



Monitoring and bioremediation of organochlorine pesticides in surface water with *Enterobacter asburiae*

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Abstract

Aim of study: One of the safest techniques regarding the remediation of contaminated water is biological remediation. This study aimed to: (i) monitoring of a collection of organochlorine pesticides (OCPs) in three agricultural drainages (Nashart, no. 9, and El-shoka), located in Kafr El-Sheikh governorate, Egypt; and ii) investigate the biodegradation potential of different bacterial isolates regarding organochlorine pesticides.

Material and methods: Analysis of OCPs was carried out by gas chromatography, Enrichment cultures were used for isolation of the bacterial strains capable of OCPs biodegradation and the most efficient isolate was identified based on morphological, biochemical and molecular characteristics.

Main Results: The determination of OCPs in water samples by gas chromatography showed varying values of OCPs ranging from 0.0 µg/L (below detection limit) to 0.0385 µg/L. A total of four morphologically different bacterial isolates were obtained, which showed a remarkable capability of OCPs biodegradation detected in mineral salt medium containing 17 OCPs active ingredients by two approaches including the analysis of the OCP residues at the end of the incubation period and measuring the bacterial growth in terms of total viable count and optical density. The bacterial isolate N2 showed the highest degradation capability when the screening process was carried out to select the most efficient isolates, which was identified according to the morphological, biochemical and molecular characterization as *Enterobacter asburiae*.

Research highlights: The biodegradation of OCPs using *E. asburiae* was proved to be a promising approach for the detoxification and removal of OCPs residues in aqueous systems.

Additional key words: biodegradation; organochlorine pesticides; Kafr El-sheikh; Egypt, water pollution; pesticide contamination; aqueous system.

Abbreviations used: D1 (Nashart drainage); D2 (drainage no. 9); D3 (El-shoka drainage); DDD (dichlorodiphenyldichloroethane); DDT (dichloro-diphenyl-trichloroethane); ECD (electron capture detector); LOD (limit of detection); LOQ (limit of quantification); MSA (mineral salt agar); MSL (mineral salt liquid); OCP (organochlorine pesticides); OD (optical density); SD (standard deviation); SE (standard error).

Authors' contributions: WAG: designing and drafting the manuscript. SSEG: acquisition, analysis and interpretation of data. EBB: revision of the manuscript for important intellectual content. MASA: coordinating the research project, conceiving and designing the experiments. MMSM: Providing important materials and chemicals for the project. NMS: supervising the work.

Citation: El-Gendy, SS; Belal, EB; Sidkey, NM; Abdelrazek, MAS; Metwaly, MMS; Gad, WA (2020). Monitoring and bioremediation of organochlorine pesticides in surface water with *Enterobacter asburiae*. Spanish Journal of Agricultural Research, Volume 18, Issue 4, e0306. <https://doi.org/10.5424/sjar/2020184-16794>

Supplementary material: (Tables S1 and S2) accompanies the paper on SJAR's website.

Received: 06 Apr 2020. **Accepted:** 22 Dec 2020.

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Funding: The authors received no specific funding for this work.

Competing interests: The authors have declared that no competing interests exist.

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Introduction

Water pollution is a major cause of global concern as it leads to the onset of numerous fatal diseases which are considered responsible for the death of over 14,000 people every day (Agrawal *et al.*, 2010). The aquatic sys-

tems contamination by pesticides is one of the most serious problems influencing the environment and human health. Pesticide contamination of surface water has been well documented worldwide and constitutes a major issue that gives rise to concerns at national, regional and global scales (Konstantinou *et al.*, 2006). Large quantities

of pesticide active ingredients (2.4 million metric tons) are applied annually worldwide to secure protection from weeds, insects, fungi, and other unwanted organisms in urban and agricultural environments (Grube *et al.*, 2011). The presence of pesticide residues at even low concentrations ($\mu\text{g/L}$ and lower) has been documented by different monitoring studies in various water resources around the world (Helbling, 2015). Although the aim behind the pesticides use is to kill or drive away different pests, their excessive usage have resulted in several toxicological impacts among the non-target species including human beings (Odukkathil & Vasudevan, 2013). Human exposure to different concentrations of pesticides in Egypt is a critical phenomenon, affecting workers, farmers, and supervisors responsible for pesticides application in the cotton fields for more than once during the season (Belal *et al.*, 2018). Most pesticides could cause adverse effects when reaching organisms, and the intensity of their toxic effect varies with time, dose, organism characteristics, the environmental presence, and pesticides characteristics.

The aquatic system contamination by organochlorine pesticides (OCPs) is usually focused on due to their toxicity and persistence in environment for long periods of time (Ashry *et al.*, 2006). OCPs are one of the persistent organic pollutants (POPs) which are characterized by the persistent bioaccumulative nature (do not break down easily in the environment) (Boudh *et al.*, 2019). OCPs are lipophilic (have an affinity for fats) and easily soluble in fat and biomagnified as they move up through the food chain (Érseková *et al.*, 2014). Due to their persistence, the OCPs pose a risk to cause adverse effects to the environment, wildlife, and human (Kallenborn *et al.*, 2012). OCP residues result in serious problems not only in the cultivated soils where they are applied, but also in the crops that systemically retain part of these residues, in non-target organisms, and finally in surface and underground water, through runoff and seepage of agricultural drainage (El-Bestawy *et al.*, 2007). Although some persistent OCPs have been banned for agricultural use during the past few decades due to public health issues, high concentrations of dichloro-diphenyl-trichloroethane (DDT) and its metabolites have been found in soil, water, and sediment samples (Miersma *et al.*, 2003; Shen *et al.*, 2005). DDT and many other pesticides of this group such as aldrin, lindane, endosulfan, etc. have been introduced as poisonous compounds for the insects and pests (Mansouri *et al.*, 2017). Some of the toxicological impact of OCPs include chronic liver damage, endocrine and reproductive disorders, immuno suppression, various cancer, inhibition of choline esterases, Parkinson's and Alzheimer's diseases (Dutra *et al.*, 2009).

Considering the toxicity and ecotoxicity of OCPs and its metabolites, the removal of OCPs from contaminated aquatic systems has become an environmental priority so that the treated water meets the acceptable quality standards. There are many techniques for the remediation or

removal of these toxic pesticides; these include chemical decomposition, photochemical decomposition, volatilization, plant uptake, absorption, and microbial decomposition (Sun *et al.*, 2018) alongside with the traditional methods of pesticides removal depending upon incineration and landfills (Benner *et al.*, 2013). These traditional water treatment processes do not effectively remove pesticides from water in addition to the expensive cost and maintenance of sophisticated devices, and requirement for a high variety of chemicals and manpower for the eradication of OCPs. Such conventional methods are not reliable for complete eradication or degradation of such pollutants due to their complexity and chemical toxicity in terms of producing an enormous amount of secondary pollutants, as well as their lethal effects on the working and local area pollution (Chandra & Kumar, 2015). Therefore, alternative techniques are needed to overcome the problems of the traditional methods for effective removal of OCPs from contaminated water resources.

Biological degradation of pesticides is emerging as a sustainable and cost-effective technology in the field of the bioremediation for the effective decontamination and detoxification of pesticide-contaminated water in the aquatic system (Fenner *et al.*, 2013; Boudh & Singh, 2019). Microorganisms can use a variety of xenobiotic compounds, including OCPs, for their growth; the mineralization and the detoxification of these compounds is called bioremediation of pesticides (Belal *et al.*, 2008).

There have been some reports on the biodegradation of OCPs, the removal of heteroatoms (like halogens) or heteroatom-containing groups is among the first stages in the biodegradation process. Bacterial biodegradation of pesticides has been studied under aerobic and anaerobic conditions, however, degradation of some pesticides may proceed under alternating aerobic-anaerobic conditions and numerous bacteria were found to be able to co-metabolically metabolize DDT by dechlorination to dichlorodiphenyl-dichloroethane (DDD) under anaerobic conditions.

Biodegradation of dieldrin and endrin has also been studied and numerous microorganisms had been isolated and characterized for their ability to metabolize DDTs notably from contaminated sites (Barragán-Huerta *et al.*, 2007; Sonkong *et al.*, 2008). The biodegradation of endosulfan in soil and water environments by indigenous microbes has been reported by Abraham & Silambarasan, (2014) by using bacterial and fungal consortium. *Enterobacter* sp. was reported for its efficient capability of bioremediation toward diverse groups of organic and inorganic contaminants including pesticides degradation and mineralization of different organic contaminants (Singh *et al.*, 2004; Panda & Sarkar, 2012; Velázquez *et al.*, 2012; Akpe *et al.*, 2015; Ikuesan *et al.*, 2016; Obi *et al.*, 2016; Khanafer *et al.*, 2017). To ensure the safety and the reliability of the bioremediation strategy using the bacterial strain *Enterobacter* sp., the process was examined under controlled conditions

prior to the application on a larger scale as the success of the bioremediation process depends not only on the high degradation ability of the biological agent but also on the stability of active microorganisms under varied conditions, such as changes in pH and temperature. As biodegradation of OCPs could occur under both aerobic and anaerobic conditions, water microorganisms may have the potential ability to degrade pesticides existing in their aquatic environments. Therefore, the recent study aims to monitor the existence of some OCPs in certain agricultural drainages located in Kafr El-Sheikh governorate, Egypt, including Nashart drainage, drainage no. 9, and El-shoka drainage which have never been monitored for OCPs residues and to evaluate the efficiency of one or more bacterial isolates in the bioremediation of OCPs. It also reports the possibility of developing a cost-effective and environmental-friendly method for the treatment of such contaminated drainages that guarantees the removal efficiency of different environmental toxicants from the polluted areas under study.

Material and methods

The most efficient OCPs degrading bacterial isolate was examined for its biodegradation capability on raw water collected from D1, D2, and D3, three drainages in Kafr El-Sheikh governorate, Egypt; that had been analyzed for detection of the 17 OCPs to assess the efficacy of the bacterial isolate to achieve the biodegradation potential of the different tested pesticides outside the laboratory-controlled conditions.

Chemicals

The tested pesticide active ingredients, supplied by AccuStandard® Inc. Co., USA, included (purity in parenthesis): HCH-delta (99.8%), endosulfan sulfate (99.4%), HCH-alpha (98.1%), HCH-gamma (lindane, 99.3%), heptachlor (99.6%), aldrin (97.6%), HCH-beta (100.0%), heptachlor epoxide (99.1%), endosulfan I (100.0%), 4,4-DDE (100.0%), dieldrin (99.1%), endrin (97.0%), 4,4-DDD (99.0%), endosulfan II (99.9%), 4,4-DDT (100.0%), endrin aldehyde (100.0%), and methoxychlor (98.8%). Methylene chloride; (99.8%), supplied by Carl Roth Co. (Germany); was used as an organic solvent.

Study area and sampling

Three sites of agricultural water drainages, located in Kafr El-Sheikh Governorate, Egypt, were selected for a collection of water samples, including Nashart drainage (D1), drainage no. 9 (D2), and El-shoka drainage (D3). The selection of those three sampling sites was attained to

a number of reasons: i) there were neither previous studies made on D1, D2, and D3 for monitoring and detection of OCPs nor any other types of pesticides and further studies on biodegradation potential and bacterial diversity; ii) the agricultural lands surrounding the three drainages were known for extensive pesticide application and subsequent draining of water loaded with pesticide residues in the aforementioned drainages.

Raw water samples were collected manually at about 30 cm underwater surface in appropriate, clean, free of analyst of interest, free of contaminants sterilized containers. Suitable amounts of raw water were collected according to each test requirements. Each lab conditions and instructions for sample collection, storage, and preservation were exactly followed and performed according to standard methods of water and wastewater. The temperature of the sample was maintained at ambient conditions before and during testing. The collected water was stored and refrigerated at 4 ± 1 °C for subsequent testing according to EPA (1995) and Eaton *et al.* (2005).

Media

Minimal medium as mineral salt liquid (MSL) and mineral salt agar medium (MSA), nutrient agar medium, and nutrient broth medium, were used as enrichment media throughout this study as described by Brunner *et al.* (1980). We used high chemical purity products of DIFCO, Becton Dickinson & Co., USA.

— MSL 5X: 15 g/L KH_2PO_4 , 33.9 g/L Na_2HPO_4 , 2.5 g/L NaCl, 5 g/L NH_4Cl , and 1000 mL distilled water;

— MSA 5X: 15 g/L KH_2PO_4 , 33.9 g/L Na_2HPO_4 , 2.5 g/L NaCl, 5 g/L NH_4Cl , 15 g/L agar, and 1000 mL distilled water;

— Nutrient agar medium is a product of OXOID: 5 g/L peptone, 5 g/L NaCl, 2 g/L yeast extract, 15 g/L agar, and 1000 mL distilled water;

— Nutrient broth medium: 5 g/L peptone, 5 g/L NaCl, 2 g/L yeast extract, and 1000 mL distilled water.

Monitoring of OCPs residues in collected wastewater samples

Extraction procedures: Firstly, the three samples were filtered to remove any large particles or impurities that may interfere with the steps of extraction and the subsequent analysis procedures. Methylene chloride was used as organic solvent. A measured volume of each sample of approximately 1 L was solvent-extracted with 60 mL methylene chloride by shaking in a separatory funnel for about 2 min. The separatory funnel was degassed at time intervals to get rid of excessive pressure resulted from shaking. Two separate layers were obtained.

This procedure was repeated three times to ensure the extraction of all OCPs present in the sample. Anhydrous sodium sulfate was added to remove any water present in the extracts. The dried extract was evaporated using a rotary evaporator adjusted at 65–70 °C until the volume reached 2 mL. A volume of 5–10 mL of n-hexane was added to the extract; then the final extract was evaporated again using the rotary evaporator until the volume reached 1 mL. The walls of the concentrator tube were rinsed with n-hexane while adjusting the volume to 5.0 mL. The extract was transferred to an appropriate-sized TFE-fluorocarbon sealed screw-cap vial and stored, refrigerated at 4 °C, until analysis by Gas Chromatography–Electron Capture Detector (GC-ECD). This method was used according to EPA (1995). Three replicates were used during the pesticides monitoring experiment. The mean, standard deviation and the standard error were calculated.

Analysis of OCPs using gas chromatography: For detection of OCPs residues, the analytes in the extracts were measured by gas chromatography model (Varian CP3800) with an electron capture detector (ECD) according to EPA method 508 (EPA, 1995). The column used through gas chromatography was Varian CP-Sil 19 CB with a length of 30 m and an inside diameter of 250 µm; the used gases

were helium which was used as a carrier gas with flow rate 2.0 mL/min and nitrogen which was used as a make-up gas with flow rate 25 mL/min. The injection volume was 2 µL and the injector temperature was 220 °C and the detector was ECD. The used temperature program involved 30 min while temperature ranged between 120, 180 and 260 °C on three steps.

Method of verification: Determination of chlorinated pesticides in water by gas chromatography with an ECD (EPA, 1995) is a validated procedure which has been published as a standard by EPA to be used for a specific application. Basic validation work has been carried out to ensure method fitness for purpose. OCPs standard with concentration 0.5 µg/L was injected and the resulted signal to noise (S/N) ratio was used to calculate the practical limit of detection (LOD) and the limit of quantification (LOQ) according to Magnusson (2014). OCPs standard at concentration 5.0 µg/L was injected and the results were used to determine measuring recovery which by comparison with method recovery criteria; 70–130% is considered acceptable. Table 1 shows verification of the method by determining LOD, LOQ and recovery. The chromatographic run conditions used in "Method verification" were based on EPA (1995).

Table 1. Validation of the analytical method (EPA method 508) to determine chlorinated pesticides (OCPs) in water by gas chromatography and determination of limit of detection (LOD), limit of quantification (LOQ) and recovery.

OCPs	LOD & LOQ					Recovery		
	Injected standard µg/L	Result µg/L	S/N ratio	LOD (S/N=3) µg/L	LOQ (LOD*3) µg/L	Prepared standard µg/L	Result µg/L	Recovery %
HCH-delta	0.5	0.49	19	0.078	0.235	5	3.85	77
Endosulfan sulfate	0.5	0.45	250	0.005	0.016	5	4.1	82
HCH-alpha	0.5	0.51	150	0.010	0.031	5	4.55	91
HCH-gamma	0.5	0.53	150	0.011	0.032	5	4.75	95
Heptachlor	0.5	0.47	250	0.006	0.017	5	4.9	98
Aldrin	0.5	0.48	30	0.048	0.144	5	5.25	105
HCH-beta	0.5	0.47	167	0.008	0.025	5	4.7	94
Heptachlor epoxide	0.5	0.52	150	0.010	0.031	5	4.35	87
Endosulfan I	0.5	0.5	150	0.010	0.030	5	4.4	88
DDE	0.5	0.52	214	0.007	0.022	5	3.95	79
Dieldrin	0.5	0.51	30	0.051	0.153	5	5.4	108
Endrin	0.5	0.48	150	0.010	0.029	5	5.5	110
DDD 4.4	0.5	0.47	19	0.075	0.226	5	5.35	107
Endosulfan II	0.5	0.48	19	0.077	0.230	5	4.2	84
DDT	0.5	0.44	15	0.088	0.264	5	4.25	85
Endrin aldehyde	0.5	0.51	17	0.092	0.275	5	4.65	93
Methoxychlor	0.5	0.48	21	0.067	0.202	5	4.95	99

S/N: signal to noise ratio

Microbial degradation of the tested pesticides

Isolation of the bacterial isolates capable of OCPs biodegradation

Enrichment cultures of bacterial isolates having the ability to degrade and mineralize certain chlorinated pesticides were established from the collected water samples from the three agricultural water drainages within Kafr El-Sheikh governorate, that were previously screened for detecting concentrations of chlorinated pesticide. A measured volume of each sample of ~ 10 mL was suspended in 90 mL sterilized mineral salt medium in a 500-mL bottle containing 10 µg/L of chlorinated pesticide standard as a sole carbon source, and then incubated at 30 °C and 150 rpm for 1 month. The great variation in the 17 tested pesticides which belong to three different groups of OCPs (cyclodienes, hexachlorocyclohexanes and DDTs), led us to prolong the period of enrichment to ensure the complete acclimatization and tolerance of the bacteria, needed to be isolated, to such a large group of the studied pesticides. This long incubation period was also to ascertain that the bacterial isolates became entirely capable of degradation of each one of the tested pesticides and to exclude any other weak isolates that could not tolerate and acclimatize this large pesticides groups. At the end of the incubation period, a volume of 10 mL of each culture was transferred into a freshly prepared 90 mL MSL medium containing the same concentration of chlorinated pesticides (10 µg/L). This process was repeated four times to isolate the largest number of isolates possessing assured highly biodegradation abilities. After the last time of the enrichment culture, dilution series up to 1:10⁻⁶ were prepared in glass test tubes containing 9 mL MSL medium, and then a measured volume of 100 µL of the enrichment culture was spread over plates of MSA medium containing chlorinated pesticides at the concentration of 10 µg/L using drigalisky triangle. The plates were wrapped in polyethylene bags and then incubated at 30 °C for 7 days. During this incubation period, the plates were monitored for the appearance of bacterial colonies. Single colonies growing on these diluted plates were isolated by picking the colonies up using a sterile inoculation loop and were further purified by the standard spatial streaking on complex agar media (Derbalah & Belal, 2008; Derbalah *et al.*, 2008; Massoud *et al.*, 2008).

Estimation of biodegradation potential of OCPs by the selected isolates in liquid culture

The selected isolates were screened for their ability to grow on and degrade the tested pesticides. MSL was prepared, distributed into 250 mL Erlenmeyer flasks, each

flask containing 50 mL of the MSL and 10 µg/L initial concentrations of the 17 OCP standard which were used as mixture not individually and then inoculated with 1 mL of culture containing 5*10⁶ CFU/mL and incubated for 7 days at 30°C. OD measurements were carried out using spectrophotometry analysis by spectrophotometer of Cecil Instruments, Cambridge, England. The total viable count of each isolate was determined by plating several dilutions of the liquid medium onto the nutrient agar medium. Bacterial populations were estimated by counting the number of colonies on plates. The growth represented in turbidity measurements was carried out by withdrawing a volume of 2 mL of each culture and reading it directly on the UV-VIS spectrophotometer. The experiment was carried out in 250 mL Erlenmeyer flasks, each flask containing 50 mL of sterilized MSL medium. Control flasks of an equal volume of liquid mineral medium and pesticide without any bacterial population were used in parallel at all intervals to evaluate the abiotic loss and another control flasks for each bacterial isolate without the pesticides to evaluate the microbial growth in pesticide absence. The percentage of degradation for the tested pesticides was also determined after 7 days using gas chromatography to evaluate the biodegradation process.

Identification and characterization

The most efficient OCPs degrading bacteria isolates were identified based on morphological and biochemical characters and 16S rRNA gene analyses.

Morphological and cultural characterization of the selected isolate

Bacterial isolates were examined for their cell shape, motility studies, and gram reaction.

— Cell shape: The purified cultures at the log phase after 72 h were microscopically examined for the cell morphological characters.

— Motility: The 72-h grown isolates were microscopically examined using cavity slides for bacterial motility.

— Gram reaction: Gram staining was carried out as mentioned by Rangaswami & Bagyaraj, (1993).

Biochemical characterization

A set of 64 biochemical characterizations tests were performed automatically for the efficiently selected isolates using VITEK 2 compact system, an automated microbiology system utilizing growth-based technology (AOAC OMA 2012.02) (Crowley *et al.*, 2012).

Molecular characterization

The most efficient bacterial isolate regarding OCP degradation was identified using 16S rRNA as described by Boye *et al.* (1999). DNA was extracted and purified using the protocol of GeneJet genomic DNA purification Kit (Thermo K0721), and polymerase chain reaction (PCR) was performed using Maxima Hot Start PCR Master Mix (Thermo K1051), then a PCR clean up to the PCR product was carried out using GeneJET PCR Purification Kit (Thermo K0701). Finally, sequencing the PCR product was performed with ABI 3730xl DNA sequencer by using forward and reverse primers through combining the traditional Sanger technology with the new 454 technology (Shokralla, 2014).

The nucleotide sequences were compared with those from the GenBank using BLASTn in the National Center for the Biotechnology Information server (<http://www.ncbi.nlm.nih.gov>). The sequence of PCR product and the reference sequences retrieved from the GenBank were aligned by ARB (<http://www.arb-home.de>) to construct the phylogenetic tree using the neighbor-joining method (based on Jukes-Cantor corrected distance). The bootstrap value was calculated based on 1,000 replications (Ludwig *et al.*, 2004; Jin *et al.*, 2010).

Biodegradation development monitoring over the incubation period

A standard concentration of 10.0 µg/L of the 17 OCPs was used to monitor the biodegradation process development over the incubation period, by growing the most efficient isolate in MSL medium containing 10 µg/L of the 17 OCPs and incubation for 7 days at 30 °C and 150 rpm. The residual concentration of each pesticide was estimated from day 2 to day 7 of incubation to monitor the chain process of biodegradation. Three replications of each experiment were carried out. The mean, the standard deviation (SD), and the standard error (SE) were calculated for each experiment day by day.

Remediation application

A volume of 1 mL of bacterial cell suspension (27×10^9 CFU) of *E. asburiae* isolate N2 was used to inoculate 50 mL of raw water from each source. The bacterial cultures were allowed for incubation with raw water at 30 °C for 7 days. The remediation application experiment was made in three replicates. At the end of the incubation period, the cultures were tested for determining the residual pesticide concentration levels using gas chromatography to evaluate the biodegradation process in raw water. The remediation application experiment was

made in three replicates. The mean, the SD and the SE were calculated.

Results and discussion

Monitoring of OCPs residues in collected wastewater samples

The analysis of certain OCPs residues in water samples collected from three agricultural water drainages (D1, D2 and D3) in Kafr El-Sheikh governorate (Egypt) accomplished by gas chromatography, showed remarkable variable values of OCPs in those sites, ranging from 0.0 µg/L (below detection limit) to 0.0385 µg/L. The results of the analysis process for D2 recorded the highest OCPs residual value for S cyclodiene endosulfan sulfate, while the lowest concentration was given to another S cyclodiene endosulfan I. Concerning these results it can also be noticed that in D3 the highest concentration of OCP residues was recorded by HCH-gamma (Lindane). Endosulfan II concentration was below the detection limit. In D1 endrin registered the greatest concentration among the other OCP residues, whereas the minimum residual value was recorded by one of S cyclodiene, aldrin. One of the OCP residues, endosulfan I, was absent in D1. Figures 1 and 2 show the concentrations (µg/L) and GC/ECD chromatograms of OCPs residues for surface water samples collected from the three different locations, respectively. The study establishes an effective biological solution that can be applied on a larger scale in such contaminated areas.

The studied OCPs could be grouped into three classes: cyclodienes, hexachlorocyclohexanes, and dichlorodiphenyltrichloroethanes (DDTs). Cyclodienes, also known as hexachlorocyclopentadiene (HCCPD), are persistent OCPs, with polycyclic structures used as insecticides including aldrin, dieldrin, endrin, endrin aldehyde, heptachlor, heptachlor epoxide, methoxychlor, endosulfan I, II, and endosulfan sulfate. Humans' exposure to its derivatives can be through direct contact with those insecticides as in farmers (application, harvesting, and handling of crops) or workers in wastewater treatment, workers in manufacturing and packaging of pesticides (Klaassen & Amdur, 2013). The use of most cyclodienes of the cyclodienes has been banned (Connell *et al.*, 2002; Perry *et al.*, 2013). The novelty of this work is comprising the study of a large group of pesticides (17 OCPs) in certain agricultural drains that were not previously monitored.

Cyclodienes have harmful effects on the central nervous systems of the target organism through interfering with (Ca^{2+} - Mg^{2+}) ATPase and GABAA receptors (Gant *et al.*, 1987). Among highly hazardous cyclodienes, aldrin and dieldrin are responsible for many disorders in higher organisms including distributions in the endocrine system

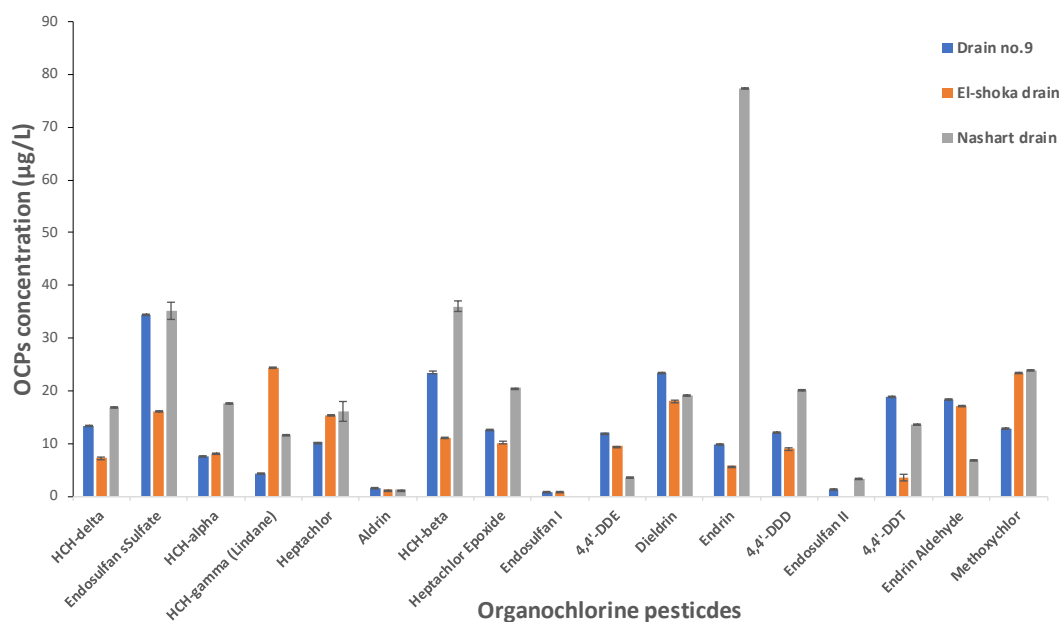


Figure 1. Concentration ($\mu\text{g/L}$) of the 17 OCPs in water samples collected from three different locations of agricultural water drainages (Nashart Drainage, Drainage no. 9, and El-shoka Drainage) in Kafr El-Sheikh governorate, Egypt. The measurements were repeated 3 times. Histogram bars show the mean and error bars show the standard deviation.

in birds and mammals, interferences with sex hormones leading to disorders in reproductive ability of males and in some cases result in cancer (Jaiswal *et al.*, 2017). As presented by the results of extraction and analysis of OCPs, it can be noticed that the concentration of cyclodienes in D2 ranged from 0.0009 to 0.03455 $\mu\text{g/L}$ for endosulfan I and endosulfan sulfate, respectively, recording the highest concentration among other cyclodienes in D2 followed by dieldrin with a concentration of 0.02345 $\mu\text{g/L}$. This elevated level of endosulfan sulfate in this water sample may indicate that it is still manufactured and used in spite of it has been forbidden. Another explanation of this elevated concentration can be attributed to the chemical transformation of endosulfan to its metabolite, endosulfan sulfate (Kwon *et al.*, 2005). Endosulfan sulfate is more persistent than the parent compound endosulfan (Abraham & Silambarasan, 2015), which is in accordance with the results of this study.

Isolation of the bacterial isolates capable of OCPs biodegradation

By applying the enrichment culture technique, a total of four morphologically different bacterial isolates possessing the ability to mineralize and degrade 17 OCPs were obtained from three drainage water samples previously collected from the three preceding described agricultural water drainages. The bacterial isolates were named N2, O1, O2, and O3.

Estimation of biodegradation potential of OCPs by the selected isolates in liquid culture

The four bacterial isolates were examined for their biodegradation capability with 17 OCPs in MSL media containing a definite concentration of the tested compounds (10 $\mu\text{g/L}$) as a sole carbon source. The four bacterial isolates were incubated with the OCPs at 30 μC for 7 days. At the end of the incubation period, the growth of bacterial isolates (in terms of total viable count and optical density [OD]) and the degradation percentage were assessed in comparison with control samples, one control is for the pesticides without any bacterial growth to assess the abiotic factors and another one for the bacterial isolates without the pesticides to evaluate the bacterial growth in absence of the tested pesticides the pesticides to assess. Regarding the bacterial isolate controls (no pesticides) it was found that no growth was detected because the medium was devoid of any carbon sources that are the main factor for the microbial growth. The control sample for OCPs showed a negligible changed concentration of the OCPs till the end of the experiment, meaning that OCPs residues are persistent. On the other hand, the pesticides incubated with the microorganisms showed the lowest concentration of the residues when compared to the initial concentrations; such findings could be attributed to the microbial action as discussed by Derbalah & Belal (2008), Derbalah *et al.* (2008) and Massoud *et al.* (2008). The reduction in pesticide concentrations after the incubation with various bacterial isolates could

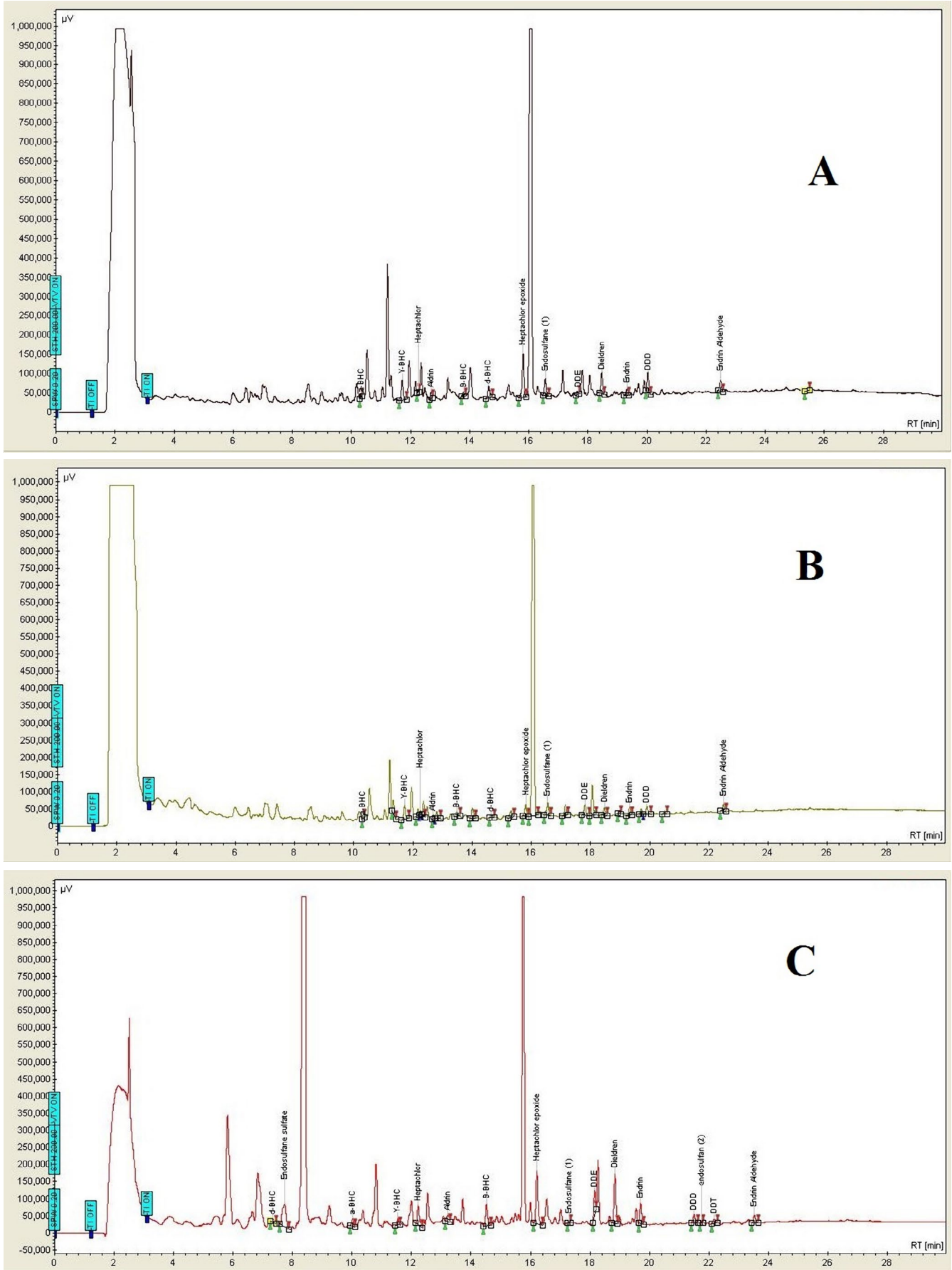


Figure 2. GC/ECD chromatograms of OCPs residues for surface water sample collected from three sites included: A) Drain no. 9, B) El-shoka drain and C) Nashart Drain.

be attributed to either biodegradation, or bioaccumulation, or both with different ratios; the bacteria could convert organic molecules into small non-toxic molecules. Thus, the chances of the secondary pollution could be avoided. Several studies showed that mineralization and co-metabolism were the main mechanisms for the further degradation of pesticides and their intermediate products (Mai *et al.*, 2001; Arora *et al.*, 2012; Ye *et al.*, 2018).

The whole bacterial degradation mechanism can be divided into three parts (Chen *et al.*, 2011). Firstly, the adsorption of the pesticide particle on the target site of bacterial cells, this occurs on the surface of the cell membrane and represents a critical dynamic equilibrium process. Secondly, the target got into the cell through the surface of the cell membrane, where the penetration rate and efficiency were related to the molecular structure of the target isomerism. Thirdly, xenobiotic target stimulated a rapid enzymatic reaction in the membrane (Chen *et al.*, 2011). Mineralization was a general term for the conversion of organic compounds into inorganic compounds under the action of soil microbes. Many chemical pesticides were analogs of natural compounds, and some microorganisms had the enzymes to degrade them. OCPs could be used as a source of microbial nutrients and then be degraded to inorganic matters, carbon dioxide, and water by microorganisms. Mineralization was an ideal way, because pesticides were completely degraded into non-toxic inorganic substances (Huang *et al.*, 2018). It was found that bacterial isolates have variable growth rates and incommensurate capability of biodegradation. As measured by total viable count and OD, the bacterial isolate N2 showed the highest records and demonstrated high biodegradation capability compared to the other three isolates that recorded less total viable count and OD measurements (data for its growth not shown). As presented in Figure 3, the bacterial isolate N2 showed exponential growth rate over

the incubation period. The growth rate expressed in terms of viable count started with count 5×10^9 CFU/mL at the first day and reached its highest count (27×10^9 CFU/mL) in the 7th day of incubation then started to decline at the 8th day which recorded 22×10^9 CFU/mL. The growth rate in terms of optical density started with 0.007 nm at the first day and reached its highest value (0.027 nm) in the 7th day of incubation, then started to decline at the 8th day which recorded 0.025 nm for the OCPs degrading N2 bacterial isolate. This elevated growth rate represented in the total viable count and OD, indicates that N2 was capable of growth and mineralization depending on the pesticide residues as a sole source of carbon.

By the analysis of OCPs residues using gas chromatography for the detection of pesticides removal percentage by the four tested bacterial isolates, it was noticed that the bacterial isolates showed different biodegradation abilities. Table 2 shows the initial concentrations of OCPs, OCPs concentrations in control samples, and OCPs concentrations in the bacterial cultures at the end of the incubation period. N2 recorded elevated levels of OCPs' biodegradation. Biodegradation of OCPs was observed following incubation. For all OCPs the degradation reached 100%, except for 4,4'-DDD, which recorded a degradation of 96.5%. Besides the results of bioremoval capability, the bacterial growth measurement values for each isolate was taken into consideration when selecting the most efficient OCPs degrading bacteria, as it was found that N2 registered the highest values of total viable count and OD compared to the other three isolates. In this point of study, there were not statistical studies. The findings regarding the biodegradation process by *E. asburiae* were in agreement with Abraham & Silambarasan (2015), who also found that *E. asburiae* is capable of mineralization and utilization of the OCPs endosulfan.

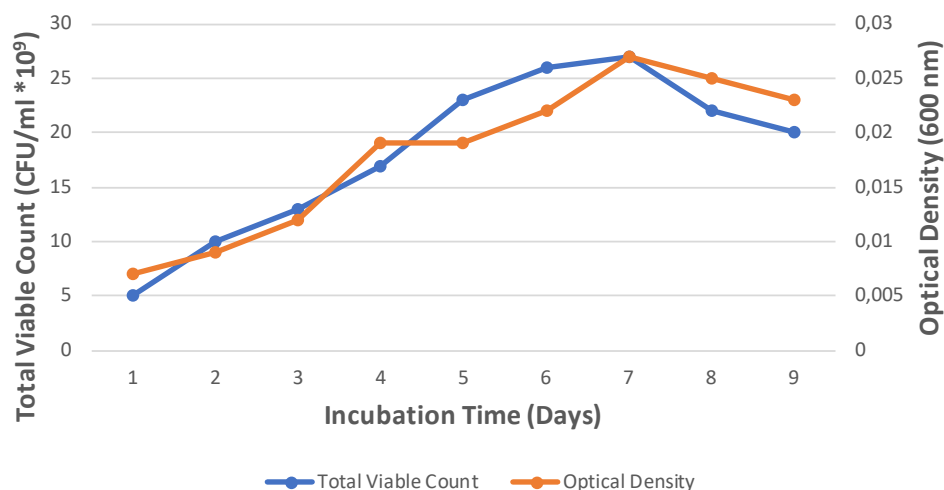


Figure 3. Growth curve by total viable count (CFU/mL) and optical density (nm) of *Enterobacter asburiae* isolate N2 at different incubation periods

Table 2. Initial concentrations of OCPs, OCPs concentrations in control samples, and OCPs concentrations in the bacterial cultures at the end of the incubation period.

OCPs	Initial conc. (µg/L)	After incubation period (µg/L)				
		Control	O1	O2	O3	N2
HCH-delta	10	8.15	6.35	< LOQ	4.35	< LOQ
Endosulfan sulfate	10	8.85	3.9	< LOQ	4.9	< LOQ
HCH-alpha	10	8.25	1.6	< LOQ	1.45	< LOQ
HCH-gamma (lindane)	10	9	3.35	< LOQ	2.9	< LOQ
Heptachlor	10	8.8	1.55	< LOQ	0.95	< LOQ
Aldrin	10	9.2	3.6	< LOQ	< LOQ	< LOQ
HCH-beta	10	9	6.5	< LOQ	3.85	< LOQ
Heptachlor epoxide	10	9.2	1.35	< LOQ	3.25	< LOQ
Endosulfan I	10	8.55	5.6	< LOQ	4.59	< LOQ
4,4'-DDE	10	8.65	0.65	< LOQ	< LOQ	< LOQ
Dieldrin	10	8.5	4.0	< LOQ	0.8	< LOQ
Endrin	10	8.3	1.1	< LOQ	0.3	< LOQ
4,4'-DDD	10	9	0.85	< LOQ	< LOQ	0.35
Endosulfan II	10	9.05	1.25	< LOQ	1.15	< LOQ
4,4'-DDT	10	9.1	0.85	< LOQ	0.35	< LOQ
Endrin aldehyde	10	8.95	< LOQ	< LOQ	< LOQ	< LOQ
Methoxychlor	10	8.3	2.3	< LOQ	2.9	< LOQ

The results of this study demonstrate the biodegradation potential of the bacterial isolates. According to the obtained degradation results, it was found that the bacterial isolate possesses promising degradation capabilities on chlorinated pesticide. Similar findings were reported by Derbalah & Belal (2008) who used an enrichment culture technique for isolation of bacterial isolates having the biodegradation potential on the aliphatic nitrogen fungicide cymoxanil. Belal *et al.* (2018) also showed that the bacterial strain *Paenibacillus* sp. was able to biodegrade the sum of 17 OCPs which are completely in agreement with the current research results. *Enterobacter* sp. potential for biodegradation of other groups of pesticides was proved by Sharma *et al.* (2014), who showed the ability of *Enterobacter* sp. to degrade imidacloprid, a potent insecticide of the neonicotinoid family.

Identification of efficient pesticide-degrading bacterial isolates

The cultural, morphological, and physiological characteristics performed for the most three efficient pesticide degrading bacterial isolates, N2, O2 and O3 revealed that N2 was gram-negative, rod-shaped, circular form, entire margin, flat elevation, with rough and cottony texture. Table 3

indicates the results of gram staining and colony characteristics of the bacterial isolates N2, O2 and O3. The results of the biochemical characterization tests as given by the VITEK 2 system are presented in Table S1 [suppl].

According to molecular characterization through the 16S rRNA analysis N2 was confirmed as *E. asburiae*, because its rDNA sequence was found to be 99% similar to that of *E. asburiae* strain JCM6051. The phylogenetic tree of N2 and related bacterial species based on the 16S rRNA sequence is provided in Figure 4. Finally, it could be stated that the morphological and biochemical characteristics and 16S rRNA identified the bacterial isolate as *E. asburiae*.

Biodegradation development monitoring over incubation period

Table S2 [suppl.] shows the biodegradation process development over the incubation period indicating that good biodegradation percentages were observed from day 5 and the biodegradation reached the highest percentages within day 7 of incubation. Similar findings were recorded by Abraham & Silambarasan (2015), who reported that the strains *E. asburiae* JAS5 and *Enterobacter cloacae* JAS7 were able to degrade endosulfan and its

Table 3. Results of gram staining and colony characteristics of the bacterial isolates N2, O2 and O3

No.	Colony characteristics	N2	O2	O3
1	Gram stain	Negative	Negative	Negative
2	Cell shape	Rod-shaped	Rod-shaped	Rod-shaped
3	Motility	Positive	Positive	Positive
4	Pigmentation	no	no	no
5	Margin	Entire	Entire	Entire
6	Elevation	Raised	Flat	Flat
7	Texture	Rough& cottony	Smooth	Smooth

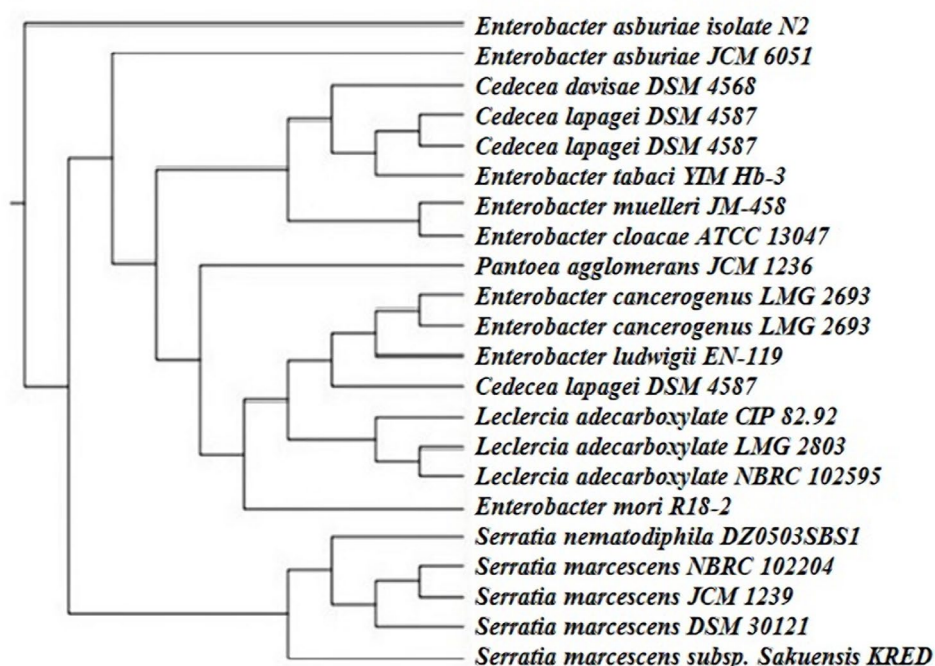
metabolites in the aqueous medium after 6 days of incubation. Sharma *et al.* (2014) also showed that *Enterobacter* sp. ATA1 was able to achieve the biodegradation of imidacloprid (a potent insecticide, after an incubation period of 72 h resulting in 30-40% degradation. Figure 5 shows GC chromatograms for the initial and the final (7th day) of the experiment which indicated full biodegradation (100%) for 17 OCPs where the biodegradation reached 96.9% for HCH-delta and 87.4% for Methoxychlor.

Remediation application

The bacterial isolate N2 of *E. asburiae* was used for a remediation application experiment on raw water collec-

ted from the three studied sources (Table 4). In case of D2 remediation experiment, *E. asburiae* showed 100% degrading potential for 15 OCPs and 98% and 91% for HCH-delta and Methoxychlor, respectively. The biodegradation potential ranged between 81% and 100% regarding D3 OCPs and between 77% and 100% with regard to D1 OCPs.

The variation of the biodegradation potential between the three drainages could be attributed to the variance of the environmental conditions between sites which can affect the biodegradation capability of *E. asburiae*. On the other hand, expected variations between natural and in-vitro conditions could explain the differences of the biodegradation potential between laboratory designated experiments and natural applications of the biodegradation process. Similar findings were given by Ramadan

**Figure 4.** Phylogenetic dendrogram obtained by distance matrix analysis of 16S rRNA sequences, showing the position of *Enterobacter asburiae* isolate N2 among phylogenetic neighbors.

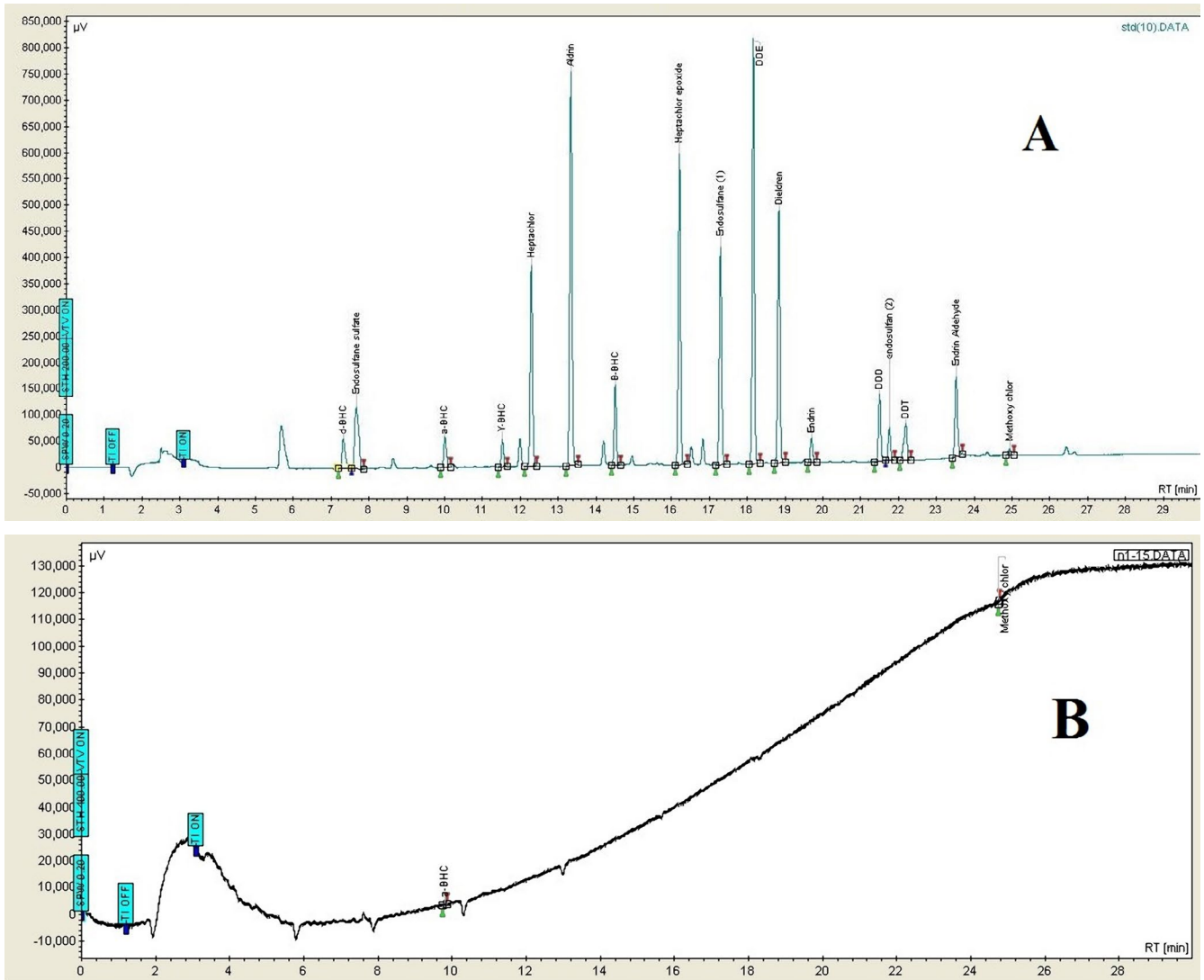


Figure 5. GC chromatogram of 17 OCPs residues to monitor the biodegradation process development: (A) initial concentrations before the incubation period and (B) final concentrations after the incubation period.

et al. (1990). The optimization of *E. asburiae* for natural applications can achieve better circumstances for the biodegradation of these polluted sites and other areas where the problem may arise from contamination with these types of pesticides.

The results of the recent study are promising, allowing the use of such biological strategies as a cleanup solution for the contaminated matrices in the areas under study and other polluted sites. Further studies are recommended for the development of the bioremediation process and making it possible to be applied in different contaminated environments to find an effective solution for the dilemma of pesticide contamination. This study recommends manufacture and usage of less toxic and less recalcitrant pesticides and rationalizing the use of pesticides with high resistance to degradation, consideration of reducing the doses of pesticides used in the agricultural field and using pesticides with other biological, agricultural, and chemical control methods,

using pesticides in forms that reduce their spread, such as capsules or slightly soluble substances, the usage of pesticides with different effective mechanisms to avoid the pests acquired immunity, the usage of suitable technology for the application of pesticides in terms of following the proper rules for storage, transport, mitigation, and addition. Finally, the considerable monitoring of different pesticide residues should be carried out on a larger scale covering the sites and drains expected to have pesticides-contamination problems.

Acknowledgement

We would like to thank the staff of the central laboratory for drinking water (in particular Dr. Hazem Fathy Taha), Kafr El-Sheikh company for water and wastewater, Kafr El-Sheikh city, Egypt, for the great effort that led to the success of this project.

Table 4. Biodegradation potential (%) of *Enterobacter asburiae* isolate N2 incubated for 7 days with water samples collected from Nashart drainage (D1), drainage no. 9 (D2), and El-shoka drainage (D3). The experiments were repeated three times.

	D1			D2			D3		
	Mean	SD	SE	Mean	SD	SE	Mean	SD	SE
HCH-delta	100	0.12	0.07	98	0.44	0.26	100	0.09	0.05
Endosulfan sulfate	99	0.59	0.34	100	0.64	0.37	100	0.81	0.47
HCH-alpha	99	0.32	0.19	100	0.26	0.15	99	0.05	0.03
HCH-gamma	99	0.3	0.17	100	0.81	0.47	100	0.36	0.2
Heptachlor	100	0.4	0.23	100	0.17	0.1	100	0.35	0.2
Aldrin	77	0.31	0.18	100	0.23	0.13	100	0.42	0.24
HCH-beta	99	0.25	0.15	100	0.17	0.1	100	0.76	0.44
Heptachlor epoxide	100	0.36	0.21	100	0.47	0.27	100	0.12	0.07
Endosulfan I	99	0.46	0.26	100	0.17	0.1	81	0.46	0.26
4,4'-DDE	97	0.32	0.19	100	0.25	0.15	100	0.06	0.03
Dieldrin	100	0.7	0.4	100	0.58	0.33	100	0.12	0.07
Endrin	100	0.72	0.42	100	0.66	0.38	100	0.25	0.14
4,4'-DDD	100	0.67	0.38	100	0.32	0.19	100	0.34	0.2
Endosulfan II	100	0.6	0.35	100	0.24	0.14	100	0.2	0.12
4,4'-DDT	100	0.74	0.43	100	0.14	0.08	100	0.64	0.37
Endrin aldehyde	100	0.55	0.32	100	0.29	0.16	100	0.2	0.12
Methoxychlor	100	0.1	0.06	91	0.4	0.23	100	0.14	0.08

SD: standard deviation. SE: standard error.

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