Importance of Biologically Treated Wastewater Re-use in Irrigation and Aquaculture Purposes
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Abstract
Treated effluent from Waste Stabilization Pond (WSP) System in Suez Governorate, Egypt represents nutrient as well as additional water source. In addition, it contains beneficial bacteria, especially nitrogen-fixers. The highest counts of asymbiotic nitrogen fixers, Azotobacter, Azospirillum and Clostridium were recorded during summer, while the lowest ones were in winter. The lowest counts of Azotobacter and Azospirillum were recorded in the anaerobic pond (AP) which maintained the highest counts of Clostridium. The most active nitrogen fixers’ isolates were identified as Azotobacter chrococcum, Azotobacter vinelandii, Azospirillum brasilense and Azospirillum lipoferm. These identified bacteria were tested for production of indole-3-acetic acid (IAA) and polyamines (PAs).

Introduction
Water re-use is seen as a promising solution to the growing deficit in water resources. In many countries, including Egypt, treated wastewater is the only water resource that is increasing as the population increases, while other sources are depleted.

According to World Bank estimates, the wastewater for More than 4,000 million people worldwide does not receive any form of treatment (Ringskog, 1999).

WSPs are frequently the wastewater treatment process of first choice in warm climates wherever land is available at reasonable cost (Mara, 2000).

Microorganisms are necessary to every agriculture system. Beneficial microorganisms is a generic term for a large group of microorganisms that contribute beneficial soil effects such as nitrogen fixation, mineralization, humus formation, disease suppression, and decomposition. Dinitrogen-fixing (diazotrophic) bacteria are potential suppliers of nitrogen for the environment. In addition, they are able to synthesize plant growth regulators (PGRs) such as 3-indole acetic acid (IAA), an auxin identical to that found in plants (Patten and Glick, 1996) and polyamines (PAs) such as putrescine (Put), spermidine (Spd), spermine (Spm) and cadaverine (Goris et al., 1998).

Polyamines are small aliphatic amines and are known to be ubiquitous in all living organisms, plant, animal and microbial cells (Davies, 1995; Cohen, 1998).
PAs participate in numerous biological processes (Tabor and Tabor, 1984), including cell replication and differentiation, and biosynthesis of nucleic acids and proteins (Bardócz et al., 1993). In addition, PAs help organisms to become more tolerant to salt stress (Bouchereau et al., 1999) and act as protective agents against the toxic effects of oxygen (Chattopadhyay et al., 2003). However, the release of PAs by N-fixing bacteria has not been well studied (Thuler et al., 2003).

Although the dual benefit of wastewater reuse for irrigation (water and nutrients) is well recognized, serious environmental problems such as nitrate leaching, build up of toxic elements in soils and plants and human health hazards from pathogenic microorganisms may develop. It is thus necessary that the negative impacts should be minimized whilst obtaining the fore mentioned benefits. Thus, the aim of this work is to study the distribution of asymbiotic N₂-fixing bacteria in WSP and to establish a qualitative and quantitative estimation of PGRs (IAA and PAs) produced by these bacteria in biologically treated wastewater reused for aquaculture and irrigation of wooden forest in Suez Governorate, Egypt.

Materials and Methods

Study area (The Suez Experimental Station, SES)

The SES is located 15 Km to the west of the City of Suez. It occupies an area of about 29 feddans (1 feddan = 4200 m²). The treatment facilities have been constructed on 11 feddans and the rest of the area was being used for irrigation methods.

The sewage treatment system is a battery of 7 waste stabilisation ponds (Table, 1): 2 square (7 x 7m), deep (3.5m) anaerobic ponds (AP). These ponds work alternately, i.e. one is operated for about 1 year, then drained off, dried and the sludge is removed, while the other pond is being used; one aerobic/facultative (FP) rectangular (75 m in length, 20 m width) and 1.5 m deep pond; 2 successive maturation (or polishing) rectangular (each is 30 m long, 9 m wide) and 1.5 m deep ponds. The retention time in each of these ponds is 12 hr in the anaerobic pond, 15 days in the aerobic/facultative pond, and 7 days in each of the maturation ponds. Thus, the retention time of this system is 29.5 days. Then the treated wastewater is pumped into 2 fish rearing ponds (FP) (70 m long, 20 m wide and 1.5 m deep). The fish in these ponds further purify the treated wastewater which is subsequently re-used for irrigation and agricultural purposes. In addition, there are two extra ponds called depuration ponds for clearing contaminants from the reared fish (Fig. 1)

Sampling

Water samples were collected seasonally (spring, 2004-winter, 2005) from each pond of the six WSP System in the Suez Experimental Station.

Bacteriological analysis

Counting of asymbiotic N₂-fixing bacteria

Most probable number (MPN) method was used for counting free living nitrogen-fixing bacteria. Modified Ashby's medium (Abdel-Malek and Ishac, 1968) was used for the aerobic free-living nitrogen-fixing Azotobacter at 30°C for 15 days. The presence of Azotobacter was detected by visible turbidity and presence of a pellicle formed over the medium surface. Semi-solid Malate medium was used for the
aerobic free-living nitrogen-fixing *Azospirillum* (Döbereiner *et al*., 1976). Anaerobic free living nitrogen-fixing *Clostridium* was grown on Modified Winogradsky’s medium (Naguib, 1961). The dilutions were pasteurized at 80°C for 15 min. before incubation to kill all vegetative cells. The presence of *Clostridium pasteurianum* was detected after 12 days by the accumulation of gases (stormy fermentation) and was confirmed microscopically for plectridial sporangia. The MPNs of the tested nitrogen fixing bacteria were calculated using tables of Cochran (1950).

**Nitrogen-fixation activity**

Efficiency of aerobic nitrogen-fixing bacteria (*Azotobacter* and *Azospirillum*) isolates was examined for fixing atmospheric nitrogen. An aliquot of 50 ml of each medium was inoculated with 1 ml of cell suspension in 250 ml conical flask and incubated at 30°C for 6 days. Then total nitrogen was measured by micro-kjeldehi according to *Page et al.* (1982).

**Identification of N-fixing bacteria**

Identification of the most active nitrogen-fixing isolates to species level was based on morphological, cultural, biochemical, physiological characteristics according to *Bergy's Manual of Determinative Bacteriology* (1994).

**Production of plant growth regulators**

The identified most active nitrogen-fixing isolates were tested for production of IAA and PAs.

**Determination of IAA**

Quantitative determination of IAA was carried out using the colorimetric method according to Pilet and Chollet (1970).

**Qualitative determination of PAs**

Putrescine production was detected by the presence of a dark red halo around and beneath isolates colonies at 0.0, 8 and 24h of incubation according to Arena and De Nadra (2001).

**Quantitative determination PAs**

The isolates showing a large dark red halo were further selected to test their abilities to produce Put, Spd, and Spm. Polyamines were extracted as described by Redmond and Tseng (1979) and their quantitative determination were performed by High Performance Liquid Chromatography (HPLC) according to Smith and Davies (1985).

**Results**

**Numbers of nitrogen-fixing bacteria**

Data in Table (2) showed that the MPN of asymbiotic nitrogen-fixing *Azotobacter* ranged from $1.6 \times 10^2$ org/ml in the anaerobic pond (AP) to $16 \times 10^6$ org/ml in both maturation ponds (PMP and SMP). On the other hand, summer season maintained the highest counts, while the lowest were in winter.

As regards *Azospirillum*, the MPN varied from $1 \times 10^2$ org/ml to $16 \times 10^2$ org/ml during winter and summer, respectively. As in case of *Azotobacter*, the anaerobic pond recorded the lowest counts. On the other hand, the highest counts of *Azospirillum* were recorded in facultative pond (FP) and the primary maturation pond (PMP) (Table 3).

On contrary, the anaerobic nitrogen-fixing *Clostridium* recorded their highest counts in the anaerobic pond ($21- 47 \times 10^2$ org/ml) compared to the other facultative and aerobic ponds. However, summer season maintained the highest counts, while
winter maintained the lowest ones (Table 4).

**Nitrogen-fixation activity**

During the present study, 20 strains were characterized, 10 of them as *Azotobacter*, while the other as *Azospirillum*. Out of *Azotobacter* isolates No.6 and 10 were the most active as regards nitrogen-fixation recording 18.9 and 18.2 ppm TN/100ml, respectively. On the other hand, *Azospirillum* isolates No.5 and 9 were the most active nitrogen-fixer recording 20.1 and 18.2 ppm TN/100ml, respectively (Table 5).

**Identification of nitrogen-fixing bacteria**

Characteristics of isolates No.6 and 10 clearly placed them in the genus *Azotobacter* and they are similar to *Azotobacter chrococcum* and *Azotobacter vinelandii*, respectively, while characteristics of isolates No.5 and 9 clearly placed them in the genus *Azospirillum* and they are similar to *Azospirillum brasiliense* and *Azospirillum lipoferm*, respectively.

**Production of IAA**

- During the present study *Azotobacter chrococcum* produced 25 µg IAA/ml, while *Azotobacter vinelandii* produced 10 g IAA/ml.
- On the other hand, *Azospirillum brasiliense* produced 35 µg IAA/ml, while *Azospirillum lipoferm* produced 40 µg IAA/ml.

**Qualitative determination of PAs**

The isolates showed a large dark red halo around and beneath isolates colonies indicating putrescine production (Photo 1).

**Quantitative determination PAs**

*Azotobacter chrococcum* was able to produce high (53.14%) and moderate (14-15%) Put and Spm, respectively, while it unable to produce Spd. On the other hand, *Azotobacter vinelandii* produced the highest Spm (90.43%) but can't produce either Put or Spd (Fig. 1a and b). *Azospirillum brasiliense* produced high Spm (66.04%) and low Spd (0.56%), while it unable to produce Put. On the other hand, *Azospirillum lipoferm* produced both low Put (1.08%) and Spm (3.17%) and it unable to produce Spd (Fig. 2a and b).

**Discussion**

Several countries have embraced wastewater re-use for drinking industrial applications, saline ingress control, groundwater recharge, irrigation and aquaculture (Grabow, 1990; Rabeh et al., 2005, 2006).

Compared to other types of re-use, the agricultural use of wastewater effluents presents the additional benefit of nutrient recycling in crop irrigation. The impetus for the use of wastewaters comes from a shortage of freshwater for irrigation, together with a need to increase agricultural production and in some cases, shortage of cash needed to purchase mineral fertilizers (Vazquez-Montiel et al., 1996).

Edwards (1990) reported that a minimum of three ponds with a minimum total detention time of 25 days will produce an effluent that is either completely pathogen-free or with only low concentrations of enteric bacteria and viruses; pathogen helminthes and protozoa will have been completely destroyed.

Depending on the prevailing environmental conditions in Egypt, the detention time in WSP should be 28 days or more to achieve effluents complying with the WHO guide-lines (El-Gohary et al., 1995).
The detention time during the present study was 29.5 days. Thus, the existing WSP System was efficient in removing both of bacterial indicators (Rabeh, et al., 2005) and pathogenic bacteria (Rabeh et al., 2006). Also, the bacteriological quality of the treated effluent from the existing WSP was suitable according to WHO both for unrestricted irrigation and fish culture (Rabeh, et al., 2005).

Wastewater, although sufficient as a source of nutrients, can present problems such as toxicity to fish, accumulation of heavy metals and toxic substances in the muscles of fish, and the potential danger of transmission of pathogens from wastewater to handlers and consumers (Edwards, 1985; Buras et al., 1987; Mara and Caimcross, 1989). In this connection, Shereif and Moaty (1995) reported that the concentrations of heavy metals in fish grown in WSP System in the Suez Experimental Station were within the acceptable limits when compared to the international legal limits for hazardous elements in fish and fishery products.

During the present study, the maximum and minimal counts of aerobic nitrogen-fixing Azotobacter were recorded during summer and winter, respectively. It is to be mentioned here that the high counts of Azotobacter might be due to the high temperature, organic and suspended matter content and the pH (8.22 - 9.4) which was suitable for the Azotobacter growth (Rabeh, 1993; Rabeh, 2001). Mahmoud et al. (1973) reported that the pH range for the growth and N2-fixation of Azotobacter was between 6.5 - 9.5.

On the other hand, the high numbers of clostridial spores were found in the anaerobic pond compared to those in the aerobic ones. This finding might be explained by the fact that Clostridia are strictly anaerobic, being unable to survive in the oxygenated waters of aerobic ponds. Moreover, presence of more deposits-rich organic matter in the former compared with that in the latter.

The levels of IAA produced by the tested organisms during the present study may be compared with that observed by Tien et al. (1979) in A. brasilense cultures and by Beijerinckia derxii (Thuler et al., 2003).

Edwards (1985) found that yields of finfish in excreta-loaded fish pond systems in the tropics should be at least 5-6 tons/ha/yr, and with good management could be as high as 10-12 tons/ha/yr, while Mara and Cairncross (1989) report yields up to 3 tons/ha/yr.

The role of dietary polyamines in mammals has been studied extensively, but literature concerning their effects on fish is scarce. On the other hand Péres et al. (1997) observed a dose-dependent effect of purified Spermine on gut maturation in sea bass larvae weaned onto dry food. Also, application of Azotobacter chroococum enhances pond productivity and fish growth and biomass in fresh water fish ponds (Garg et al., 1998). Thus, the high counts of the tested nitrogen fixing bacteria and their production of PAs in the studied biologically treated wastewater might enhance its re-use in aquaculture in Suez, Governorate, Egypt.

Conclusion
1- Waste stabilization ponds (WSPs) are low-cost (usually least-cost), low-maintenance, highly efficient, entirely natural and highly sustainable. Only solar energy is used in this method which is reasonable for mainly in developing countries.

2- However, attention should also be paid to protecting aquaculture workers
and populations living nearby the ponds from contact with the pond water and to ensuring that high standards of hygiene are maintained during fish handling and gutting.

3- The safe, controlled and efficient re-use of treated wastewater has the potential to meet some of the urgent demands for water in Egypt.

4- Treated effluent from Waste Stabilization Pond (WSP) System was suitable for reuse in aquaculture and irrigating a wooden forest in Suez Governorate, Egypt.

5- It is thus necessary that the negative impacts should be minimized whilst obtaining the fore mentioned benefits.

Fig. (1). Stabilization Pond System of Suez Experimental Station
Fig. (2). Polyamines produced by (A) *Azotobacter chroococcum* and (B) *Azotobacter vinelandii*

Fig. (3). Polyamines produced by (A) *Azospirillum brasilense* and (B) *Azospirillum lipoferm*
Photo (1). Dark red halo around and beneath isolates colonies indicating putrescine production
Table (1) Dimensions and retention time of the Stabilisation Pond System of Suez Experimental Station

<table>
<thead>
<tr>
<th>Ponds</th>
<th>Dimensions (m)</th>
<th>Retention Time</th>
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<tbody>
<tr>
<td></td>
<td>Length</td>
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<tr>
<td>FP</td>
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<td>75</td>
<td>20</td>
</tr>
<tr>
<td>SFP</td>
<td>75</td>
<td>20</td>
</tr>
</tbody>
</table>

AP: Anaerobic pond  
FP: Facultative pond  
PMP: Primary maturation pond  
SMP: Secondary maturation pond  
PFP: Primary fish pond  
SFP: Secondary fish pond

Table (2). Most probable number (MPN) of *Azotobacter* (×10⁴/ml) in Stabilisation Pond System of Suez Experimental Station.

<table>
<thead>
<tr>
<th>Season</th>
<th>Ponds</th>
<th>Spring</th>
<th>Summer</th>
<th>Autumn</th>
<th>Winter</th>
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<tbody>
<tr>
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<td>1.6</td>
<td>0.018</td>
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<tr>
<td>FP</td>
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<td>160</td>
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<td>0.047</td>
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</tr>
<tr>
<td>PMP</td>
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<td>160</td>
<td>1.6</td>
<td>0.054</td>
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</tr>
<tr>
<td>SMP</td>
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<td>1.6</td>
<td>0.054</td>
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</tr>
<tr>
<td>PFP</td>
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<td>0.033</td>
<td></td>
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<tr>
<td>SFP</td>
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<td>33</td>
<td>0.11</td>
<td>0.032</td>
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</tr>
</tbody>
</table>

Table (3). Most Probable number (MPN) of *Azospirillum* (×10⁴/ml) in Stabilisation Pond System of Suez Experimental Station.

<table>
<thead>
<tr>
<th>Season</th>
<th>Ponds</th>
<th>Spring</th>
<th>Summer</th>
<th>Autumn</th>
<th>Winter</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP</td>
<td>2.2</td>
<td>1.6</td>
<td>0.018</td>
<td>0.01</td>
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<tr>
<td>FP</td>
<td>9.2</td>
<td>160</td>
<td>1.6</td>
<td>0.047</td>
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<tr>
<td>PMP</td>
<td>1.6</td>
<td>160</td>
<td>1.6</td>
<td>0.054</td>
<td></td>
</tr>
<tr>
<td>SMP</td>
<td>16</td>
<td>54</td>
<td>1.6</td>
<td>0.054</td>
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<tr>
<td>PFP</td>
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<tr>
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Table (4). Most Probable number (MPN) of *Clostridium* \((\times 10^2/m)\) in Stabilisation Pond System of Suez Experimental Station.

<table>
<thead>
<tr>
<th>Season Ponds</th>
<th>Spring</th>
<th>Summer</th>
<th>Autumn</th>
<th>Winter</th>
</tr>
</thead>
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</tr>
<tr>
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<td>0.21</td>
<td>0.14</td>
</tr>
<tr>
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<td>0.21</td>
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Table (5). Nitrogen-fixation (total nitrogen) activity by nitrogen-fixing isolates

<table>
<thead>
<tr>
<th>No. of Isolates</th>
<th>Total nitrogen (ppm)</th>
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<tr>
<td></td>
<td>Azotobacter</td>
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<td>15.4</td>
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<tr>
<td>2</td>
<td>12.6</td>
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<tr>
<td>3</td>
<td>16.1</td>
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<td>17.5</td>
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<td>10</td>
<td>18.2</td>
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References


