Role of Bacteria as Bioindicators for Organochlorine Pesticides Residues in Groundwater

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Abstract: The present study was performed to spot the light on the potential role of bacterial community in groundwater environment as bioindicators of organochlorine pesticides (OCPs) pollution. Groundwater samples were collected in summer and winter seasons, 2013 from hand pumps on Twelve (12) observation wells located in El-Rahawy rural area in Giza governorate, Egypt. Determination of oxygen demanding substances showed detectable levels of biochemical oxygen demand (BOD) and chemical oxygen demand (COD) mostly in winter than summer season, indicating susceptibility of these wells to organic pollutants. The COD/BOD ratio mean value (1.3) revealed the potential biodegradable conditions favored by microbial activities in summer season. Qualitative and quantitative assay of OCPs (n=18) showed that DDTs (dichlorodiphenyltrichloro ethanes) were the most predominating OCPs group, followed by cyclodienes and HCHs (hexachlorocyclohexanes). Both β-HCH & δ- HCH were not detected in any sample during the period of survey. The residual contaminants of total organochlorine pesticides (ΣOCPs) were maximum in winter (0.471 ng/L) compared to summer season (0.083 ng/L), but still being within safety permissible limits. Bacteriological assessment of groundwater quality reported non-ignorable levels for bacterial indicators, rendering these wells not suitable for drinking purposes. Characterization of major classes of bacterial community reported 48% for Gram-negative rods, followed by Gram-positive rods (44%), and Gram-positive cocci group (8%). Applying the pollution induced community tolerance (PICT) approach revealed identification of three bacterial strains showing recognizable tolerance abilities to high concentrations of OCPs mixture. *Pseudomonas aeruginosa* was the most compatible and tolerant isolate to concentrations ranged between 25 mg/L and 150 mg/L, followed by *Bacillus pseudomycoides* and *Bacillus amyloliquefaciens*. The Pearson's correlation coefficient between different concentrations of OCPs mixture and percentages of tolerant bacterial isolates showed highly significant correlation ($P<0.01$) at 0.992 with *pseudomonas aeruginosa* which was confirmed by linear regression analysis. The study concluded the importance of bacterial community in groundwater environment as bioindicators of OCPs pollution and suggested *Pseudomonas aeruginosa*, *Bacillus pseudomycoides* and *Bacillus amyloliquefaciens* as promising bacterial strains for bioremediation of contaminated spots. Recommendations were directed to encourage PICT studies and control pesticides application to minimize groundwater quality degradation. Setting standards and laws regarding the minimum depth and distance of wells from pollution sources could practicaly maintain good level of environmental hygeine.
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1. Introduction

Pesticides, the most cost-effective means of pest and weed control, have always been recognized as a magic tool for the maintenance of crops yield and economic viability. In the last 50 years, pesticides have greatly increased the quantity of food for the growing world population. However, concerns about their adverse effects among the different forms of life and ecosystems have also grown (Estevez *et al*., 2008). It has been estimated that, approximately 90% of agricultural pesticides never reach their target organisms; instead they are widely dispersed through air, soil and water (Hussaini *et al*., 2013).

Among these, are the organochlorine pesticides (OCPs) which are categorized as persistent organic pollutants with long half life. Their presence has been linked to several environmental and ecosystems disturbance, as well as chronic health effects such as cancer, neurological and teratogenic complications (Vaccari *et al*., 2006). The problems goes beyond the locality where they are used, as they can travel long distances and move downwards until reaching the water table at detectable concentrations. In fact, decontaminating polluted areas is a very complex task, and the damages are practically irreparable (Gavrilescu, 2005).

In response to the aforementioned hazards, OCPs were prohibited from use throughout the world for more than 20 years ago (Fenik *et al*., 2011). In Egypt, they have been banned since late 1996, after being used for more than 50 years (Nasr *et al*., 2009). Inquires about their environmental impacts have promoted the necessity to investigate the fate of these toxic compounds for rational decision-taking

regarding their authorization and/or decontamination if possible.

Groundwater is considered one of the most important water resources that has been recognized susceptible to OCPs pollution. In Egypt, groundwater represents a significant alternative to River Nile for drinking, irrigation and other domestic purposes, particularly in rural areas as well as some cultivated lands to which the Nile is not reachable (Mostafa *et al*., 2013). OCPs contamination to groundwater is likely to be non-point sources via run off, atmospheric depositions and leaching due to agricultural applications. Commonest cause of pollution is attributed to close proximity of septic tanks or drains to wells (Onunkwo and Uzoije, 2011). To study the fate and transport of OCPs in groundwater, very low detection limits must be reached. The analysis is recognized as complex and cost-prohibitive, and requires both high performance analytical instruments as well as efficient sample preparation, which could be prone to errors due to very low concentrations of compounds.

In this sense, researchers have developed the concept of bioindicators and pointed out their role in monitoring hazardous chemical contaminants even in trace amounts. Bioindicators reflect both biotic and abiotic levels of contamination, either through accumulating contaminants in higher concentrations or presenting alterations in their numbers, metabolic activities, physiological, morphological or behavioral trends (Fontanetti *et al*., 2011).

Studying groundwater ecosystem using microbial ecology can help in evaluating environmental stressors such as OCPs. In fact, aquifer microbiota plays an important role in maintaining ecosystem quality. The ability of groundwater to recover from chemical contaminants is primarily dependent on the presence of a microbial community with the ability to remove or bioremediate them. It can also indicate changes in the resource availability and the presence of pollution. Using bacteria as proxies to address this topic is warranted by their central role and responses to massive pollution events, even after long periods of time (Griebler and Lueders, 2009; Grenni, 2011).

Among the different possible modes of bacterial responses to perturbations by pesticides, that of tolerance is perhaps the most elusive, and has not been given the adequate level of attention so far. Tolerance may be defined as the ability of communities to withstand toxic insults inflicted by pollutants on the ecosystem, and survive under the resulting conditions. The idea underlying "Pollution induced community tolerance" at the bacterial level can be used as a qualitative and quantitative measure of the degree of ecological disturbance (Blanck, 2002; Aliasgharzad *et al*., 2011).

The present study is concerned to identify and quantify the organochlorine pesticides (OCPs) residues in some selected groundwater wells in El-Rahawy rural area in Egypt, and to address the nature and diversity of associated bacterial community. The potential role of bacteria as bioindicators of these toxic compounds will be investigated in terms of pollution induced community tolerance **(**PICT**)** approach.

2. Materials and Methods

2.1. Study area and sampling procedure

For conducting this study, El-Rahawy village in Giza governorate was chosen as representative for the Egyptian rural areas that depend mainly on groundwater supply. This area is characterized by high density of population and wide agricultural lands in which, about 43% of irrigation water is extracted from groundwater. Some private hand pumps are used to serve drinking water requirements. An important main drain, namely El- Rahawy drain passes through this village. This drain lies between latitudes 30º 11’ N to 30º 12’ N and longitudes 31º 1’ E to 31º 2’ E. It is about 12.41 km^2 with an average length of 5.2 Km. It receives agricultural and domestic wastes in addition to sewage of El-Giza governorate and discharges these wastes directly into Rosetta branch of River Nile (Gaber *et al*., 2013). Groundwater samples subjected for investigation were collected from hand pumps on observation wells located on both sides along El-Rahawy drain and south of Rosetta branch. Twelve (12) groundwater wells of average depth 48.4 m were selected as given in Table 1 and illustrated by Figure 1. About 58.3% of these wells (GW4, GW7, GW8, GW9, GW10, GW11 and GW12) are used illegally by local residents for irrigation, while 41.7% (GW1, GW2, GW3, GW5 and GW6) are used for drinking and domestic purposes. Sampling procedure was carried out in two different seasons (summer and winter, 2013) according to Standard Methods for Examination of Water and Wastewater (APHA, 2005). In all cases, well water pumps were operated for some time before taking the samples for purging purpose. For chemical analysis, samples were collected in clean glass containers (1.5 liter capacity).

For bacteriological examinations, at least three independent samples (n=72) were collected from each well in clean sterilized glass containers. All samples were stored in an iced cooler box and delivered immediately to the Central Laboratory for Environmental Quality Monitoring, National Water Research Center “CLEQM-NWRC” where they have been analyzed within 6h.

Table 1: Description and locations of investigated groundwater wells south Rosetta branch

Site code	Description	Location	
		Latitude (˚N)	Longitude (˚E)
GW1	East El Rahawy drain at 4.9 km	30˚ 11’ 14.36”	31˚ 02’ 53.19”
GW2	East El Rahawy drain at 4.5 km	30˚ 11’ 13.52”	31˚ 02’ 50.85”
GW3	West El Rahawy drain at 4.7 km	30˚ 11’ 14.43”	31˚ 02’ 71.06”
GW4	West El Rahawy drain at 4.6 km	30˚ 11’ 13.61”	31˚ 02’ 49.79”
GW5	East El Rahawy drain at 2.6 km	30˚ 11’ 36.36”	31˚ 02’ 74.19”
GW6	East El Rahawy drain at 2.1 km	30˚ 11’ 52.52”	31˚ 02’ 79.85”
GW7	West El Rahawy drain at 2.8 km	30˚ 11’ 31.36”	31˚ 02’ 69.19”
GW8	West El Rahawy drain at 2.4 km	30˚ 11’ 41.84”	31˚ 02’ 71.22”
GW9	East El Rahawy drain at 1.3 km	30˚ 12’ 05.76”	31˚ 02’ 13.29”
GW10	East El Rahawy drain at 0.8 km	30˚ 12’19.86”	31˚ 02’ 08.56”
GW11	West El Rahawy drain at 1.1km	30˚ 12’ 07.97”	31˚ 02’ 16.13”
GW12	West El Rahawy drain at 0.5 km	30˚ 12’23.16”	31˚ 02’ 03.48”

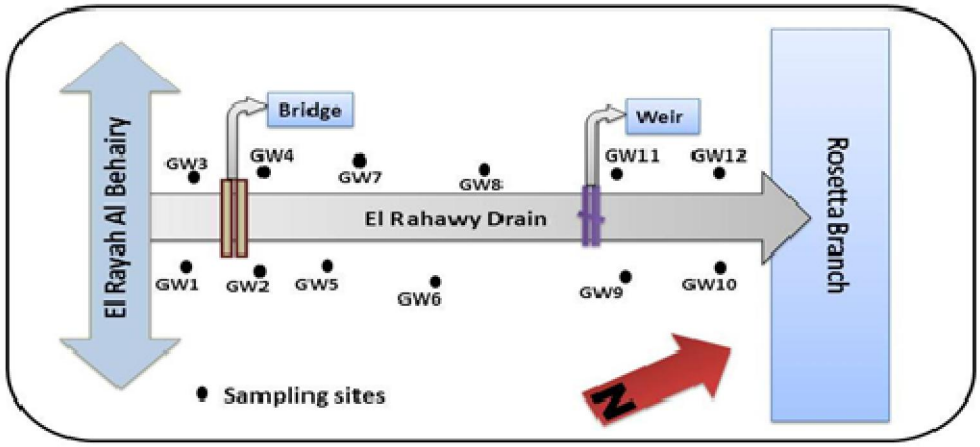

Figure 1: Schematic diagram for groundwater sampling locations.

2.2. Chemical analysis

2.2.1. Biochemical oxygen demand (BOD)

Determined using ORION BOD fast respirometry system model 890 with a measuring range 0-4000 mg/L at 20^{o}C incubation in a thermostatic incubator chamber model WTW.

2.2.2. Chemical oxygen demand (COD)

Spectrophotometer DR/2010 model 690 with COD reactor HACH was used. The organic matter in the sample is oxidized to carbon dioxide and water by boiling with a mixture of sulphuric acid and potassium dichromate. The amount of dichromate (oxidant) consumed in the breaking down of organic matter is measured calorimetrically.

2.2.3. Organochlorine pesticides

Standards and reagents

All chemicals and reagents used in this study were of high purity quality and were of analytical grade. n-Hexane and dichloromethane of special grade for pesticide residue analysis were purchased from Sigma-Aldrich, Germany. Organic solvents particularly dichloromethane which is toxic, were handled with care observing safety precautions, using efficient fume hoods and wearing protective gloves. Other materials used throughout the experimental procedure, such as cotton wool, filter paper and anhydrous sodium sulphates (Na_2SO_4) as well as Silica gel (60-100 mesh ASTM) were purchased from Merck, Germany. The individual reference pesticide standards (ISO 9001Certified) used for GC analysis of the organochlorines were purchased from Dr. Ehrenstorfer GmbH of Augsburg in Germany. A standard solution of each OCP was prepared in a proper way depending on being solid or liquid, to give a 100 µg/mL stock solution in n-hexane, which was stored at -20°C in glass bottles with PTFE-faced screw caps. Dilutions were prepared from the stock solutions and stored in the refrigerator at +4°C. A standard mixture solution containing all 18 pesticides was prepared with the appropriate concentrations of each pesticide, and stored at -20°C. For qualitative and quantitative interpretation of results, a concentration of 1.0 µL mixture of OCPs was used as

internal standard for OCPs standard mixture and in the real sample final solutions (Abbaccy *et al*., 2003).

Extraction procedure

Liquid-liquid extraction was used for the extraction of OCPs residues from water samples. One liter of each water sample was extracted with 60 ml dichloromethane in a 2-L separatory funnel. The mixture was shaken manually for 5 min, followed by collection of the lower organic layer. The extraction was repeated twice each time with 60 mL dichloromethane. The pooled 180 mL dichloromethane extracts were dried over anhydrous sodium sulfate and filtered. The solvent was evaporated to dryness under vacuum at ≤40˚C and 350 mbar. The residues were dissolved in 1 mL n-hexane containing 1 µL as internal standard (Golfinopoulos *et al*., 2003).

Calibration and quantification

Calibration curves were prepared from a stock solution of 10.0 mg/L of OCPs dissolved in hexane by serial dilution to reach calibration concentrations of 5, 10, 20, 40 and 50 µg/ L. Each calibration solution was analyzed in threefold by GC-ECD. The peak areas of the corresponding analytes were plotted against the calibration concentrations and the regression coefficient was calculated reaching a mean of r^2 = 0.9993 for all analytes. The minimum detection limits of the method used for extraction of OCPs residue from water is 0.01 ng/L (Rezaee *et al*., 2006).The retention times obtained for the components of the mixture are based on a signal-to-noise ratio of 3:1, the retention times were as follows: α-HCH (11.511 min), γ-HCH (13.288 min), heptachlor (14.514 min), aldrin (16.215 min), β-HCH (16.38 min), δ- HCH (17.311 min), heptachlor epoxide (18.221 min), endosulfan I (19.282 min), p,p'-DDE (20.145 min), dieldrin (20.721 min), endrin (21.523 min), p,p'-DDD (23.112 min), endosulfan II (23.337 min), p,p'-DDT (23.887 min), endrin aldehyde (25.037 min), methoxychlor (26.597 min), endrin ketone (26.786 min) and endosulfan sulfate (28.824 min).

GC-ECD analysis

A Hewlett-Packard 5890 series II GC with ECD and HP-A1773 (length of 6 m, 0.25 mm I.D. and 0.25 µm film thickness) capillary column was used with helium as the carrier gas and nitrogen as auxiliary gas. Conditions of the GC were: injector temperature 250˚C; detector temperature 320 ˚C; oven temperature 90˚C; initial temperature 90˚C; initial time 2 minutes; ramp 1, 30˚C min^{-1}; temperature 1, 180 min^{-1}; time 1, 0.0 minute; ramp 2, 30˚C min^{-1}; temperature 2, 270 ˚C, time 2, 0.0; final time 35 minutes; purge time 0.75 minutes; injection split less (Fatoki and Awofolu, 2003).

2.3. Bacteriological analysis

2.3.1. Bacterial indicators of groundwater quality

Water samples from different groundwater wells were assayed for traditional bacterial indicators according to standard methods recommended by APHA (2005). The Heterotrophic Plate Count (HPC) at 37 ^{o}C was determined by spread plate method No. 9215C on plate count agar medium, and the count was computed as CFU/mL. For enumeration of Total Coliforms (TC), Fecal Coliforms (FC) and Fecal Streptococci (FS), the membrane filter technique was applied. Water samples of appropriate volumes were filtered through sterile, surface girded "Sartorius" membrane of pore size 0.45 um and diameter 47mm, according to standard methods No. 9222B, 9222D and 9230C on: M-Endo agar LES, M-FC agar and M-Enterococcus agar, respectively.

All media used were purchased in a dehydrated form, Difco, USA. Results were recorded as Colony Forming Unit (CFU/100 mL) using the following equation:

$$Colonies\ /100\ ml = \frac{counted\ \ colonies}{ml\ of\ sample\ \ filtered} \times 100$$

2.3.2. Characterization and classification of bacterial community

Colonies developed from all tested water samples were randomly selected according to differences in their cultural and morphological characteristics and re-streaked twice on Nutrient agar plates to ensure purity. Pure isolates were maintained on Nutrient agar slants at 4^{0}C for further investigations as described below:

Microscopic examinations:

To differentiate Gram positive and negative bacteria as well as shape and arrangement of cells, the bacterial cells were cultured over night on Nutrient agar plates. Each bacterial isolate was smeared with a drop of water on a cleaned, grease-free glass slide and air-dried. The prepared slides were Gram stained and examined under a light microscope with 100x oil immersion objectives and the picture was captured. The presence, shape and position of spores -if present- were examined microscopically by preparing smears of pure bacterial culture of 48 h age stained by Malachite green for 5 minutes on water bath and safranin as a counter stain for 30 seconds (Collins and Lyne, 2004; Cheesbrough, 2006).

2.3.3. Isolation of OCPs tolerating bacteria from groundwater samples

A standard mixture solution containing all screened OCPs (n=18) was prepared with the appropriate concentration of each pesticide. Dilutions were prepared from the stock standard mixture to give four different concentrations (25, 50, 100 & 150

mg/L). 0.5% v/v of each concentration was supplemented under aseptic conditions to mineral salts agar medium used for bacterial isolation in which pesticides served as a sole source of carbon and energy for bacterial growth. The pH value was adjusted at 6.5 and the media was sterilized by autoclaving at 121^0C for 20 minutes (Barragan-Huerta *et al*., 2007). The prepared media were poured in sterile Petri dishes and allowed to solidify. 0.1 mL of undiluted and serially diluted groundwater samples were plated using spread plate method and the plates were incubated at 30^0C for 24 h. Three replicates were prepared for each dose of pesticides including the zero doses (control without pesticides addition). The growth of OCPs tolerating bacteria was determined by enumerating the developed viable colonies. Pure and separate bacterial colonies were selected, isolated and purified on the basis of morphological and cultural characteristics for subsequent identification process.

2.3.4. Identification of OCPs tolerating bacteria using Biolog profiling system

Pure bacterial isolates were characterized and identified by determining their utilization profiles on microtiter plate designed to test the ability of an organism to oxidize 95 different carbon sources using BIOLOG GIN III system, Biolog Inc., California, USA. Bacterial isolates were grown for 24 h. at 33±2 °C on Biolog Universal Growth Agar medium (BUG, 57 g l-1 purified water) supplemented with 5% sheep blood, according to Civilini (2009). The test yields a characteristic pattern of wells, which represents the "Metabolic finger print" of the inoculated organism. The pattern of wells is then keyed into the Biolog's Microlog Computer Program, which automatically cross-references the pattern to the standard library of species. If an adequate match is found, an identification of the isolate is made.

2.4. Statistical analysis

Results obtained were analyzed using SPSS software statistical program version 17. The correlation coefficient between different concentrations of OCPs and the corresponding percentages of tolerant bacterial isolates were compared at significant levels 0.01 and 0.05 (Levesque, 2007). Linear regression analysis was used to confirm the degree of correlation.

3. Results and Discussions

3.1. Determination of oxygen demanding substances

In the present study, biochemical oxygen demand (BOD) and chemical oxygen demand (COD) were used to reflect the possible oxygen burden in the investigated groundwater wells. Measuring BOD involves determining the amount of dissolved oxygen required by bacteria to decompose organic material in water through aerobic biochemical action. Mean while, COD constitutes the amount of oxygen consumed for complete chemical oxidation of organic constitutes to CO_2 and water in the presence of strong oxidizing agent (Duruibe *et al.*, 2007). Results demonstrated in Table 2 showed the seasonal variation in BOD and COD values. The BOD ranged between 10-18 mg/L and 12-21 mg/L, while COD fluctuated between 11-31mg/L and 18-42 mg/L in both summer and winter seasons, respectively. Clearly, BOD and COD levels were higher in winter than summer season, and all recorded values violate the permissible limits recommended by WHO (2004) (5 mg/L BOD and 10 mg/L COD) for drinking water quality, thus indicating susceptibility of these wells to organic pollutants, most probably due to close proximity to El-Rahawy drain.

Table 2: preliminary screening for organic load in groundwater samples during summer and winter seasons

Site Code	Summer			Winter		
	BOD (mg/L)	COD	COD/BOD	BOD (mg/L)	COD	COD/BOD
GW1	18	31	1.7	21	42	2
GW2	18	26	1.4	19	38	2
GW3	16	21	1.3	20	36	1.8
GW4	16	19	1.2	18	34	1.8
GW5	14	19	1.4	17	32	1.9
GW6	13	17	1.3	16	33	2.1
GW7	12	17	1.4	14	28	2
GW8	12	15	1.3	16	24	1.5
GW9	11	14	1.3	15	21	1.4
GW10	12	12	1	13	19	1.5
GW11	10	12	1.2	12	19	1.6
GW12	10	11	1.1	12	18	1.5

BOD: Biochemical oxygen demand; COD: Chemical oxygen demand; GW: groundwater samples.

Accordingly, COD/ BOD ratio could be a crucial factor to describe the biodegradability level by which organic matter in an aquatic system is readily broken down. Ratio of 2.0 to 2.1 was found to be optimum for biodegradable environment, while higher (3-5 fold) COD/BOD ratio reveals the intensive non- biodegradability and persistent nature of organic substances in the water body (Wentzel *et al.*, 2003). As presented in Table 2, COD/BOD ratio ranged between 1-1.7 in summer season with a mean value of 1.3, while in winter season the given records were 1.4-2.1 with a mean value of 1.8. The above data revealed the potential biodegradable conditions in investigated wells which was much better in summer than winter season. This finding was supported by the fact that, microbial activities for biodegradation are usually favored by increase in temperature (mean water temperature in summer was 27.6 ^{o}C) compared to cold seasons (mean water temperature in winter was 19.2 ^{o}C). The expected role of naturally – occurring bacteria as bioremediators of groundwater environment could interpret our results and agree with those reported by Debarati *et al*. (2005) and Hussain *et al*. (2009).

3.2. Qualitative and quantitative evaluation of organochlorine pesticides (OCPs)

OCP is a common name for a group of Pesticides consisting of benzene and chlorine (Pandit *et al.*, 2005). As demonstrated in Table 3, a total number of eighteen (18) organochlorine compounds were qualitatively and quantitatively evaluated in twelve (12) groundwater wells from study area. Their residual concentrations (ng/L) were expressed as minimum, maximum and mean values in both summer and winter seasons.

Table 3: Concentrations of OCPs residues in groundwater during summer and winter seasons.

	Concentrations (ng /L)					
OCPs	**Summer**			**Winter**		
	Min.	**Max.**	**Mean± SE**	**Min.**	**Max.**	**Mean± SE**
p,p'- DDT	0.000	0.034	0.005±0.0027	0.001	0.061	0.011±0.005
p,p'- DDE	0.000	0.003	0.0004±0.0003	0.000	0.031	0.009±0.003
p,p'- DDD	0.000	0.014	0.002±0.0012	0.000	0.04	0.004±0.004
ΣDDTs	0.000	0.051	0.008±0.004	0.001	0.132	0.024±0.010
α- HCH	0.000	0.001	0.0002±0.0001	0.000	0.003	0.001±0.0003
β- HCH	0.000	0.000	0.000±0.000	0.000	0.000	0.000±0.000
γ- HCH	0.000	0.013	0.003±0.0012	0.000	0.032	0.008±0.003
δ- HCH	0.000	0.000	0.000±0.000	0.000	0.000	0.000±0.000
ΣHCHs	0.000	0.014	0.003±0.0013	0.000	0.035	0.009±0.003
Aldrin	0.000	0.001	0.0001±0.00008	0.000	0.004	0.001±0.0004
Dieldrin	0.000	0.003	0.0007±0.0003	0.000	0.012	0.004±0.0012
Endrin	0.000	0.001	0.0001±0.00008	0.000	0.003	0.001±0.0003
Endrin aldehyde	0.000	0.002	0.0003±0.00019	0.000	0.006	0.001±0.0003
Endrin ketone	0.000	0.011	0.0021±0.001	0.000	0.042	0.009±0.0042
Heptachlor	0.000	0.001	0.0001±0.00008	0.000	0.005	0.001±0.0004
Heptachlor epoxide	0.000	0.000	0.000±0.000	0.000	0.001	0.0002±0.0001
Endosulfan I	0.000	0.012	0.004±0.001	0.001	0.036	0.015±0.0031
Endosulfan II	0.000	0.007	0.001±0.0006	0.000	0.015	0.004±0.004
Endosulfan sulfate	0.000	0.013	0.004±0.001	0.001	0.036	0.014±0.0033
Methoxychlor	0.000	0.000	0.000±0.000	0.000	0.001	0.0002±0.0001
ΣCyclodienes	0.000	0.036	0.013±0.0039	0.002	0.114	0.05±0.0128
ΣOCPs	0.000	0.083	0.026±0.008	0.003	0.471	0.109±0.0396

N.B: OCPs with concentrations below detection limit were converted to zero value for statistical analysis Purposes.

Generally, no significant differences were recorded among the investigated wells in the same season period. This observation was supported as given from the recorded mean concentrations ± SE. Out of 18 evaluated compounds, only two (β-HCH & δ- HCH) were not detected in any sample during the period of study. Mean while, among the other 16 compounds the DDTs (dichlorodiphenyltrichloro ethanes) were the most predominating OCPs group, followed by cyclodienes and HCHs (hexachlorocyclohexanes). Maximum recorded total concentrations were (0.051 & 0.132 ng/L), (0.036 & 0.114 ng/L), (0.013 & 0.032 ng/L) in both summer and winter seasons, respectively for the three groups in the same order. The highest residual concentrations in summer season were recorded for p,p'-DDT (0.034

ng/L) followed by endosulfan sulfate (0.013 ng/L) and γ-HCH (0.013 ng/L). In winter season, p,p'-DDT increased to reach a maximum of 0.061 ng/L, followed by endrin ketone (0.042 ng/L) and γ-HCH (0.032 ng/L). Residual contaminants of total organochlorine pesticides (ΣOCPs) were maximum in winter (0.471 ng/L) compared to summer season (0.083 ng/L) and the levels decreased generally in investigated wells towards the direction of Rosetta branch, being maximum during winter season in groundwater well encoded GW4. Figure 2 illustrates the GC/ECD chromatogram of OCPs residues in the standard sample, while Figure 3 demonstrates that of GW4 in winter.

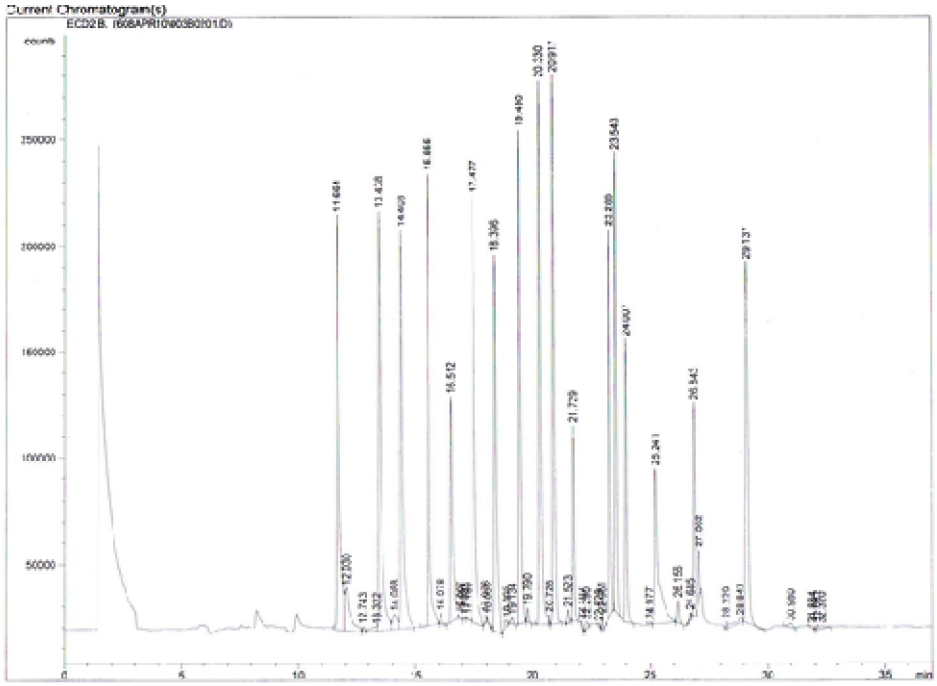

Figure 2: GC/ECD chromatogram of standard OCPs

In view of previous data, it could be seen that although an Egyptian Ministerial Decree Prohibited the import and use of OCPs in 1996, some of these toxic compounds are still illegally applied and are detectable in water bodies even in trace amounts (Barakat, 2004). The presence of residual concentrations could be attributed to their long persistence and stability in the environment for decades. It has been cited that, degradation of DDTs in soil is 75 – 100 % in 4 - 30 years. It is resistant to light, atmospheric oxygen and weak inorganic acids, but readily decomposed to the biologically inactive p,p'-DDE (Kirman *et al*., 2011). Consequently, p,p'-DDT and its metabolites (p,p'-DDE & p,p'-DDD) were detected in our study.

On the other hand, endrin (a member of cyclodienes group) is an alicyclic chlorinated hydrocarbon that is rapidly converted to the epoxide form (endrin aldehyde and endrin ketone). The detection of residual concentrations of these compounds suggests the presence of renewable sources of cyclodienes in study area surrounding investigated wells, presumably due to leaching from agricultural lands (Stenemo *et al*., 2005) and impact of El-Rahawy drain, which has been reported to be more polluted by OCPs than associated water courses (EL-Barbary *et al.*, 2008).

Concerning HCHs group, data available in Table 3 showed that γ-HCH and α- HCH were the only detected isomers in this study. The HCHs concentrations had the least contribution among ΣOCPs in both seasons. These findings are in complete harmony with the chemical and biological Properties of HCHs group, being less persistent in the environment, having higher water solubility, vapor pressure and biodegradability along with lower lipophilicity and particle affinity (Tang *et al.*, 2007). Indeed, it seems clear that seasonal variation play a non- ignorable role in directing the levels of OCPs in groundwater aquifers. Total OCPs increased in winter with increasing pollution load during low flow period and winter closure, as well as the rainy conditions which help leaching of pesticides from surrounding environment (El-Sebae, 1989; El-Bouraie *et al.*, 2011; Ezzat *et al.*, 2012). Contrastively, the high temperature in summer season and increase in solar radiation are promoting factors for microbial activities, leading the increase in biodegradation rate

and decrease in pesticides concentrations (Estevez *et al.*, 2008). It is worth mentioning that, although the above recorded values of OCPs in groundwater studied area were still within safety permissible limits as given by the Canadian Water Quality Guidelines for irrigation and fresh water use (CWQGS, 2005), yet these low levels would represent a potential hazard as sources for continuous bioaccumulation and biomagnification in the food chain.

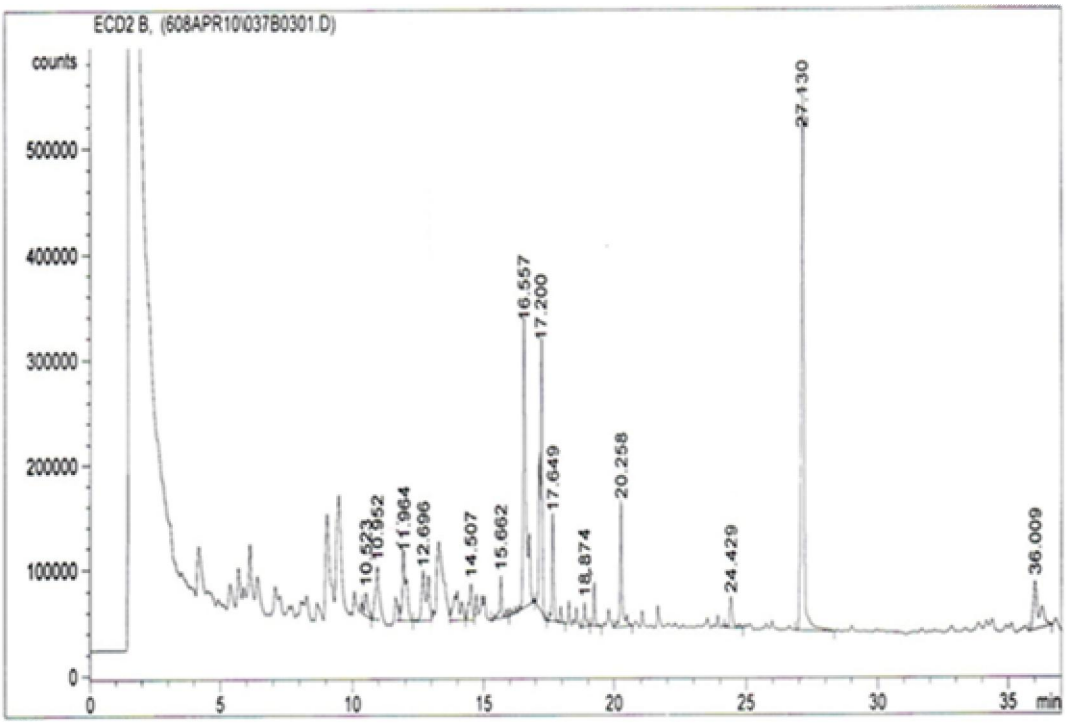

Figure3: GC/ECD chromatogram of OCPs in groundwater sample (GW4) in winter season.

3.3. Bacteriological assessment of groundwater quality

Groundwater has traditionally been considered to be the least water resource susceptible to contamination by indicator bacteria or human pathogens. As groundwater flows through an aquifer, it is naturally filtered by soil. This filtering combined with the long residence time underground implies that groundwater is usually free from disease causing microorganisms. A source of contamination close to a well, however, can defeat these natural safe guards (Francy *et al.*, 2000; Aksu and Vural, 2004; Edmunds and Shand, 2008). Bacterial indicators; Heterotrophic Plate Count (HPC), Total Coliforms (TC), Fecal Coliforms (FC) and Fecal Streptococci (FS) were typically used to assess the sanitary quality of groundwater wells in the present study. Data given in Table 4 declared obvious bacterial contamination in all sampled locations. In summer season, the mean bacterial counts ± SE for HPC, TC, FC and FS were respectively, 496±18.2 CFU/mL, 548±14.6 CFU/100mL, 283±6.4 CFU/100mL and 41±5.3 CFU/100mL. While in winter season, the levels were increased to 585±18.8 CFU/mL, 709±14.0 CFU/100mL, 336±3.5 CFU/100mL and 71±9.1 CFU/100mL for the same indicators in the same order (Figure 4).

The above presented results are in sharp contrast with the Egyptian Standards of Ministry of Health (2007), USEPA (2009) and WHO (2011) which stated that, TC, FC and FS should not be detected in any 100mL of water samples meant for drinking purposes. The presence of these bacterial groups signifies the possible presence of pathogenic bacteria contributing to water-borne diseases (AL-Khatib and Hassan, 2009). In our view, concentrations of bacterial contamination observed in studied groundwater are actually functions of several active factors including; local residents in area of survey are mostly lacking adequate level of environmental hygiene, leachate from surrounding agricultural lands served by animal manure as fertilizers and human wastes from septic tanks, as well as the effect of distance between these wells and a major pollutant source (EL-Rahawy drain). In this respect, it was estimated that coliforms could travel through a distance of 70.07 m (232 ft) from a sewage source to the surrounding groundwater (Adetunji and

Odetokun, 2011). Our results matched to a great extent with those obtained from some other private wells in the same area of study (Mostafa *et al*., 2013) as well as worldwide investigations (Emmanuel *et al*., 2009; Ramirez *et al*., 2010; Mile *et al*., 2013).

Table 4: Bacteriological analysis of groundwater samples during summer and winter seasons

Site code	Summer				Winter			
	HPC	TC	FC	FS	HPC	TC	FC	FS
	CFU/mL	CFU/100mL			CFU/mL	CFU/100mL		
GW1	550	598	314	80	660	780	346	88
GW2	580	612	302	48	505	659	324	51
GW3	600	632	294	57	640	759	341	70
GW4	480	549	284	43	680	784	352	130
GW5	510	562	278	35	520	673	321	40
GW6	540	564	291	50	635	727	351	120
GW7	430	498	235	17	500	637	336	64
GW8	460	511	274	25	515	659	348	90
GW9	500	553	254	19	600	718	326	55
GW10	475	541	278	33	630	721	317	20
GW11	425	487	307	52	580	698	329	58
GW12	400	467	279	27	550	687	341	68

N.B.: Values are means of 3 independent samples (n=72)
HPC: Heterotrophic plate count; TC: Total coliforms; FC: Fecal coliforms; FS: Fecal streptococci; GW: Groundwater samples

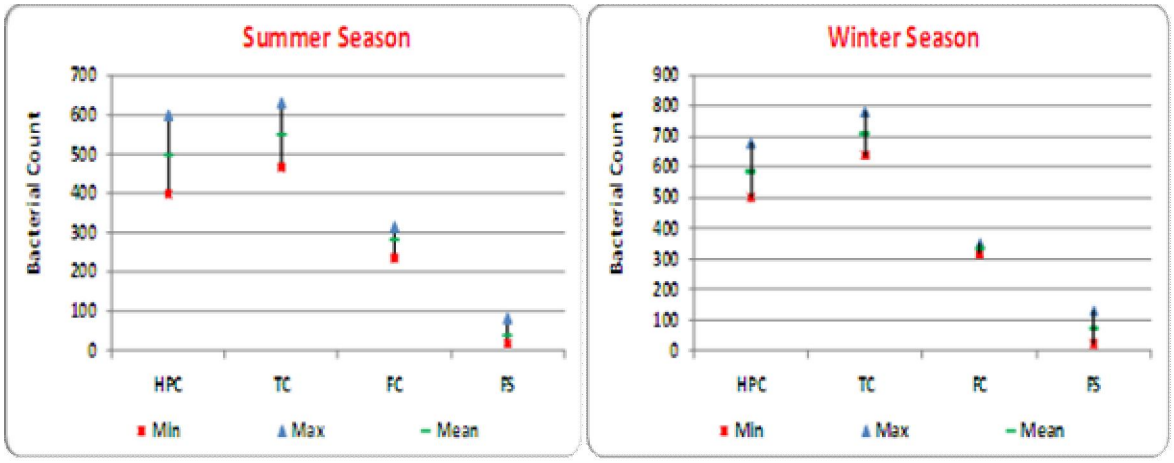

Figure 4: Seasonal variation of bacterial indicators in groundwater samples**.**

3.4. Characteristics of bacterial community in ground water

Apart from accidental and unusual microbial contamination cases in groundwater, scientific concepts and methods limited our knowledge of groundwater microbiology until the 1970's, assuming that groundwater environment is devoid of life. However, since 1990 several reviews have explored the suitability of groundwater habitats for microbial growth and demonstrated the role of bacterial flora in several natural activities of transformations and biodegradations (Griebler and Lueders, 2009).

In the present investigation, out of 72 processed water samples during two different seasons, 258 bacterial isolates were recovered from heterotrophic plate count according to differences in their cultural and morphological characteristics as well as microscopical examinations. As shown in Figure 5, the most predominating bacterial group was the Gram-negative rods (125 isolates, representing 48%), followed by Gram-positive rods (113 isolates, representing 44%), compared to the low incidence of Gram-positive cocci group (20 isolates, representing 8%) only.

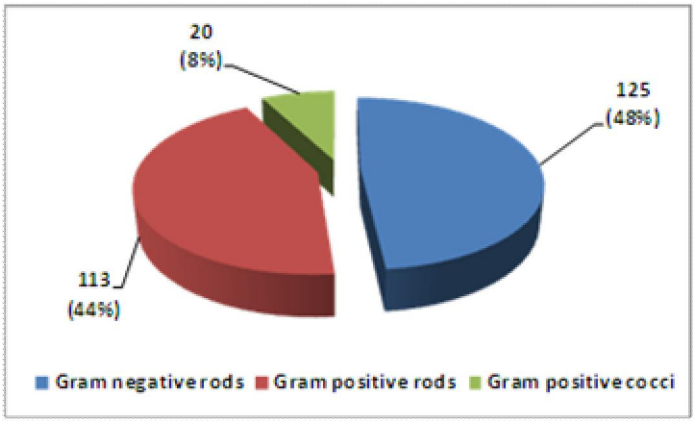

Figure 5: Number and percentages of bacterial flora isolated from groundwater samples.

The nature of bacterial community elucidated in this study correlates well with our earlier results demonstrated in Table 4, and signifies their origin. In this respect, Chapelle (2001) mentioned that Gram-negative bacteria are extensively found in shallow groundwater systems. They are either natural inhabitants in soil or derived from surrounding environment impacted with fecal contamination. Meanwhile, the Gram-positive bacteria have been widely reported to play a major role in modifying groundwater chemistry. Several genera belonging to bacterial groups addressed in our study have been documented for their biodegradation and bioremediation activities for many organochlorine pesticides (Singh *et al.,* 2000; Zhang and Quiao, 2002; Kumar and Philip, 2006). These findings are in harmony with the COD/BOD ratios reported in this study, and revealed the potential biodegradable conditions in investigated wells.

3.5. Bacteria as bioindicators of OCPs pollution

3.5.1. Identification and taxonomic characterization of OCPs tolerant bacteria

In the present investigation, a standard mixture solution containing all screened OCPs (n=18) was supplemented in different concentrations (25, 50, 100 & 150mg/L) to mineral salts agar medium used for bacterial isolation from groundwater samples. Results presented in Table 5 showed that, only three morphologically distinguishable bacterial colonies were observed (SM1, SM2 & SM3) as being tolerant to OCPs mixture at high concentrations. The Biolog system was used to assist in their phenotypic identification and suggested that isolate SM1 had a close relation to *pseudomonas aeruginosa*, while isolates SM2 & SM3 were closely related to *Bacillus amyloliquefaciens* and *Bacillus pseudomycoides*, respectively as compared to standard Biolog's identification data base. As shown in Plate 1, *pseudomonas aeruginosa* (SM1) are Gram-negative rods, flat and irregular with characteristic yellow to green fluorescent. *Bacillus amyloliquefaciens* (SM2) are Gram-positive, spore former rods, round and irregular creamy in color. *Bacillus pseudomycoides* (SM3) are Gram-positive, spore former rods with characteristic rhizoid creamy growth.

3.5.2. Pollution induced community tolerance (PICT) approach

So far, only few studies have tracked the topic of pesticides contamination by applying the pollution induced community tolerance (PICT) approach. To our knowledge, most studies have been directed to soil ecosystem, however using bacterial community in groundwater have not been adequately addressed (Aliasgharzad *et al*., 2011; Imfeld and Vuilleumier, 2012).

Depending on their type and concentrations, OCPs mixture applied in our study had different effects on the growth response of the three bacterial isolates. It is seen from Table 6 and Figure 6 that *Pseudomonas aeruginosa* (SM1) was the most compatible and tolerant isolate when exposed to varying concentrations of OCPs mixture. The number of developed colonies increased from 30 (63.8%) to 127 (92.7%) indicating that OCPs stimulated the growth of isolates in the range of 25mg/L to 150 mg/L compared to the control test (without pesticides addition). Similarly, Imfeld and Vuilleumier (2012) mentioned that co-tolerance may develop in presence of pesticides mixture. Contrastively, OCPs concentrations in the range of 25mg/L and 50mg/L were only stimulatory to both *Bacillus pseudomycoides* (SM3) and *Bacillus amyloliquefaciens* (SM2). 100mg/L was the critical concentration for both isolates at which the number of tolerant isolates decreased by 21.7% and 25%, respectively. *Bacillus pseudomycoides* sill could survive a dose of 150mg/L; however reduction in bacterial count reached 44.4%.

Table 5: Biolog system identification of bacterial isolates tolerant to OCPs

ID Code : SM1
ID Result Species ID: *Pseudomonas aeruginosa*

	PROB	SIM	DIST	Organism Type	Species
==>1	0.992	0.612	7.080	GN-NENT	Pseudomonas aeruginosa
2	0.004	0.002	9.066	GN-NENT	Pseudomonas nitroreducens/azelaica
3	0.002	0.001	9.317	GN-NENT	Pseudomonas fulva
4	0.002	0.001	9.403	GN-NENT	Pseudomonas citronellolis

<X>:Pos; <X—:Mismatched Pos; X:Neg; X++:Mismatched Neg; {X}:Borderline; -X:Less Than A1 well

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0	0	0	0	{75}	{75}	0	0	<250>	<250>	<250>
B	0	{75}	0	{75}	0	{75}	0	0	0	<250>	<250>	<250>
C	{75}	0	<250>	{75}	0	{75}	{75}	{75}	{75}	<250>	<250>	<250>
D	0	{75}	0	0	{75}	{75}	{75}	0	0	<250>	<250>	<250>
E	{75}	<250>	{75}	{75}	<250>	<250>	<250>	<250>	{75}	<250>	<250>	<250>
F	{75}	{75}	{75}	{75}	{75}	{75}	0	{75}	0	<250>	<250>	<250>
G	{75}	{75}	0	{75}	{75}	{75}	0	{75}	{75}	<250>	{75}	<250>
H	{75}	{75}	{75}	{75}	{75}	{75}	<250>	{75}	{75}	<250>	{75}	0

ID Code : SM2
ID Result Species ID: *Bacillus amyloliquefaciens*

	PROB	SIM	DIST	Organism Type	Species
==>1	0.995	0.614	6.885	GP-RODSB	Bacillus amyloliquefaciens
2	0.002	0.001	9.032	GP-RODSB	Bacillus subtilis ss spizizenii
3	0.002	0.001	9.117	GP-RODSB	Bacillus subtilis ss subtilis
4	0.001	0.000	9.508	GP-RODSB	Bacillus licheniformis

<X>:Pos; <X—:Mismatched Pos; X:Neg; X++:Mismatched Neg; {X}:Borderline; -X:Less Than A1 well

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	<250>	<250>	<250>	<250>	<250>	<250>	<250>	<250>	<250>	<250>	<250>
B	<250>	<250>	<250>	<250>	<250>	<250>	<250>	{75}	{75}	<250>	<250>	<250>
C	{75}	{75}	{75}	<250>	<250>	<250>	<250>	<250>	<250>	<250>	0	0
D	<250>	<250>	{75}	<250>	<250>	<250>	<250>	<250>	{75}	0	{75}	0
E	<250>	<250>	<250>	<250>	<250>	<250>	<250>	<250>	<250>	0	<250>	0
F	<250>	<250>	{75}	<250>	<250>	{75}	<250-	<250>	<250>	0	{75}	{75}
G	{75}	<250>	<250>	<250>	<250>	<250>	<250>	<250>	<250>	{75}	<250>	<250>
H	<250>	{75}	0	{75}	0	<250>	{75}	<250>	<250>	0	<250>	0

ID Code: SM3
ID Result Species ID: *Bacillus pseudomycoides*

	PROB	SIM	DIST	Organism Type	Species
==>1	0.964	0.607	7.145	GP-RODSB	Bacillus pseudomycoides
2	0.009	0.005	8.826	GP-RODSB	Bacillus licheniformis
3	0.005	0.002	9.079	GP-RODSB	Bacillus subtilis ss spizizenii
4	0.002	0.001	9.452	GP-RODSB	Bacillus subtilis ss subtilis

<X>:Pos; <X—:Mismatched Pos; X:Neg; X++:Mismatched Neg; {X}:Borderline; -X:Less Than A1 well

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	<250>	<250>	<250>	<250>	<250>	<250>	<250>	{75}	{75}	{75}	{75}
B	{75}	{75}	<250-	<250>	<250>	<250>	{75}	{75}	{75}	{75}	0	{75}
C	<250>	<250>	<250>	{75}	{75}	{75}	{75}	{75}	{75}	{75}	0	0
D	<250>	<250>	{75}	<250>	<250>	{75}	{75}	{75}	{75}	0	{75}	0
E	{75}	{75}	<250>	<250>	<250>	<250>	<250>	{75}	{75}	0	{75}	0
F	{75}	<250>	{75}	<250>	<250>	{75}	<250>	{75}	{75}	0	0	0
G	{75}	{75}	{75}	<250>	{75}	{75}	{75}	<250>	{75}	{75}	{75}	{75}
H	{75}	{75}	{75}	{75}	{75}	{75}	{75}	{75}	{75}	<250>	<250>	{75}

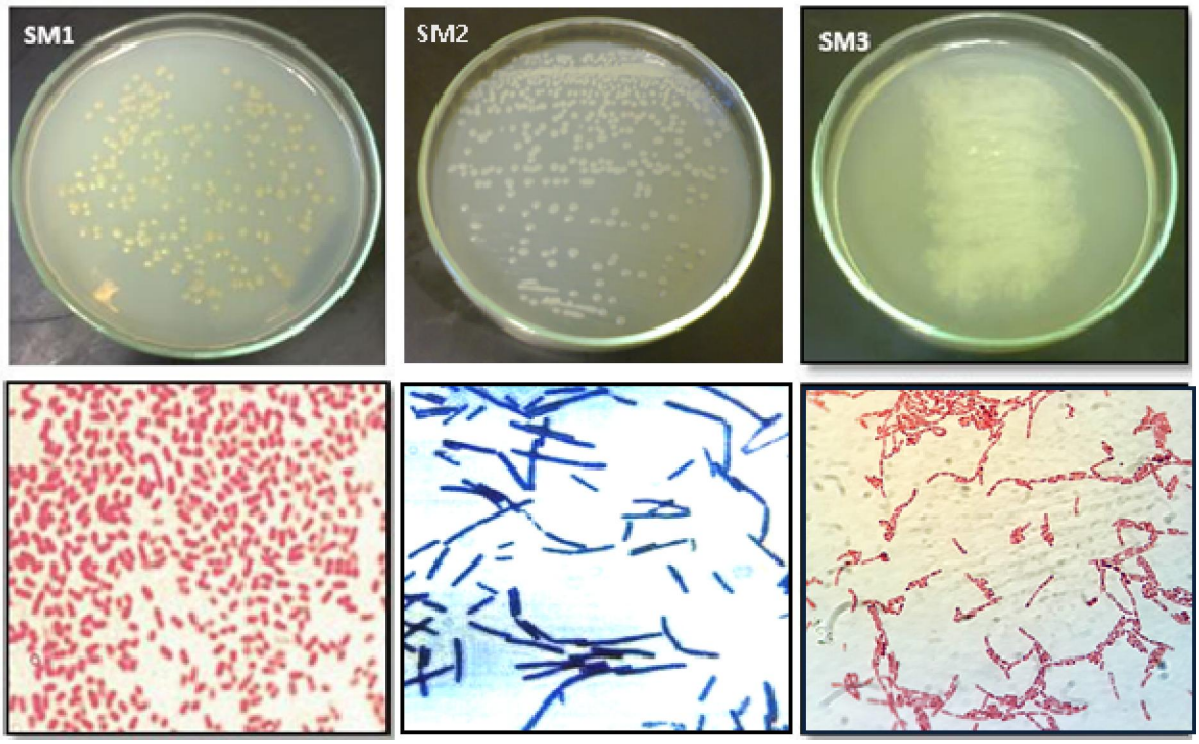

Plate 1: Morphological shapes and microscopic view of Gram-stained bacterial cells (magnification x 100).
SM1: *Pseudomonas aeruginosa*; SM2: *Bacillus amyloliquefaciens*; SM3: *Bacillus Pseudomycoides*

Table 6: Compatibility of bacterial isolates with different concentrations of OCPs

OCPs (mg/L)	Bacterial isolates						Total
	SM1		SM2		SM3		
	No.	%	No.	%	No.	%	
25	30±1.0	63.8	4±0.3	8.5	13±0.3	27.7	47
50	67±0.8	65.7	12±0.2	11.8	23±0.8	22.5	102
100	113±0.8	80.7	9±0.3	6.4	18±0.8	12.9	140
150	127±0.8	92.7	0±0.0	0	10±0.5	7.3	137
control	5±0.3	100	0±0.0	0	0±0.0	0	5

N.B.: Values are means of 3 replicate tests ± SE

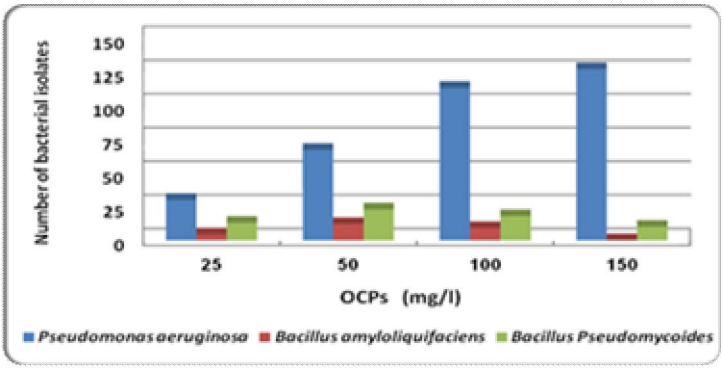

Figure 6: Effect of OCPs concentrations on number of tolerant bacterial isolates from groundwater samples.

As given in Table 7, the Pearson's correlation coefficient between different concentrations of OCPs mixture and percentages of tolerant bacterial isolates showed highly significant correlation (P<0.01) at 0.992 with isolate SM1 (*Pseudomonas aeruginosa*). Based on principles learned from earlier investigations, pseudomonades are a vast heterogeneous group of bacteria that are metabolically versatile, possessing active and extraordinary catabolic pathways for mineralization and biodegradation of about 127 different organic compounds including chlorinated pesticides (Jilani and Khan, 2004). This adaptability makes them ideal candidates for inhabiting aerobic aquifer (Chapelle, 2001).

Table 7: Correlation coefficient between different concentrations of OCPs and percentages of tolerant bacterial isolates

		Bacterial isolates %		
Variable	OCPs	SM1	SM2	SM3
OCPs	1.00			
SM1	0.992**	1.00		
SM2	-0.879	-0.925	1.00	
SM3	-0.991**	-0.979*	0.827	1.00

** Correlation is significant at the 0.01 level; * Correlation is significant at the 0.05 level

On the other hand, a highly significant negative correlation (P<0.01) at -0.991 was observed with isolate SM3 (*Bacillus pseudomycoides*). The same negative correlation but without significance was recorded for isolate SM2 (*Bacillus amyloliquefaciens*), particularly at concentrations higher than 50mg/L. this indicates that, high doses of OCPs mixture were inhibitory to the growth of these two isolates, most probably due to suppression of catabolic enzymes. Such observation could be side-stepped taking into consideration that the starting stimulatory doses for the growth (25 & 50 mg/L) were relatively high compared to the detected low residues in the groundwater samples. Accordingly, *Pseudomonas aeruginosa* could be categorized as being highly adapted tolerant strain, while both *Bacillus pseudomycoides* and *Bacillus amyloliquefaciens* are moderately adapted tolerant strains. The previous correlations were confirmed by linear regression analysis as illustrated in Figure 7and supported by data from recent studies, which concluded the ability of *Pseudomonas* and *Bacillus* strains to tolerate and bioremediate several OCPs among which are; DDT, Lindane and Endosulfan (Kumar and Philip, 2006; Barragan-Huerta *et al.*, 2007; Hussaini *et al.*, 2013).

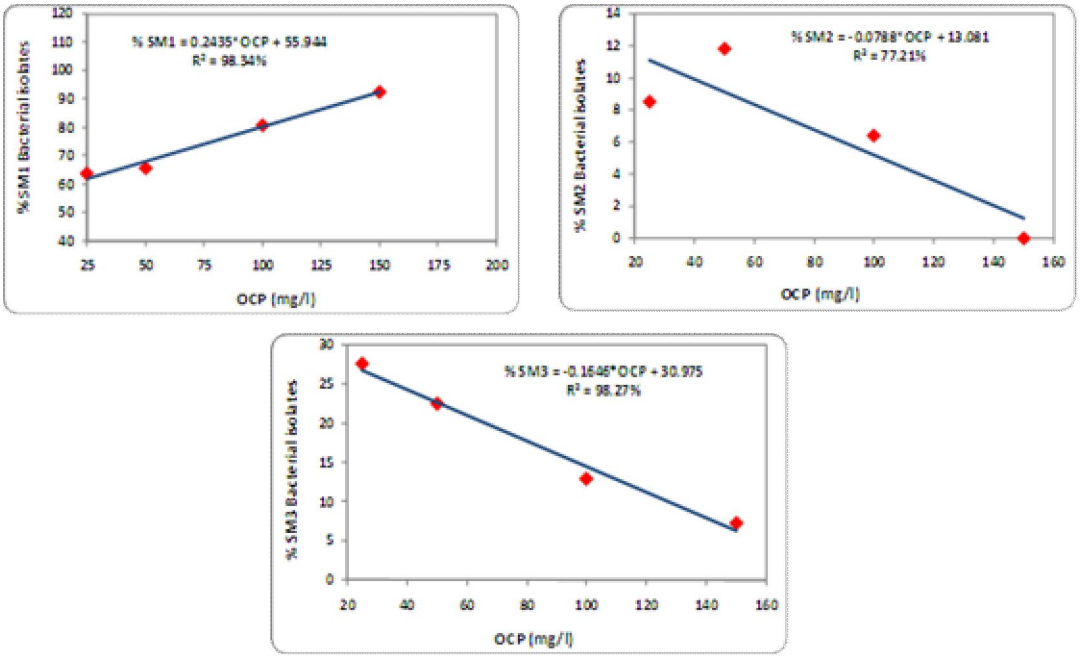

Figure 7: Regression analysis between OCPs concentrations and percentages of tolerant bacterial isolates.

In view of previous results, three different mechanisms are therefore suggested as causes of the increased tolerance observed in current study; an immediate toxic effect killing sensitive species, followed by a selection phase for OCPs tolerant strains due to different competitive abilities of surviving bacteria, and finally adaptation of bacteria due to physiological and /or genetical changes (Aliasgharzad *et al*., 2011). The growth of the three isolates at different levels of OCPs doses implies that they possess the appropriate catabolic enzymes capable of utilizing these contaminations as a sole source of carbon and energy. This ability is directly linked to their long-term adaptation to these contaminants in the aquatic environment, and reveals the history of OCPs application in Egypt even after long ban period. Indeed, it seems possible to predict that groundwater wells in this survey had been subjected to chronic levels of OCPs pollution, and that bacterial community in this environment played a master role in their biodegradation and bioremediation. In this respect, PICT approach can establish causal linkage between contaminants and effects. Our interpretations are in consistent with those reported by Schroll *et al*. (2004); Imfeld and Vuilleumier (2012) and Pesce *et al*. (2013).

Conclusions and Recommendations

The present study highlights the role played by bacterial community as potential bioindicators for some OCPs polluting groundwater environment. The pollution induced community tolerance (PICT) was found to be successful as a qualitative and quantitative measure for the degree of ecological disturbance, even after long ban period of these toxic compounds in Egypt. This approach also opens new prospects for developing ecological indicators capable of maintaining the effects of pesticides pollution in aquatic environment, and reflecting history of their application. The study also suggested *Pseudomonas aeruginosa*, *Bacillus pseudomycoides* and *Bacillus amyloliquefaciens* as promising bacterial strains for bioremediation of OCPs contaminated spots, relative to their recognizable tolerance abilities. In general, controlled pesticides application and wastes disposal practices are highly recommended to minimize groundwater quality degradation. Precautions should be taken by setting standards and laws regarding the minimum depth and distance of private wells used by local residents from pollution sources to maintain good level of environmental hygeine.

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