonical correspondence analysis between most abundant phytoplankton species, bacteria, virus and physico-chemical parameters; b) Summary of CCA analysis between most abundant phytoplankton species, bacteria and -chemical parameters (without virus); c) Summary of CCA analysis between most abundant phytoplankton species, bacteria and physico-chemical parameters (plus virus as explanatory variable) . The study period was from 18th April to 19th June of 2006.

	a)		b)		c)	
	Axes 1	Axes 2	Axes 1	Axes 2	Axes 1	Axes 2
Eigenvalues	0.139	0.067	0.137	0.055	0.151	0.064
Species-environment correlations	0.897	0.824	0.893	0.780	0.926	0.799
Cumulative percentage variance						
of species data	32.8	48.6	32.3	45.2	35.5	50.7
of species-environment relation	51.3	76.0	56.4	79.0	52.8	75.3
Sum of all unconstrained eigenvalues	0.425		0.425		0.425	
Sum of all canonical eigenvalues	0.272		0.243		0.286	
Variance explained by the CCA	**64.0%**		**57.2%**		**67.3%**	
Variance explained by the first two axes	**48.5%**		**45.2%**		**50.6%**	

Bacteria, Virus and virus-to-bacterium ratio

The concentration of viruses through the spring bloom decrease from 1,27 x10^9 viruses mL^{-1} after the Chlorophyta bloom to 5.40 x10^7 viruses mL^{-1} during the peak of Cyanobacteria, with a mean of 2.70 x10^8 viruses mL^{-1}(Figure 3c).

Although, total cells followed the same pattern of variation, viruses counts were, on average, higher than bacterial counts, yielding mean virus-to-bacterium ratio of 1.6 (range 0.3 to 7.2), the virus-to-bacterium ratio was frequently low, showing values below 1 in 43 % of the cases.

The canonical correspondence analysis

Results from CCA ordination of phytoplankton species that occurred more them 5%, heterotrophic bacteria, virus and environmental variables (Table 2a, b; Figure 4a) lead to the conclusion that water temperature (WT) and volatile solids (VS) were strongly correlated with the first CCA axis while dissolved oxygen (O_2), pH, ammonia (NH_4^+), phosphates (PO_4^{3-}) and nitrates (NO_3^-) were correlated with the second axis. These two axes alone explained 48.5% of the total phytoplankton, bacterial and viral variance (Table 2a). On the negative side, the first axis is defined by the green algae *Tetraedron minimum* (TETm), *Scenedesmus oahuensis* (SCEo), *Scenesdesmus acuminatus* (SCEa), *Pediastrum boryanum* (PEDb), *Scenedesmus* sp. (SCE), *Scenesdesmus spinosus* (SCEs), but also by the cyanobacteria *Microcystis* sp. (MYCS) and total viral concentration (Vir). The positive side of the first axis is defined by the euglenophyte *Euglena* sp. (EUG.), *Lepocinclis salina* (LEP.), *Trachelomonas* sp. (TRA) and *Trachelomonas nigra* (TRAn), the chlorophyte *Botryococcus braunii*

(BATR), *Pediastrum tetras* (PEDt), and the cyanobacteria *Aphanizomenon flos-aquae* (APHA) and *Merismopedia* sp. (MERI), but also by the total bacteria concentration (Bac). Along the second axis, the positive area is defined by the cyanobacteria *Microcystis* sp. (MYCS) and by the cryptophyte *Cryptomonas* sp. (CRYP) and the negative side is defined by chlorophyte: *Coelastrum pseudomicroporum* (COEL), *Scenedesmus disciformes* (SCEd), *Pediastrum duplex* (PEDd) and *Tetraedron caudatum* (TETc).

In the second CCA virus densities were used as explanatory variables (Table 2c and Figure 4b). The first two axes explained 50.6% (a higher percentage then the obtained for the first diagram) of the total phytoplankton and bacterial variance. This analysis was preceded by a CCA (Table 2b) using the same data (without virus) (the first two axes explained 45.2% of the total variance). The ordination diagram obtained (Figure 4b) shows that water temperature and volatile solids are still strongly correlated with the first CCA axis, but immediately followed by viral abundance. The second axis shows correlations with pH, dissolved oxygen and conductivity.

DISCUSSION

Sampling in this study was done in a two by two day collecting strategy, and a very dynamic phytoplankton community was observed for all the analysed taxa.

Early in the sampling period Chlorophyta division was predominant. With the increase in water temperature, conductivity, pH and dissolved oxygen values this predominance was progressively transferred to Cyanobacteria. Within Chlorophyta division, there was a variation in species composition, being the genus *Phacotus* (*Phacotus lenticularis*), *Coelastrum* (*Coelastrum pseudomicroporum*, *Coelastrum reticulatum*), *Pediastrum* (*Pediastrum boryanum*, *Pediastrum duplex*, *Pediastrum simplex*, *Pediastrum tetras*), *Scenedesmus* (*Scenedesmus acuminatus*, *Scenedesmus acutus*, *Scenedesmus oahuensis*) and *Tetraedron* (*Tetraedron caudatum*, *Tetraedron minimum*) the major representatives. The maximum value of Chlorophyta abundance was 2.71×10^4 cells mL^{-1} (sample 2, April 20th) and the minimum was 3.71x10 cells mL^{-1} (sample 15, May 16th). Cyanobacteria dominance has begun only when Chlorophyta started to decline, i.e., after April 20th (sample 2). *Microcystis* cf. *pulveria* and *Chroococcus limneticcus* were present throughout the sampling period. In June 9th (sample 27), a maximum value of Cyanobacteria abundance was observed (8.34×10^3 cels mL^{-1}), coinciding with an *Aphanizomenon flos-aquae* maximum. This observation is in agreement with de Figueiredo *et al*. [2] that describe Cyanobacteria dominance from late spring until early autumn in this shallow lake. The development of the dense *A. flos-aquae* bloom was preceded by the lowest concentrations of nitrogen, indicating that this cyanobacterial strain is not very dependent on nitrogen availability, probably due to its N-fixing capability [33]. However, the availability of phosphate appeared to be required for the bloom development, the *Aphanizomenom flos-aquae* bloom observed in this study was precede by an increase in phosphates concentration. Phosphorus and temperature dependence has been reported for this species [2, 34, 35, 36]. In freshwater systems, phosphorus and nitrogen often act as limiting nutrients and is known that a sharp increase in one or both of these elements can result in an algal bloom [2].

During this study, a marked increase in the number of culturable bacteria associated with the high values of chlorophyll *a* and with an increase in organic matter availability was found. This observation is supported by statistical analysis where significant correlations between chlorophyll *a* and culturable bacteria (r=0.4779, p<0.05) and chlorophyll and ammonia, total and volatile solids (r=0.3628, p<0.05, r=0.8671, p<0.05, r=0.8718, p<0.05 respectively), were found.

The abundance of heterotrophic aerobic bacteria in the water column depends on the availability of organic matter and is indirectly linked to the chlorophyll *a* concentration. Ammonia- and nitrite-oxidising bacteria together are responsible for the process of nitrification (which is the oxidation of ammonium to nitrate), this relation is supported by significant correlations between ammonia and bacteria (r= 0.3610, p<0.05). The higher number of heterotrophic aerobic bacteria occurred during the bloom, which is in agreement with the high values of organic matter registered at this period. The maximum values of chlorophyll *a*, total and volatile solids were observed during the bloom period, decreasing when the senescence period began. There were significant correlations between chlorophyll and total and volatile solids (r=0.8671, p<0.05, r=0.8718, p<0.05, respectively).

VBR is a common index used to examine viral-bacterial dynamics in aquatic systems [9]. Generally, high VBR values may have resulted from either a high infection rate or a long persistence of viruses in the plankton [37]. In addition, high concentration of phytoplankton and cyanobacteria may also result in high VBR value [9]. The mean VBR of 1.6 (range 0.3 to 7.2) is somehow below the general range (i.e., 3 to 10) reported for pelagic environments [7]. The possible explanation for our results is that viral dynamics might result from bacteriophages, algal viruses and other viruses together (cyanophages e.g. Gons *et al*. [38], cyanophage control may fail in natural water bodies exhibiting predominance of filamentous cyanobacteria). Viruses are important members of the freshwater ecosystems [12]. Using a multivariate analysis with the viruses as explanatory variable we found they have an important influence on the phytoplankton assemblage and bacteria.

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STUDY ON THE ANTIMICROBIAL, CYTOTOXIC, AND GENOTOXIC ACTIVITIES OF THE ESSENTIAL OIL OF *Artemisia dracunculus* L.

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ABSTRACT

The antimicrobial, cytotoxic and genotoxic activities of the essential oil of *Artemisia dracunculus* whose many biological activities are already known have been investigated. Antimicrobial screening was carried out by using the Kirby and Bauer disc diffusion method. We found that the essential oil of *Artemisia dracunculus* showed antimicrobial activity against some bacteria and fungi. The cytotoxic and genotoxic activities of five concentrations of the essential oil were tested with cytokinesis-block micronucleus assay (CBMN) on human peripheral lymphocytes *in vitro*. The numbers of nuclear buds were found to be statistically important while there was no significant increase in the micronuclei number. Furthermore, this essential oil has shown substantial cytotoxic activity in human lymphocytes.

KEYWORDS: *Artemisia dracunculus*, antimicrobial activity, cytotoxicity, genotoxicity, lymphocytes

INTRODUCTION

Artemisia dracunculus (Russian tarragon, estragon or Tarhun) is a small shrubby perennial plant of the large genus *Artemisia*. An ethanolic extract of *A. dracunculus* has been shown to have anticoagulatory and antihyperlipidemic activities in rats. Since the extract of *A. dracunculus* decreases serum cholesterol by 15% and serum trigylcerides by 25% in rats, it was considered to help to lower the incidence of coronary disease in humans [1]. An ethanolic extract of *A. dracunculus* called TARRALIN™ is used as an agent to correct the hyperglycemia associated with the diabetic conditions [2]. It was reported that three alkamides isolated from *A. dracunculus* showed insecticidal activity against rice weevil (*Sitophilus oryza* L.) and grain borer (*Rhyzopertha dominica*) [3]. In Iranian traditional medicine, the dried aerial parts of this plant were used orally to treat epilepsy [4]. Furthermore, antifungal, antibacterial, antitumor and DNA damaging effects were reported for the essential oil of *A. dracunculus* [5-8]. Essential oil of *Artemisia dracunculus* consists predominantly of estragole (ES). It was shown that estragole is a naturally occurring genotoxic carcinogen in experimental animals [9]. However, the genotoxicity and antimicrobial activity of essential oil *of A. dracunculus* has not yet been much explored.

In this study, we investigated the cytotoxic and possible genotoxic activities of *A. dracunculus* essential oil on human peripheral lymphocytes *in vitro,* but also antimicrobial activity of essential oil of *A. dracunculus* against some bacteria and fungi.

MATERIALS AND METHODS

Plant material

The aerial parts of *Artemisia dracunculus* (Compositae), were collected from Bayburt province in Turkey. Identification of the species was confirmed by a botanist.

Extraction of the essential oil

Air-dried aerial parts of the plants were hydro-distilled using a Clevenger-type apparatus.

Gas chromatography (GC) and GC-MS analysis conditions

GC analysis was carried out using a HP 6890 Series GC System equipped with an Innowax FSC capillary column (60 m x 0.25 i.d. x 0.25 μm film thickness) and FID detector. Nitrogen was used as carrier gaswith a flow-rate of 1 ml min^{-1}. The oven temperature was kept at 70 ^{o}C for 10 min and programmed to 180 ^{o}C at a rate of 2 ^{o}C/min, and kept constant at 180 ^{o}C for 30 min. The injector and detector temperatures were 250 ^{o}C.

A Hewlett-Packard GC-MS system with an Innowax FSC capillary column (60 m x 0.25 i.d. x 0.25 μm film thickness) was used with helium as carrier gas (flow-rate 1 ml min^{-1}). The GC oven temperature was kept at 60 ^{o}C and programmed to 260 ^{o}C at a rate of 5 ^{o}C/min, and kept constant at 260 ^{o}C for 40 min. The mass spectrometry (MS) analysis involved the use of a quadrupole analyzer with the ionization energy set at 70eV. From total ion current (TIC)

chromatogram, the mass spectra of the essential oil components were screened using the in-house Wiley GC/MS library of Essential Oil Constituents, and the components identified are listed in Table 1. Relative percentages of the separated compounds were calculated from the data.

Microorganisms

In this study, the following microorganisms were used: *Aeromonas hydrophila* and *Yersinia enterocolitica* (from Ankara University, Faculty of Veterinary, Ankara, Turkey), *Bacillus cereus* (NRRL B-3711), *Bacillus subtilis* (NRRL B-744), *Proteus vulgaris* (NRRL B-123), *Enterococcus faecalis* (NRRL B-14617), and *Klebsiella pneumoniae*, *Cladosporium* sp., and *Salmonella typhimurium* (available in our department), *Listeria monocytogenes* (from Ankara University, Faculty of Agriculture, Ankara, Turkey), *Pseudomonas syringae* pv. *tomato* (TPPB 4212), *Alternaria tenuissima, Alternaria alternata, Alternaria citri, Drechslera sorokiniana,* and *Penicillum notatum* (from Trakya University, Department of Biology, Edirne, Turkey), *Fusarium moniliforme*, *Fusarium oxysporum,* and *Fusarium culmorum* (from the Agriculture Research Center, Eskişehir, Turkey), *Candida albicans*, *Candida glabrata*, *Aspergillus fumigatus,* and *Aspergillus parasiticus* (from Osmangazi University, Faculty of Medicine, Eskişehir, Turkey), *Staphylococcus aureus* (ATCC 6538), *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922), *Aspergillus niger* (ATCC 9807) and *Fusarium solani* (ATCC 12820) (from American Type Culture Collection, USA). Bacteria and yeasts were kept on nutrient agar and yeast extract agar plates at 4 ^{o} C, respectively. Fungal test cultures were sub-cultured on potato dextrose agar (PDA) for 5-7 days at 25 ^{o}C.

Antimicrobial screening and determination of minimum inhibitory concentration

The Kirby and Bauer disc diffusion method [10] was employed for the determination of antimicrobial activity and MIC values of the essential oil of *Artemisia dracunculus* against test bacteria and fungi. To be brief, a stock solution of the essential oil (100 mg/ml) in hexane was prepared firstly. Then, 2.5 ml solution was taken from the stock solution and diluted two-fold with hexane nine times. Using these diluted solutions, 60 sterilized-filter paper discs (6 mm in diameter) for each solution were prepared. The substrate contents of these discs varied from 4100 to 8 µg/disc.

A suspension of the test microorganism (0.1 ml of 10^8 cells per ml) was spread on the nutrient agar (for bacteria) and potato dextrose agar (for yeasts and filamentous fungi). Filter paper discs were placed on the inoculated plates. These plates, after keeping at 4 °C for 2 h, were incubated at 37 °C for 24–48 h for bacteria, and at 30 °C for 48–96 h for the yeasts and filamentous fungi. Pure hexane solvent-treated and dried discs were used as control discs. The diameters of the inhibition zones were measured in mm. The MIC values were determined as the lowest concentration at which the visible growth of each organism on the agar plate was inhibited, and all the tests were performed in duplicate.

CBMN Assay

The micronucleus test was performed as described by Rothfuβ *et al*. [11]. The peripheral blood from two healthy male donors was added to 2.5 ml chromosome medium B and incubated at 37 ^{o}C for a total time of 72 h. The cells were treated with 6 different concentrations (varying from 10 to 0.05 µl/ml) of *A. dracunculus* essential oil for 24 h. A negative (DMSO, 20 µl/ml) and positive controls (Mitomycin C, 0.3 µl/ml and EMS, 200 µl/ml) were also employed. After 44 h from initiating of the cultures, Cytochalasin B was added, at a final concentration of 6 µl/ml. Then the cells were harvested after 72 h, treated with a hypotonic solution (0.4% KCl) and fixed three times with a fixative (methanol/glacial acetic acid). The slides were air-dried and stained with 5% Giemsa. The frequency of micronucleus (MN), nuclear buds (NBuds), nucleoplasmic bridges (NPBs) and apoptotic cells (ApC) was determined by analyzing 1000 binucleated cells for each treatment [12]. Statistical differences between MN, NBuds, NPBs and ApC of the treated cells and MN, NBuds, NPBs and ApC of the solvent controls were evaluated by Dunnett test in ANOVA. The cell proliferation index (CPI) was calculated as follows: (B+2P) / (M+B+P), where M, B, P are the number of cells that have not yet entered the first mitosis (M, mononucleated), and the number of cells that have divided once (B, binucleated) and twice (P, plurinucleated), respectively. (M+P+P) represents a total of, at least, 1000 cells scored.

RESULTS AND DISCUSSION

The GC-MS analysis was used to determine the composition of *A. dracunculus* essential oil. Eleven compounds were identified in the oil. The major compound of the essential oil was methylchavicol (estragole) comprising 78.96%. The principal components and their percentages of essential oil are shown in Table 1.

TABLE 1
Main components of *A. dracunculus* essential oil.

Main components	(%)
Methylchavicol (estragole)	78.96
Limonene	3.65
cis-β-ocimene	3.51
trans-β-ocimene	3.40
Spathulenol	1.51
α-pinenes	1.21
Methyl eugenol	1.20
Carvacrol	0.60
Eugenol	0.23
β-pinenes	0.16
trans-anethole	0.16

I*n vitro* antimicrobial spectrum of essential oil was found to be effective against both bacteria and fungi (Table 2). The growth inhibitions of the test microorganisms

TABLE 2
Antimicrobial activity of the essential oil of *A. dracunculus*.

Microorganisms	DD[a]	MIC (µg)
A .hydrophila	-	-
B. cereus	15	1025
B. subtilis	11	128
E. coli	14	2050
K. pneumoniae	14	2050
L. monocytogenes	9	4100
P. vulgaris	-	-
P. aeruginosa	-	-
P. syringae	-	-
S. typhimurium	12	2050
S. aureus	11	1025
S. faecalis	15	2050
Y. enterocolitica	8	1025
C. albicans	19	4100
C. glabrata	16	2050
A. niger	8	2050
A. parasiticus	10	2050
A. fumigatus	9	4100
D. sorokiniana	13	1025
F. solani	11	1025
F. oxysporum	11	1025
F. moniliforme	12	2050
F. culmorum	10	2050

DD[a], disc diffusion (diameter of zone of inhibition including disc diameter of 6 mm); -, not active.

ranged from 128 to 4100 µg, with the lowest MIC value against *B. subtilis* at 128 µg, followed by *B. cereus*, *S. aureus*, *Y. enterocolitica, D. sorokiniana*, *F. solani*, *F. oxysporum* with MIC values at 1025 µg. The highest MIC value was observed for *L. monocytogenes*, *C. albicans,* and *A. fumigatus* at 4100 µg. *A. dracunculus ''*Piemontese*''* exhibited inhibitory effects on all bacteria, except for *S. aureus* and *S. epiderimidis* [13]. However, our essential oil of *A. dracunculus* exhibited also antimicrobial activity against *S. aureus.* Similarly, Lopes-Lutz *et al.* [14] found that *Artemisia* oils had an antimicrobial effect on *S. aureus* and *S. epiderimidis*.

In another study, the essential oil of several *A. dracunculus* species have been found to possess *in vitro* anti-microbial activity against *E. coli, Pseudomonas aeruginosa, Staphylococcus aureus, Enterococcus faecalis,* and *Yersinia enterocolitica* [15].

Table 3 shows the number of micronuclei (MN), mitotic index (MI), and cell proliferation index (CPI), and Table 4 shows nuclear buds (NBuds), nucleoplasmic bridges (NPBs) and apoptotic cells (ApC) in cytokinesis-blocked lymphocytes treated with *A. dracunculus* essential oil. High concentrations of the essential oil (10, 5, 1 µg/ml) induced cytotoxic effects in human lymphocytes. The total number of cells having nuclear buds (NBuds) increased in treated lymphocytes with increasing concentrations of the essential oil. A significant increase of cells having nuclear buds (NBuds) was observed at 0.5 and 0.1 µg/ml of *A. dracunculus* essential oil. The number of micronuclei was higher in the acid-treated lymphocytes compared to those in control although the differences were not statistically significant. CPI was used as a measure of cytotoxicity and/or inhibition of cyt-B-blocked cell proliferation, because it substantially expresses the ratio of divided cells to still undivided ones. We also observed that CPI significantly decreased in treated lymphocytes with *A. dracunculus* essential oil at high concentrations.

There is only a limited amount of relevant data in the literature on genotoxicity of *A. dracunculus* essential oil. In a previous study, genotoxic properties of essential oils from *A. dracunculus* were tested with the *Bacillus subtilis* rec-assay and *Salmonella*/microsome reversion (Ames) assay. As a result, essential oil of *A. dracunculus* was found to be active in rec-assay but it was not considered to be mutagenic in Ames *Salmonella* assay. In rec-assay, DNA damaging activity was demonstrated to be due to the estragole component of the oil [8]. Cytotoxic and genotoxic effects of *A. dracunculus* ethanolic extract (TARRALIN) were inves-

TABLE 3 - Results of the CBMN test for MN, MI and CPI in human lymphocytes after 24 hours treatment with*A. dracunculus* oil

Dose (µl/ml)	Donor 1 No.of MN[a]	Donor 2 No.of MN[a]	MNnumber mean ± SD[b]	MI (%)[c]	CPI (%)[d]
Tarragon oil					
10	Tox	Tox	Tox		
5	Tox	Tox	Tox		
1	Tox	Tox	Tox		
0.5	12	15	13.5±2.12	11.3*	0.43*
0.1	8	11	9.5±2.12	16.5	0.68*
0.05	6	4	5.0±1.41	20.2	0.92
Controls					
DMSO 20	4	7	5.5± 2.12	26.3	1.81
MMC 0,3	55	68	61,5±9.19*	9.6	1.24

DMSO; Dimethyl sulphoxyde, MMC; Mitomycin C
[a] Number of micronucleated binucleated cells /1000 binucleated scored.
[b] MN frequency is average between the two experiments per 1000 binucleated scored.
[c] Mitotic index (MI) is ratio between mitotic figures and the number of stimulated nuclei.
[d] Cell proliferation index (CPI) values are calculated as explained in Materials and Methods.
* $p < 0.01$ the respective solvent control

TABLE 4 - Results of the CBMN test for NBuds, NPBs and ApC in human lymphocytes after 24-hours treatment with *A. dracunculus* oil.

Dose (µl/ml)	NBuds, mean ± SD[a]	NPBs mean ± SD[b]	ApC mean ± SD[c]
Tarragon oil			
10	Tox	Tox	Tox
5	Tox	Tox	Tox
1	Tox	Tox	Tox
0.5	85±16.97**	3±1.41	6.5±2.12
0.1	50±11.31*	1.5±0.70	5.5±3.53
0.05	15±2.82	1.0±0.70	3.0±1.41
Controls			
DMSO 20	5±2.82	0.0±0.0	2.0±1.41
EMS 200	32±2.82*	5.5±2.12	3.5±2.12

DMSO; Dimethyl sulphoxide, EMS; Ethyl methane sulphonate
[a]Nuclear buds frequency is average between the two experiments per 1000 binucleated scored.
[b]Nuclear bridges frequency is average between the two experiments per 1000 binucleated scored.
[c]Apoptotic cell frequency is average between the two experiments per 1000 binucleated scored..
** $p< 0.001$, * $p< 0.01$ the respective solvent control

tigated with rats *in vivo*, and with Ames test *in vitro,* respectively. It was found that *A. dracunculus* extract was non-toxic and non-genotoxic in that study [2]. No experiment has been performed so far about the genotoxicity of *A. dracunculus* essential oil by micronucleus assay performed on human lymphocytes *in vitro*.

In this study, we have observed that *A. dracunculus* essential oil has cytotoxic and nuclear budding activities in human lymphocytes. *A. dracunculus* essential oil exhibited a direct cytotoxic effect against normal human lymphocytes through a decrease in the rate of cell proliferation index (CPI). Since we did not observe an increase in micronucleus formation in our experiments, we conclude that the observed nuclear budding activities were not caused by chromosomal damage.

The nature of nuclear buds has been not fully understood so far. It has been suggested that nuclear buds in lymphocytes could represent gene amplification, and amplified DNA is localized selectively to specific sites at the periphery of the nucleus and eliminated via nuclear budding [16]. Some investigators consider nuclear buds in lymphocytes as indicators of genotoxicity [17]. It was shown that the increased levels of micronuclei and nuclear buds were observed in human lymphocytes exposed to herbicide Deherban A, ethyl-methane sulphonate (EMS) or cyclophosphamide (CPA) [18].

On the other hand, it was reported in some studies that the frequency of nuclear buds, but not that of micronuclei, was significantly increased due to the different factors in exfoliated cells [19, 20]. Montero *et al*. [21] found a significant increase of the number of nuclear buds, but not of micronuclei, in buccal epithelium of adolescents living in an extremely polluted central area of Mexico City. It was claimed that nuclear buds in exfoliated cells are not similar to those in lymphocytes, and nuclear buds in exfoliated cells most likely are not associated with genotoxic activity but with degenerative processes in the first layer of epithelial cells. Our data showed that the formation of the nuclear buds in human lymphocytes is not due to genotoxic activity of *A. dracunculus* essential oil.

The results reported here can be considered as information on antimicrobial, cytotoxic and genotoxic properties of *A. dracunculus* essential oil in the Turkish flora.

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EVALUATION OF BIOLOGICAL TREATMENT OF INDUSTRIAL WASTEWATER USING AEROBIC AND ANAEROBIC RESPIROMETRY

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ABSTRACT

This paper describes studies on an aerobic and anaerobic treatment of an industrial wastewater, contaminated with phenol (560 mg L^{-1}) and formaldehyde (480 mg L^{-1}), using both aerobic and anaerobic respirometry.

Returned activated sludge (RAS) was the source of biomass used in the aerobic studies, digester biomass (DB) was used in anaerobic experiments. Respirometric tests demonstrated that the aerobic system corroborated with the Haldane model for the inhibitory wastes, while data obtained during the anaerobic experiments complied with the Monod model for non-inhibitory wastes. Aerobic treatment of the wastewater/sewage mixture (1:1) removed 46% of formaldehyde and 48% of phenol. For the same wastewater content, the anaerobic removal of formaldehyde and phenol was 73% and 76%, respectively.

Results of respirometric tests, biokinetic calculations, and chemical analyses indicated that the anaerobic process was a preferred technology for the investigated wastewater treatment. It has been demonstrated that the anaerobic respirometry can be successfully applied for an investigation of anaerobic processes in a way similar to the aerobic respirometry.

KEYWORDS: aerobic respirometry, anaerobic respirometry, wastewater treatment.

INTRODUCTION

For a long time, aerobic processes have dominated the wastewater treatment industry [1]. The increasing energy prices and decreasing available land for sludge disposal have motivated the use of anaerobic treatment as an attractive alternative [2, 3]. As both aerobic and anaerobic treatments have some advantages and disadvantages, selecting one of these processes for a specific application is not easy. The quality of water to be treated, particularly concentrations of inhibitory substances and their biodegradability under aerobic and anaerobic conditions, are the most important issues, which should be considered. Both of them can be addressed using respirometric tests and biokinetic models. Recent advances in modeling of activated sludge processes [4] and development of new automated respirometers provided environmental scientists and engineers with convenient tools for designing and optimization of wastewater treatment systems.

Since most of the sewage and wastewater treatment plants apply suspended growth activated sludge technology, there are many literature reports describing an application of aerobic respirometric tests [1, 5, 6]. There are, however, very few reports about an application of the anaerobic respirometry [7, 8].

Both phenol and formaldehyde are toxic and inhibitory substances to unacclimated activated sludge. They can be efficiently treated under aerobic and anaerobic conditions in full-scale treatment plants but their presence at elevated concentrations (>300 mg L^{-1}) can inhibit both, nitrification and denitrification [9-13]. Most of the studies on the phenol and formaldehyde biodegradation were conducted using these compounds separately, as a single carbon source, and applying acclimated inoculum [14, 15]. No report on evaluation of aerobic and anaerobic treatments of wastewater contaminated with both these pollutants using respirometry has been published.

This paper describes an application of both aerobic and anaerobic respirometry for the studies on the aerobic and anaerobic treatment of industrial wastewater contaminated with phenol and formaldehyde.

MATERIALS AND METHODS

Biomass and water collection and preparation

Industrial wastewater was collected from a chemical plant manufacturing phenolic resins. Municipal sewage, returned activated sludge (RAS) and biomass from a digester (DB) were collected from a local wastewater treat-

TABLE 1 - Analyses of wastewater, sewage, returned activated sludge (RAS) and digester biomass (DB).

Parameter	Units	Wastewater	Sewage	RAS*	DB*
pH	(St units)	8.2 ± 0.1	7.3 ± 0.1	7.6 ± 0.1	7.8 ± 0.1
Alkalinity	mg $CaCO_3$ L^{-1}	280 ± 5	96 ± 2	_	_
COD	mg L^{-1}	2250 ± 5	205 ± 2	_	_
SCOD	mg L^{-1}	1900 ± 5	190 ± 2	30 ± 2	68 ± 2
TSS	mg L^{-1}	110 ± 1	12 ± 1	2800 ± 5	3600 ± 5
VSS	mg L^{-1}	90 ± 1	9 ± 1	2100 ± 5	2800 ± 5
Ammonia–N	mg L^{-1}	8.0 ± 0.05	8.3 ± 0.1	_	_
Total phosphorus	mg L^{-1}	0.9 ±0 .02	4.2 ± 0.05	_	_
Phenol	mg L^{-1}	560 ± 0.5	<0.5	_	_
Formaldehyde	mg L^{-1}	480 ± 0.5	<0.5	_	_

Note: * – analyses of RAS and DB after acclimatization

ment plant. RAS and DB were acclimatized to the studied wastewater/sewage mixtures before tests for 6 days and 20 days, respectively. Analyses of wastewater, sewage, RAS and DB are presented in Table 1. Oxygen and nitrogen were medical grade from Praxair, Mississauga, ON, Canada. All chemicals were of analytical grade, purchased from Anachemia Science, Lachine, QC, Canada.

Equipment

The respirometric system used in the study included modified Comput-OX, N-CON 12 channel respirometer equipped with CTOX computer program for data collection and instrument control (N-CON Systems Co. Crawford, GA, USA). The system operated 12 reactors, each 1L volume. Reactors were connected to a pure oxygen supply system for aerobic tests or to a vacuum pump (Air Cadet, Cole-Parmer, Vernon Hills, IL, USA) for anaerobic tests. Reactors were kept in a water bath with a precision temperature control ± 0.5° C, set up at 25° C (for aerobic tests) or at 35° C (for anaerobic tests). The content of the reactors was mixed with high-speed magnetic mixers (~1200 rpm) to ensure the homogeneity. The VSS, TSS, COD, ammonia and phosphorus analyses were conducted according to the Standard Methods [16]. Concentrations of phenol and formaldehyde were determined using Lachat QuickChem Model 8500 flow injection analyzer (Lachat Instruments, Milwaukee, WI, USA) applying the EPA 420.2 and EPA 556 methods [17] respectively. The pH was monitored (but not adjusted) using Orion 3-Star Portable pH/ISE Meter with Triode pH/ATC electrode (Thermo Scientific, Waltham, MA, USA).

Procedures

During aerobic tests, ten reactors were filled (to the total volume of 500 mL) with 10 ml of RAS and mixtures of municipal sewage with different concentrations (from 0% to 100%) of wastewater. Two control reactors contained no RAS but 500 mL sewage and 500 mL wastewater, respectively. The system recorded the mass and the volume of oxygen as well as the time when it was delivered to each reactor, and it calculated the oxygen uptake rates (OUR). Content of the reactors after the tests was analyzed for TSS, VSS, SCOD, phenol and formaldehyde.

During anaerobic tests, the reactors were filled (to the total volume 500 mL) with 50 mL of digester biomass (DB) and mixtures of municipal sewage with different concentrations (from 0% to 100%) of the wastewater. Two control reactors contained no DB, but 500 mL sewage and 500 mL of the wastewater, respectively. The reactors were connected to the vacuum and purged with pure nitrogen to remove oxygen. Subsequently, they were connected to the respirometer and the test was initiated. The system recorded the volume of the biogas as well as the time when it was generated in each reactor, and it calculated the gas production rates (GPR). Content of the reactors after the tests was analyzed for TSS, VSS, SCOD, phenol and formaldehyde.

Respirometric data for reactors containing RAS (in aerobic tests) or DB (in anaerobic tests) were normalized by subtracting data from the control reactors. Tests were conducted in duplicate and the results were averaged.

Biokinetic calculations

Two general biokinetic models [5] were used to evaluate biokinetic parameters:

the Monod model for non-inhibitory wastes:

$$\mu = \frac{\mu_{\max} S}{(K_s + S)} \quad (1)$$

and the Haldane model for inhibitory wastes:

$$\mu = \frac{\mu_{\max} S}{\left(S + K_s + S^2/K_i\right)} \quad (2)$$

where:

μ - specific growth rate, calculated from the equation (3):

$$\mu = \ln(X_t/X_o)/(t - t_o) \quad (3)$$

where: X_t – concentration of biomass at time t

X_o – initial concentration of biomass at t_o

μ_{max} – maximum specific growth rate

S – substrate concentration as SCOD in mg L^{-1}

K_s – saturation constant, is the substrate concentration as soluble chemical oxygen demand (SCOD mg L^{-1}) that produces 0.5 μ_{max}

K_i – inhibition constant, is the concentration of the inhibitory substance as SCOD in mg L^{-1}

Two other parameters were also calculated:

$$\mu_{ci} = \frac{\mu_{max}}{\left(1 + 2\sqrt{\frac{Ks}{Ki}}\right)}$$

$$Sci = \sqrt{Ks \cdot Ki}$$

where:

μ_{ci} - critical rate constant for investigated system, is the practical maximum growth rate

S_{ci} - critical saturation constant, is the concentration of inhibitory substance as SCOD mg L^{-1} for which critical rate constant can be achieved.

Respirometric data were analyzed following the procedure described by Rozich and Gaudy [5]. Biokinetic calculations were performed using Mathcad 2001 computer program (MathSoft Inc. Cambridge, MA, USA).

RESULTS AND DISCUSSION

Aerobic respirometric tests

Aerobic respiration (and oxygen consumption) is linked to the key metabolic processes that take place in the cell and its growth at different rates is directly related to the substrate removal. Measurements and interpretation of the biological oxygen consumption rate under well-defined experimental conditions are the basis for the aerobic respirometry [5]. This method was applied to evaluate an effect of the investigated industrial wastewater on the activity of RAS. Table 2 shows the content of the wastewater and the initial SCOD value in the reactors during this set of experiments.

TABLE 2 - Results of aerobic respirometric tests with sewage and sewage/wastewater mixtures.

Parameter	Wastewater content (%)									
	0	5	10	20	30	40	50	60	80	100
Initial SCOD (mg L^{-1})	190 ± 5	275 ± 5	361 ± 5	532 ± 5	703 ± 8	874 ± 8	1045 ± 10	1216 ± 10	1558 ± 10	1900 ± 10
Final SCOD (mg L^{-1})	29 ± 2	75 ± 2	120 ± 4	221 ± 5	360 ± 5	442 ± 8	604 ± 8	795 ± 10	1083±15	1390 ±15
SCOD Removed (%)	84.7 ± 5	72.7 ± 5	66.7 ± 5	58.5 ± 6	48.8 ± 8	49.4 ± 5	42.2 ± 5	34.6 ± 4	30.5 ± 5	26.8 ± 4
OUR_{max}* Time (h)	25 ± 0.15	28 ± 0.15	33 ± 0.16	37 ± 0.18	40 ± 0.16	42 ± 0.16	46 ± 0.22	50 ± 0.20	58 ± 0.20	65 ± 0.20
OUR_{max}* (mg $L^{-1}h^{-1}$)	9.8 ± 0.4	9.5 ± 0.4	9.2 ± 0.3	8.8 ± 0.5	8.3 ± 0.3	7.7 ± 0.3	7.4 ± 0.4	7.2 ± 0.3	6.8 ± 0.5	6.3 ± 0.3

Note: * – OUR_{max} = maximum of the oxygen uptake rate (OUR)

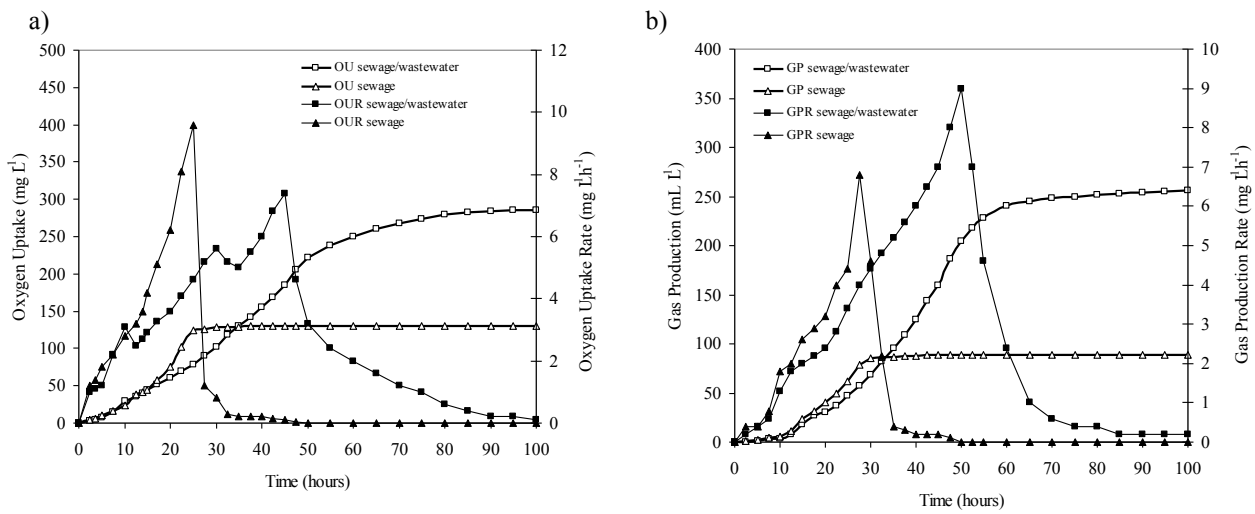

FIGURE 1 – a) Oxygen uptake (OU) and oxygen uptake rate (OUR) versus time plots for aerobic treatment of sewage and sewage/wastewater (1: 1) mixture. b) Gas production (GP) and gas production rate (GPR) versus time plots for anaerobic treatment of sewage and sewage/wastewater (1:1) mixture.

Plots of the oxygen uptake (OU) by RAS and the oxygen uptake rate (OUR) versus time in the sewage and in the sewage/wastewater mixture (1:1) are presented in Fig. 1a. During the treatment of the sewage, the OU increased quickly to the final value of 145 mg L^{-1}. The OUR reached the maximum of OUR_{max} = 9.8 mg $L^{-1}h^{-1}$ after 25 h. For the sewage/wastewater mixture the OU increase was slower and the final maximum OU value (OU_{max} = 286 mg L^{-1}) was achieved after about 60h. This higher final OU was probably due to a much higher organic load in the sewage/wastewater mixture (initial SCOD = 1045 mg L^{-1}) than that in the sewage (initial SCOD = 190 mg L^{-1}).

The OUR plot for the sewage/wastewater (1:1) mixture had two peaks, at 5.6 mg $L^{-1}h^{-1}$ and 7.4 mg $L^{-1}h^{-1}$, likely related to the consecutive phases of the biological degradation of the wastewater constituents. The maximum OUR (OUR_{max}) value was lower than the OUR_{max} for the sewage. Similar pattern was observed for the plots obtained for other sewage/wastewater mixtures (Table 2). On increasing the wastewater content from 0% to 100%, the SCOD removal was reduced from ~85% to ~27%, and the OUR_{max} decreased from about 10 mg $L^{-1}h^{-1}$ to 6.3 mg $L^{-1}h^{-1}$, while the OUR_{max} time increased from 25h to 65h. These data suggested that the wastewater had an inhibitory effect on the activity of RAS in the sewage.

Based on the respirometric data and chemical analyses, the growth rate was calculated using the procedure described by Rozich and Gaudy [4]. Non-linear regression analyses indicated a good fit to the Haldane Model for inhibitory wastes (Fig. 2a). Inhibitory constant K_i = 985.92 mg L^{-1} as well a substantial difference between critical rate μ_{ci} = 0.027 h^{-1} and μ_{max} = 0.044 h^{-1} indicated a strong inhibitory effect. This effect occurred for the value of SCOD > S_{ci} = 313.88 mg L^{-1} which corresponded to ~10% content of wastewater (with 48 mg L^{-1} of formaldehyde and 56 mg L^{-1} of phenol). As it can be seen from Fig. 2a, for the SCOD > K_i = 985.9 mg L^{-1}, corresponding to the sewage/wastewater ratio ~1:1 (with the initial concentrations of phenol and formaldehyde ~280 mg L^{-1} and ~240 mg L^{-1}, respectively), the investigated aerobic system could be upset. These data suggest a synergetic inhibitory effect of these pollutants since a successful aerobic treatment of wastewater contaminated with phenol at concentration above 400 mg L^{-1} has been reported [18]. Synergetic inhibitory effect of phenol with other substances has been previously reported [19].

Anaerobic respirometric tests

Anaerobic respiration (and biogas generation) is also related to the substrate removal and to the cell growth, and many available respirometers can monitor not only oxygen uptake but also the gas generation. According to Speece [ref. 2, p. 250], the same concept can be applied to monitor the activity of both aerobic and anaerobic processes. Thus, anaerobic respirometric tests were conducted with the same concentrations of the investigated wastewater (Table 3) as those used for the aerobic tests but, instead of RAS, a biomass from an anaerobic digester (DB) was used.

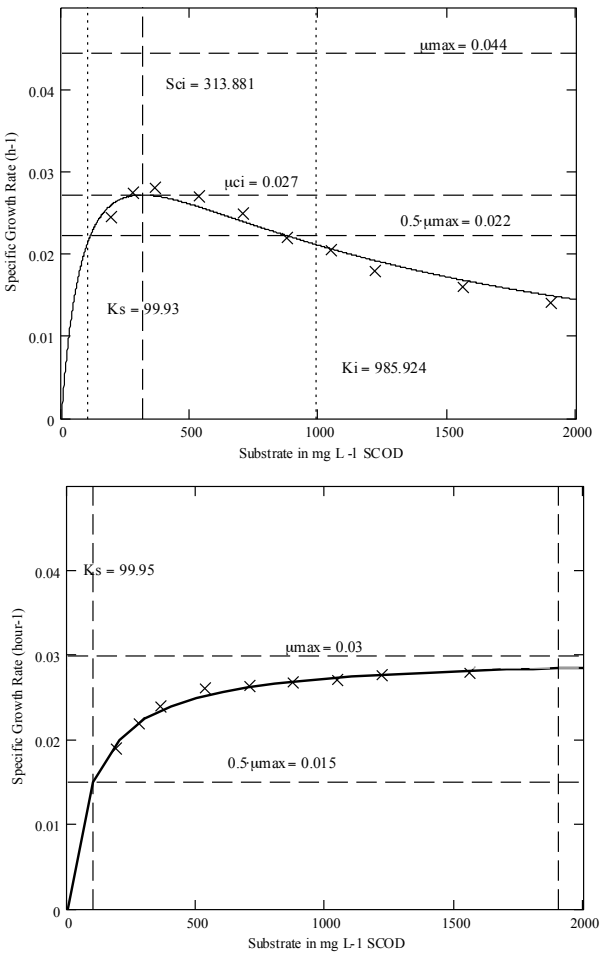

FIGURE 2 - a) Non-linear regression curve (Haldane model for inhibitory wastes) for aerobic treatment of sewage with wastewater. b) Non-linear regression curve (Monod model for non-inhibitory wastes) for anaerobic treatment of sewage with wastewater.

Respirometric plots for the gas production (GP) and the gas production rate (GPR) for the sewage and the sewage/wastewater mixture (1:1) are presented in Fig. 1b. They show an increase in the GP value to the maximum of GP_{max} = 88 mL L^{-1} for the sewage and to 256 mL L^{-1} for the sewage/wastewater. The maximum GPR for the sewage, (GP_{max} = 6.8 mL $L^{-1}h^{-1}$, was achieved after 28h, while the GPR_{max} for the sewage/wastewater (1:1) was 9.1 mL $L^{-1}h^{-1}$ and it occurred after 52h.

The sequence of the GPR maxima for the sewage and sewage/wastewater (1:1) was similar to that observed for the OUR maxima. However, contrary to the OUR data (Fig. 1a), the GPR_{max} value for the sewage was lower than the GPR_{max} value for the sewage/wastewater (1:1) (Fig. 1b), suggesting that the wastewater did not inhibit the activity of the anaerobic biomass. As it is presented in Table 3, increasing the wastewater content in the sewage resulted in an increase of the GPR_{max} what is opposite to the effect on the OUR_{max} shown in Table 2. The GPR_{max} values for the

TABLE 3 - Results of anaerobic respirometric tests with sewage and sewage/wastewater mixtures.

Parameter	Wastewater content (%)									
	0	5	10	20	30	40	50	60	80	100
Initial SCOD ($mg\ L^{-1}$)	190 ± 5	275 ± 5	361 ± 5	532 ± 8	703 ± 10	874 ± 10	1045 ± 10	1216 ± 10	1558 ± 10	1900 ± 10
Final SCOD ($mg\ L^{-1}$)	30 ± 2	46 ± 2	74 ± 4	140 ± 5	183 ± 5	288 ± 8	387 ± 10	496 ± 10	665 ± 10	840 ± 10
SCOD Removed (%)	84.2 ± 7	83.3 ± 8	79.5 ± 5	73.7 ± 5	73.9 ± 5	67.0 ± 4	62.9 ± 5	59.2 ± 5	57.3 ± 5	55.8 ± 5
GPR_{max}* Time (h)	28 ± 0.14	30 ± 0.14	34 ± 0.16	39 ±0.16	42 ± 0.16	46 ± 0.18	50 ± 0.16	53 ± 0.16	60 ± 0.18	66 ±0.20
GPR_{max}* ($mg\ L^{-1}h$)	6.8 ± 0.3	7.3 ±0.4	7.5 ± 0.3	8.2 ± 0.4	8.6 ± 0.3	8.8 ± 0.3	9.1 ± 0.4	9.2 ± 0.3	9.2 ± 0.4	9.3 ± 0.3

Note: * – GPR_{max} = maximum of the gas production rate (GPR)

sewage/wastewater mixture containing more than 40% of wastewater are very similar (8.8 – 9.3 mg L^{-1} h^{-1}), indicating that the anaerobic system was relatively stable.

Gas production (GP) data and chemical analyses of reactor contents (SCOD, VSS) were used to calculate the growth rate. As it is shown in Fig. 2b, the obtained non-linear regression curve fitted well to the Monod model for non-inhibitory wastes. These results are consistent with the literature [2] according to which anaerobic systems show a better resistance to inhibitory substances. The calculated value of the maximum specific growth rate μ_{max} = 0.030 h^{-1} (or 0.72 day^{-1}) is comparable with the values 0.49 ± 0.21 day^{-1} reported in the literature [20] for acetoclastic methanogenesis at 22°C.

Phenol and formaldehyde removal during aerobic and anaerobic treatment

Figure 3 presents the removal of SCOD, phenol and formaldehyde from the selected wastewater/sewage mixtures (5%, 10%, 20% and 50%), during the aerobic and anaerobic tests.

The results demonstrate that the removal of all these parameters was better under the anaerobic condition. The effect was more pronounced for the phenol and formaldehyde removal. During the aerobic treatment of the sewage/wastewater mixture 1:1 (with the SCOD value close to the value of the inhibitory constant K_i), only 46% of formaldehyde and 48% of phenol were removed. For the same wastewater content, the anaerobic removal of formaldehyde was 73% and phenol removal was 76%.

Comparison of aerobic and anaerobic treatments

Comparison of the respirometric data, biokinetic calculations and the results of chemical analyses indicate that the investigated wastewater inhibited the activity of RAS (Haldane fit, Fig. 2a), but it did not affect the activity of DB (Monod fit, Fig. 2b). Removal of the parameters of concern from the sewage/wastewater (1:1) system was much better under the anaerobic conditions. Biokinetic parameters suggested that a reliable aerobic operation would be possible using the 10% – 20% content of the wastewater, and that the application of the sewage/wastewater ratio higher than 1:1 would upset the treatment system. The anaerobic treatment was expected to be more stable even with 100% of the wastewater content. Consequently, reactors for the anaerobic treatment system could be substantially smaller. The obtained results suggested that the anaerobic treatment of the investigated wastewater would be a preferred choice.

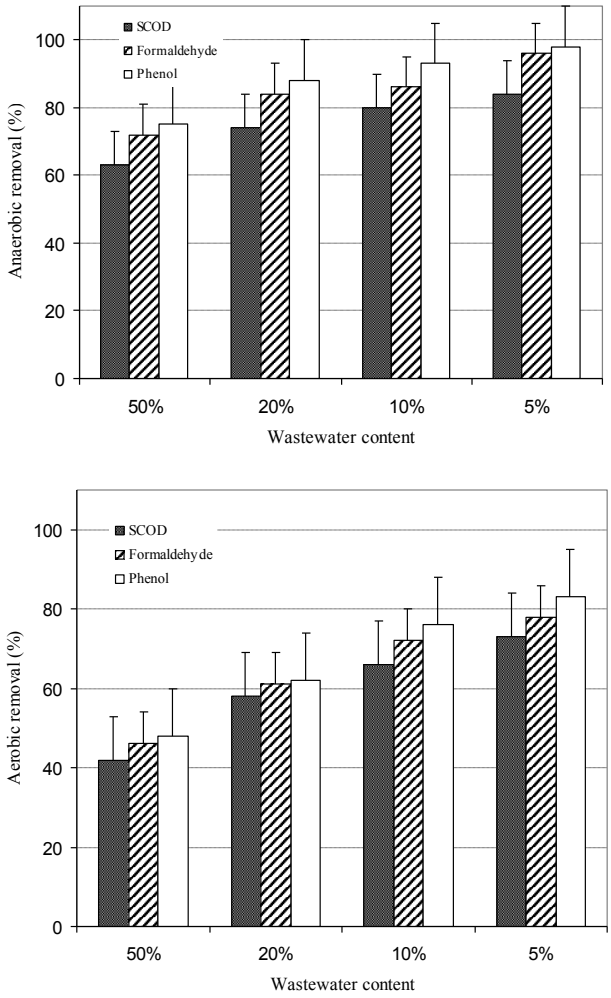

FIGURE 3 - Removal of SCOD, phenol and formaldehyde from selected mixtures of wastewater/sewage (5%, 10%, 20% and 50%) during a) aerobic treatment and b) anaerobic treatment. Time 100h.

CONCLUSIONS

Results of the described respirometric and chemical experiments indicate that the anaerobic method would be a preferred treatment of the investigated wastewater, contaminated with phenol (560 mg L^{-1}) and formaldehyde (480 mg L^{-1}).

Respirometric tests demonstrated that the aerobic system corroborated with the Haldane model for the inhibitory wastes, while the data obtained during anaerobic experiments complied with the Monod model for non- inhibitory wastes. Aerobic treatment of the wastewater/sewage mixture (1:1) removed 46% of formaldehyde and 48% of phenol. For the same wastewater content, the anaerobic removal of formaldehyde and phenol was 73% and 76%, respectively.

Results of the anaerobic respirometric tests indicate that this technique can be successfully applied for an investigation of anaerobic processes in a similar way to the aerobic respirometry. Respirometers capable of running both aerobic and anaerobic tests can be used as powerful and convenient tools for scientists and engineers to determine the most suitable technology for a particular wastewater treatment.

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PERSISTANT ORGANIC POLLUTANTS (POPs) IN AGRICULTURAL SOILS OF SYRIAN MEDITERRANEAN REGION: A PRELIMINARY STUDY

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ABSTRACT

The present paper deals with the determination of selected persistent organic pollutants (POPs) in soil samples. Five sets of 20 soil samples were collected from five Syrian agricultural field soils (potatoes, gherkins, olives and cotton) near the city of Sarakeb. Every soil sample was analysed for residues of POPs after Soxhlet extraction, clean up and determination by gaschromatography (GC) combined with electron capture detector (ECD) as well as mass spectrometry (MSD). Lindane, Polychlorinated Biphenyls (PCB), Heptachlor, Aldrin, Chlordane, Endosulfane, Dieldrin, Endrin and Mirex could not be detected (limit of quantitation 0.1 µg/kg dry wt., limit of detection 0.02 µg/kg dry wt.). The chlorinated organic compound Hexachlorobenzene (HCB) and the insecticide 4,4´-DDT with its derivative products 4,4´-DDE and 4,4´-DDD were found at concentrations between < 0.1 µg/kg dry wt. and 17.2 µg/kg dry wt. The study supports the activities of Stockholm Convention on Persistent Organic Pollutants and the National Implementation Plan of Syria and now the first international data for Syrian agricultural soils are available.

KEYWORDS: Persistent organic pollutants (POPs), Syrian agricultural soils, residue analysis, GC/ECD/MS

INTRODUCTION

Up to now no data are available in literature about the occurrence of POPs in the agricultural soils as well as in urban areas and industrial sites in Syria. A scientific cooperation between the Department of Chemistry of the Tishreen University (Syria) and Martin-Luther-University Halle-Wittenberg (Germany) presents a study that supports the activities to realize the Stockholm Convention on Persistent Organic Pollutants and the National Implementation Plan of Syria and delivers first data for Syrian agricultural soils.

POPs are found in all environmental media and in all regions of the globe. They are ubiquious organic compounds of anthropogenic origin that resist photolytic, chemical and biological degradation. They are characterized by low water solubility and high lipid solubility, resulting in bioaccumulation in fatty tissues of living organisms. Their different chemical properties lead to a more or less efficient long-range transport of different POPs, leading to a "global fractionation". In aquatic systems and soils they adsorb strongly to solids, notably the organic matter, avoiding the aqueous phase. POPs can have a half-life of years or decades in soil/sediment and several days in the atmosphere [1, 2].

POPs have the tendency to enter the gas phase under environmental temperatures. Hence, they may volatilise from soils, vegetation and water bodies into the atmosphere and because of their resistance to breakdown reactions in air they travel long distances before being re-deposited. POPs can partition between particles and aerosols depending on ambient temperature and the physico-chemical properties of the chemical. The bulk of the burden of POPs in the environment resides in soils and sediments where they primarily partition into organic matter. The processes influence POPs concentrations/burdens in soils and sediments, including biodegradation, volatilisation and “ageing” effects which can produce non-extractable residues over time [3].

POPs are a class of synthetic organic chemicals that are amongst the most widespread of all environmental pollutants, and found worldwide in air, water, soil, food and the fatty tissues of humans and animals [4, 5]. For example, DDT can travel to long distances and accumulate in animal and human bodies due to its solubility in fats. In the soil POPs may be either degraded by soil microorganisms, adsorbed by soil organic matter or re-emitted by volatilisation.

Traditionally, the nature and amounts of POPs in solid environmental samples such as sediments and soils are determined by exhaustive extraction of the sample using liquid extraction techniques, followed by gas chroma-

tographic analysis. One of the most frequently used liquid–solid extraction method is Soxhlet extraction. It was developed in the late 19th century [6], but is still in routine use in many laboratories.

MATERIALS AND METHODS

Sampling consisted of five sets of 20 soil samples. They were collected in July and August 2008 on clear dry days during field visits of five fields (potatoes, gherkins, olives and two times cotton) of the city of Sarakeb in Syria. The collected samples were prepared in order to determine trace concentrations of selected POPs including HCB, Lindane, PCB, Heptachlor, Aldrin, Chlordane, Endosulfane, 4,4´-DDE, Dieldrin, Endrin, 4,4´-DDD, 4,4´-DDT and Mirex. The certified standards were purchased from Dr. Ehrenstorfer (Germany). All used solvents (toluene, dichloromethan, n-hexane) were of analytical grade (Carl Roth GmbH, Germany).

Soil samples were dried for 16 h at 105°C. Two duplicates of 10 g of each dried soil sample were extracted over 20 h by Soxhlet extration with 160 ml toluene. The extracts obtained were concentrated to about 2 ml using a rotation evaporator. The clean up was carried out with a column containing 10 g florisil (Merck Ltd.) which was rinsed previously with 40 ml n-hexane : dichlormethane (1:1) after drying for 4 h at 120°C and furthermore for 4 h at 180°C. It was eluted with 80 ml of the same solvent mixture and as a first step the eluate was concentrated to about 2 ml, followed by further drying using argon gas. The dried sample was dissolved in 1 ml n-hexan containing 20 pg/µl internal standard (PCB-209). An aliquote of the resulting solution was transferred to a GC vial with insert (150 µl).

The concentrations of POPs were analyzed by GC/ECD (5890 Series II, Hewlett Packard) and GC/MSD (6890/5973, Hewlett Packard), both systems equipped with a similar capillary column, VF-5ms., 30 m x 0.25 mm i.d. x 0.25 µm film thickness (Varian). The temperature programme was as follows: 60°C (2 min) 20°C/min → 120°C (0 min) 4°C/min. → 280°C (5 min). Other analytical parameters were for GC/ECD: Injector 250°C, detector 300°C, make up gas N_2. GC/MSD (selected ion monitoring, SIM): Injector 250°C, transfer line 280°C. A 1 µl aliquot of the sample extract was injected using splitless mode. The carrier gas for both instruments was helium with a flow rate of 1 ml/min. All samples were processed twice as a duplicate and measured parallel with GC/ECD and GC/MSD (SIM). Recovery tests were carried out for quality assurance. Blank soil samples were spiked at three residue levels (0.20, 2.0 and 20.0 µg/kg dry wt.) with four independent parallel determinations. The total recovery ranged between 72.5% and 116.2 %. The limit of quantitation was 0.1 µg/kg dry wt. and the limit of detection ranged between 0.02 and 0.1 µg/kg dry wt., depending on the compound and the kind of detection (ECD/MSD).

RESULTS AND DISCUSSION

Polychlorinated Biphenyls (PCB) were used as insulating fluids in transformers, hydraulic oils and many other applications. It can be found in many soils in urban area as well as in agricultural soils (sewage sludge). In the selected Syrian soils no residues of PCB could be detected (< 0.1 µg/kg dry wt.), whereas the background levels of PCB in Germany were 1-10 µg/kg dry wt. Agricultural soils in the United Kingdom ranged between 1.7 and 1199 µg/kg dry wt. (average value 6.5 µg/kg dry wt.) [7]. Our investigations indicated the absence of PCB in the selected soils. To get more detailed information about the occurrence of PCB in Syrian ecosystem, soil samples of urban areas, industrial sites and near waste dumps must be analysed.

Hexachlorobenzene (HCB) was used as fungicide and wood preservative as well as additive for different industrial products (insulating materials, PVC). A study in Leipzig-Halle region (Germany) showed that residues in soil were between 0.6 and 3.7 µg/kg dry wt. [8], whereas the HCB level in Syrian soil samples from the Mediterranean region was about 0.3 ± 0.1 µg/kg dry wt. (Table 1). The chlorinated pesticides Lindane, Heptachlor, Aldrin, Chlordane, Endosulfane, Dieldrin, Endrin and Mirex could not be detected in the agricultural soils (< 0.1 µg/kg dry wt.).

TABLE 1 - Determination of POPs in soil samples of Syrian agriculture [µg/kg dry wt.]

Sample/ substance	HCB	4,4´-DDE*	4,4´-DDD	4,4´-DDT
Potatoes	**0.2**[+] (0.2, 0.2, 0.2, 0.2)	**17.2** (19.4, 12.8, 16.9, 19.5)	**0.6** (0.5, 0.5, 0.7, 0.7)	**0.7** (0.9, 0.8, 0.4, 0.7)
Olives	**0.3** (0.4, 0.2, 0.3, 0.3)	**9.5** (14.5, 8.8, 6.1, 8.5)	**0.2** (0.4, 0.2, 0.1, 0.1)	**0.3** (0.4, 0.2, 0.2, 0.2)
Gherkins	**0.3** (0.3, 0.5, 0,3, 0,3)	**10.7** (10.9, 9.1, 11.5, 11.4)	**0.2** (0.1, 0.1, 0.2, 0.2)	**0.5** (0.3, 0.4, 0.6, 0.6)
Cotton A	**0.2** (0.2, 0.3, 0.2, 0.2)	**0.3** (0.2, 0.3, 0.4, 0.4)	**< 0.1** (< 0.1, < 0.1, < 0.1, < 0.1)	**0.2** (0.2, 0.3, 0.2, 0.2)
Cotton B	**0.4** (0.3, 0.4, 0.4, 0.4)	**0.2** (0.2, 0.2, 0.2, 0.3)	**< 0.1** (< 0.1, < 0.1, < 0.1, < 0.1)	**0.2** (0.2, 0.1, 0.2, 0.1)

* The deviation of double determination of 4,4´-DDE in soil samples for potatoes, olives and gherkins was in the range of 2.2 to 20.7 %.
[+] Each value is the mean of 4 samples.

This result indicates that these selected pesticides are not used today in these selected fields. To elucidate the overall occurance in Syrian soils more samples from different regions of the country must be taken and more detailed data about the previous and recent use of chlorinated pesticides in agriculture should be collected and integrated in an optimized sampling strategy.

The insecticide 1,1,1-trichloro-2,2-bis(p-chlorophenyl)-ethan (4,4´-DDT) was/is extensively applied in agriculture and forestry. In most western countries the use was permitted in the 1970th. Nevertheless in the last years still concentrations of 4,4´-DDE up to 100 µg/kg dry wt. in soil samples in eastern Germany [2, 8] were detected. Similar values can be found in agricultural soils from Alabama, USA [9]. In the presented study the insecticide 4,4´-DDT with its derivative products 4,4´-DDE and 4,4´-DDD ranged between < 0.1 and 17.2 µg/kg dry wt. (Table 1). Only agricultural soil samples (potatoes, gherkins, olives) showed elevated residues. The degradation of 4,4´-DDT in soil can vary between 2-20 years. The results showed that the ratio between parent product (DDT) and its degradation product (DDE) ranged between 0.032 and 0.047, which means that the use of DDT in the studied soil samples was at least 20 years ago.

CONCLUSIONS

Our study is a spot check which gives limited information about POPs in Syrian environment. In future an extended strategy must be developed for agricultural soils as well as for soils from urban areas, industrial sites and waste treatment facilities. An analytical strategy for POPs in Syria is very important in order to control the levels of POPs in food chain (representative food and feed). Most analytical methods use GC/MSD(SIM) to determine POPs in samples of different origin. A simultaneous screening with GC/ECD can give enhanced information about the presence of non-target compounds like Dioxins (OcCDD/OcCDF), which are not discriminated by clean up procedure.

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