CHAPTER - 3
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Bacterial ecology of atrazine contaminated site

3.1. Introduction

Environmental pollution in India, as elsewhere in the world, is a major issue of public policy and research. Unprecedented economic growth during the last fifty years has led to extensive environmental degradation of all parts of the ecosystem (Prasad, 1996). To meet the increasing demand for food, use of synthetic pesticides in modern agriculture has become an inevitable choice. Irrespective of the dose and mode of application, most of these pesticides eventually reach soil and water ecosystem that could lead to undesirable consequences.

Currently, India is the largest manufacturer and consumer of pesticides in Southern Asia. On an average, approximately 500 g ha\(^{-1}\) of pesticides is used in India (Prasad, 1996). Among the pesticides, herbicides constitute a major portion of synthetic chemicals that are employed in farmland. With shortage of farm labour and escalating labour cost, use of herbicides is on the raise. Usually herbicides are applied in the rates of 0.5 to 15 pounds per acre, the rate
depending upon the compound, crop and soil. Organic soil sterilants are applied at 10 to 100 pounds per acre with most application being at 25 to 30 pounds. A large percentage of it ends up in the soil, whether directly applied or not. Persistence and leachability of these herbicides are extremely important and are influenced by soil texture, chemical composition, organic matter content, pH, moisture and temperature (Khan, 1978; Barreda et al. 1991 and Felding, 1992).

Soil microorganisms and microbial process are vital for the sustenance of soil fertility. As it is well recognized, soil microorganisms are the crucial component that determines the continuance of various biogeochemical cycles of macro and microelements. Hence, any detrimental effect of the soil-applied chemical on these biota would have serious consequence on the basis of nutrient availability in that ecosystem.

Tenacity and undesirable effects of herbicides and their residues are frequently reported (Federle et al., 1990 and Bezbaruah et al., 1995). Continuous exposure of soil to herbicides tends to enrich the population of microorganisms that are resistant to it. Such selective enrichment of herbicide resistant and degrading microbial population was reported by many researchers (Verma and Bagle, 1987; Kuwatsuka and Miwa, 1989; Burnet and Hodgson, 1991; Jumila et al., 1993 and El-Fantroussi et al., 1999).

Atrazine, s-triazine herbicide (2-chloro 4-ethyl amino - 6-isopropyl amino - 1,3,5-triazine) is a moderately persistent herbicide. It is currently being used worldwide to control weeds in sugarcane, corn, maize, sorghum and
orchards (Vermeulen et al., 1982). Its half-life in soil ranges from 1.5 to 6 months (Goring et al., 1975). Atrazine is one of the most extensively used herbicide due to its phytotoxic potentials, but its non-target effects on micro and macroflora is indeed a cause of concern. Its toxicity on plants and animals (Pacakova and Kozaková, 1978), persistent nature (Roseboom and Harbold, 1980) and its mobility in soils and water (Topp et al., 1994) explicitly reinforces the negative potentials of this xenobiotic.

Recalcitrant nature of atrazine was well documented and so its residues. Marriage et al. (1975) had reported the recovery of residual atrazine in the surface soil of peach orchard. Its non-target effect was also found to be significant as they had reported a marked reduction (38%) in subsequent oat plants yield. Reduction in soil fertility and production efficiency could very well be influenced by the deleterious impact of atrazine and its metabolites on soil micrflora. Any change in microbial population and composition would have direct bearing on the recycling of the organic and inorganic constituent of the soil. As little attention has been paid on this aspect pertaining to atrazine, an attempt was made in the present investigation to understand the microbial status of a soil system in a sugarcane field that was on constant exposure to atrazine.

3.2. Materials and Methods

Distribution of aerobic and facultative bacteria in a sugarcane field was analyzed. Simultaneously, atrazine resistant population of these bacteria was also enumerated. As representative of beneficial microbes, Azotobacter sp.
Azospirillum sp. and Phosphate Solubilizing Bacteria (PSB) were also included in this distribution studies. Discrete colonies from both total heterotrophic and atrazine resistant bacterial population were characterized and identified upto generic level.

3.2.1. Sampling station

Azhwarkurichi, a Panchayat block belonging to Ambasamudram Taluk, Tirunelveli District, Tamil Nadu, India is basically an agricultural village. Rice and sugarcane are the two important agricultural produce of this place. In tune with modern and aggressive agricultural practices, various farm chemicals viz., pesticides, herbicides, insecticides, fertilizers etc., are used in these fields. A sugarcane field in this area with regular applications of atrazine (2 – 5 kg/ha) was selected in the present investigation.

3.2.2. Sample collection

A plot (one acre) in the sugarcane field was selected for sampling. Soil samples (100 gms each) were collected from four corners and center in the selected area in gamma rays irradiated, pre-sterilized polythene bags and transported to the laboratory in an ice pack (4°C). These samples were mixed in the laboratory aseptically and used in this study.

Water samples (100 ml) were collected from the entry and exit points of the irrigation channel in the chosen field. These samples were collected in a sterile 250 ml Erlenmeyer flasks and transported to the laboratory in an ice pack (4°C). Both the soil and water samples were subjected to microbial analyses within 6 hours of collection. Sampling and microbial analysis of the
samples were carried out for 10 cycles over a period of 15 months. Replicates (triplicates) and suitable controls were maintained in all experiments.

3. 2. 3. **Enumeration of total heterotrophic bacterial population**

Soil and water samples collected from the sugarcane field were decimally diluted with sterile distilled water. From dilutions $10^{-2} - 10^{-7}$, 1.0 ml was poured plated with sterile Nutrient Agar (NA) medium (Himedia, Mumbai, India) in pre-sterilized petri plates. The inoculated plates were incubated at 37°C in an inverted position for 24 - 48 hrs. After incubation, bacterial density was counted from plates with the countable range of cells (30 - 300) and expressed as colony forming units (cfu) gm$^{-1}$ or ml$^{-1}$.

3. 2. 4. **Enumeration of *Azotobacter* sp. load**

Soil and irrigation water samples were collected from the chosen sugarcane field and serially diluted as described in section 3. 2. 2. and 3. 2. 3. From $10^{-4} - 10^{-3}$ dilutions, 0.1 ml was spread on sterile air-dried Ashby's medium (Hi-media, Mumbai, India) in petri plates. These plates were incubated at 25 - 28°C for 7 - 10 days in a bacteriological incubator. After incubation, bacterial load was counted from plates with the countable range of cells (30 - 300) and expressed as cfu gm$^{-1}$ or ml$^{-1}$. White elevated translucent colonies were pure cultured by quadrant streaking in air-dried Ashby's media plates and stored in agar slopes of same media at 4°C to keep the bacterial strains viable.
3. 2. 5. Enumeration of *Azospirillum* sp. load

Soil and water samples collected from the sugarcane field were enumerated for *Azospirillum* sp. population employing three tube MPN technique. Nitrogen Free Sodium Malate (NFSM) medium was employed for this purpose (Kreig and Deberener, 1984). The inoculated tubes were incubated at RT for 5 – 7 days. The characteristic growth of *Azospirillum* sp. was recorded from the formation of white pellicle 2 cm just above the bottom of the medium in the test tube. The *Azospirillum* sp. load in the sample was calculated from the MPN table (Environmental Protection Agency, 1978).

3. 2. 6. Enumeration of Phosphate Solubilizing Bacterial (PSB) population

The soil and water samples collected from the chosen sugarcane field was analysed for PSB population. Initially, the samples were homogenized with sterile diluent (distilled water) and from this serial dilutions were made with 9 ml of sterile diluent. From $10^4$ - $10^5$ dilutions, 0.1 ml was withdrawn aseptically and spread plated on sterile air-dried, Pikovskaya's medium (Hi-media, Mumbai, India) plates. These plates were inverted and incubated at 37°C for 3 - 5 days. Bacterial out growth with zone of utilization (zone of clearance around bacterial colonies) of complex phosphate incorporated in the media was enumerated.

3. 2. 7. Enumeration of Atrazine resistant bacterial load

The sugarcane field soil and water samples were enumerated for atrazine resistant heterotrophic bacteria, *Azotobacter* sp. *Azospirillum* sp. and PSB. In the first two sampling cycles, atrazine was incorporated at 3 concentrations viz.
0.01%, 0.1% and 1.0% (Al) in the respective nutrient or selective media for the enumeration of atrazine resistant bacteria. Atrazine at 1.0% (1000 ppm) concentration was completely inhibitory to all the bacteria with the absence of growth even on prolonged incubation. On the other hand, no significant difference in bacterial count was appreciated between 0.01% and 0.1% atrazine concentration. Hence, 0.1% (Al) atrazine concentration was used in all the subsequent sampling cycles to enumerate atrazine resistant bacterial population.

NA for heterotrophic bacteria. Ashby's medium for Azotobacter sp. NFSM for Azospirillum sp. and Pikovskaya’s medium for PSB were prepared, autoclaved and cooled to 45°C. Filter sterilized atrazine (Rallis, Bangalore) was incorporated at 0.1% (Al : v/v) into these sterile molten media separately adopting aseptic techniques. These atrazine-incorporated media were used to enumerate atrazine resistant bacteria.

Data pertaining to the occurrence of total and atrazine resistant population of various bacterial groups in the soil and water samples were analysed statistically to find out the relationship between the two by using linear correlation method.

3.2.8. Characterization of total heterotrophic and atrazine resistant heterotrophic bacterial isolates

After enumeration, morphologically discrete colonies from the agar plates described in section 3.2.3. and 3.2.7. were pure cultured and stored in respective agar slopes. Fresh broth cultures were generated from these agar slope stock cultures and characterized on the basis of morphological.
biochemical and physiological characteristics. Pure cultures of heterotrophic and atrazine resistant heterotrophic bacterial population were identified up to generic level on the basis of the identification scheme of Aiso and Simudu (1962) (Chart. 1). The Bergey’s manual of systemic bacteriology (1989) was also referred in the identification procedure.

3. 2. 9. Characterization of beneficial bacterial isolates

*Azotobacter* sp. and *Azospirillum* sp. isolated and pure cultured from Ashby’s and NFSM medium plates respectively were characterized up to generic level as per the differential tests recommended in the Bergey’s manual of systematic bacteriology (Chart. 2 and 3 respectively). PSB were identified and characterized on the basis of their morphological, physiological and biochemical properties (Chart. 1).
Chart 1. Scheme for the identification of heterotrophic bacteria
(Aiso and Simudu. 1962)

GRAM STAINING

Positive
- Cocci small
- Small rods
- Spore forming rods
  - Non motile
  - Non spore
  - Micrococcus sp.
  - Corynebacterium sp.
  - Bacillus sp.

Negative
- Sensitivity Towards Penicillin (2.5 mg/disc)
  - Negative
  - Positive
    - Pigmentation Test
      - Non-fermentative
        - Fermentative
          - No pigment
          - Pseudomonas sp.
          - Achromobacter sp.

Acid and Nogas
- Acid and gas
  - Non-Luminous
    - Luminescent
      - Kovac's Oxidase Test
        - Flavobacterium sp.
        - Cytophaga sp.

Yellow or Orange pigment

Positive
- Vibrio sp.
- Photobacterium sp.
- Acrononas sp.

Negative
- Enterobacteriaceae
Chart. 2. Scheme for the characterization of *Azotobacter* sp.

(Tchan and New. 1984)

White, transleuscent colonies in Ashby's Medium

\[ \downarrow \]

Gram negative

\[ \downarrow \]

Bacilli

\[ \downarrow \]

Motile

\[ \downarrow \]

Catalase, Oxidase +ve

\[ \downarrow \]

Urease +ve

\[ \downarrow \]

H₂S production +ve

\[ \downarrow \]

Indole +ve

\[ \downarrow \]

Citrate -ve

\[ \downarrow \]

No Starch/Gelatin hydrolysis

\[ \downarrow \]

Fermentative. Acid and no gas

\[ \downarrow \]

*Azotobacter* sp.
Chart. 3. Scheme for the characterization of *Azospirillum* sp.

(Kreig and Doberciner, 1984)

White colonies in NFSM Medium  
\[ \downarrow \]  
Gram negative, spirilli  
\[ \downarrow \]  
Motile  
\[ \downarrow \]  
Catalase, Oxidase, Urease, +ve  
\[ \downarrow \]  
No H$_2$S production  
\[ \downarrow \]  
Indole, Citrate +ve  
\[ \downarrow \]  
No Starch, Gelatin hydrolysis  
\[ \downarrow \]  
Fermentative, Acid and no gas  
\[ \downarrow \]  
*Azospirillum* sp.
Chart 4. Enumeration of soil and water samples for physiologically different bacterial groups

(Protocol followed in this study)

Field soil and water samples

- Decimally diluted with glass distilled water

  - Dilutions $10^{-1}-10^{-2}$
  - Dilutions $10^{-3}-10^{-5}$
  - Dilutions $10^{-6}$

  - Pourplated with NA
  - Spread plated on Ashby’s medium
  - Incubated at RT - 5 - 7 days
    - Pellicular growth at the bottom of the tube
    - Incubated - RT - 3 - 5 days
    - Bacterial colonies with zone of clearance around their outgrowth

  - Total Heterotrophic Bacterial Population [THBP] (Cappuccino and Sherman 1999)
  - White, translucent colonies
    - Azotobacter sp. load
      - Tchan and New (1984)
      - Phosphate Solubilizing Bacterial load (PSB) (Cappuccino and Sherman 1999)

For the enumeration of atrazine resistant population of these bacterial groups, respective media were incorporated with atrazine (0.1%) concentration and used.
3.3. Results

Atrazine exposed sugarcane field was screened for the distribution of different microbial communities. This included, Total Heterotrophic Bacterial Population (THBP), *Azotobacter* sp. *Azospirillum* sp. PSB and atrazine resistant population of these microorganisms. Field soil and irrigation channel water samples were used for this purpose. Ten sampling cycles were carried out over a period of 15 months and the results are presented in Tables 4–7 and Fig. 3–4. The protocol adopted in this investigation is shown in Chart 4.

The initial THBP was in the order of $9.1 \times 10^6$ cfu gm$^{-1}$ in the field soil samples but at the end of the experiment period, a sharp drop in THBP ($4.8 \times 10^4$ cfu gm$^{-1}$) was recorded (Table 4). *Azotobacter* sp. and *Azospirillum* sp. bacterial densities ranged from $3.5 \times 10^1$ to $1.20 \times 10^3$ cfu gm$^{-1}$ and from $4.5 \times 10^6$ to $1.62 \times 10^4$ cfu gm$^{-1}$ respectively. The densities of *Azotobacter* sp. and *Azospirillum* sp. were found minimum during the 3rd and 5th cycle respectively. The maximum bacterial counts for both were noticed during 9th and 6th cycles respectively. The minimum bacterial density of ($8.5 \times 10^2$ cfu gm$^{-1}$) was observed during the 4th cycle and the maximum density ($9.0 \times 10^3$ cfu gm$^{-1}$) was noticed during the 10th cycle. In general, the densities of beneficial microflora *viz.* *Azotobacter* sp. *Azospirillum* sp. and PSB were relatively uniform throughout the experiment period with marginal fluctuations that could be correlated with the absence of vegetation *etc.* (Table 4).

The minimum and maximum THBP load in the inlet water sample was $4.0 \times 10^4$ and $5.0 \times 10^6$ (cfu ml$^{-1}$) at 2nd and 4th cycles respectively. In the case
of water samples drawn from the outlet, minimum THBP density was recorded in the 1st cycle (39 \times 10^6 \text{ cfu ml}^{-1}) and the maximum was observed in the 4th cycle (56 \times 10^7 \text{ cfu ml}^{-1}). The \textit{Azotobacter} sp. recorded in the water samples seems to be drawn mainly from the soil as only four sample cycles provided enumerable load of the bacteria in the inlet water. In this the lowest \textit{Azotobacter} sp. density (10 \times 10^1 \text{ cfu ml}^{-1}) was recorded in the 10th cycle and the maximum (280 \times 10^7 \text{ cfu ml}^{-1}) at the 4th cycle. The minimum and maximum \textit{Azotobacter} sp. densities in outlet water samples were recorded in the 9th (35 \times 10^3 \text{ cfu ml}^{-1}) 4th and 10th (120 \times 10^3 \text{ cfu ml}^{-1}) cycles respectively. \textit{Azospirillum} sp. densities were found to be consistent (10^3 \text{ cfu ml}^{-1}) throughout the study period irrespective of the sample (inlet or outlet). Variations in densities of PSB were observed to follow the pattern of that of THBP, while the inlet revealed relatively low concentration (10^1 \text{ cfu ml}^{-1}) with moderate increase (290 \times 10^3 \text{ cfu ml}^{-1}) in the 8th cycle. The highest PSB population in the outlet was recorded in the 10th cycle (75 \times 10^3 \text{ cfu ml}^{-1}).

Contrary to the sharp reduction in THBP in soil, a ten-fold increase in atrazine resistant heterotrophic bacterial population over the initial load was recorded at the end of the experimental period (Table 6). Data presented in Table 6 reveal a slow increase in the atrazine resistant heterotrophic bacterial density with the minimum of 32 \times 10^2 \text{ (cfu gm}^{-1}) in the 1st cycle and 96 \times 10^3 \text{ cfu gm}^{-1} in the 8th cycle. The occurrence of atrazine resistant \textit{Azotobacter} sp. was moderate with low enumerable load during 4th to 6th cycle. Similar inferences can be drawn in the case of atrazine resistant \textit{Azospirillum} sp. with
both having the average densities of $10^1$ cfu gm$^{-1}$. As that of atrazine resistant HBP a steady rise in atrazine resistant PSB population was recorded from $90 \times 10^2$ cfu gm$^{-1}$ (1$^{st}$ cycle) to $80 \times 10^3$ cfu gm$^{-1}$ in the 9$^{th}$ cycle.

As recorded in the case of THBP in irrigation water, the inlet sampling point yielded very low load of atrazine resistant heterotrophic bacteria. In two sampling cycles, no enumerable load of atrazine resistant heterotrophic bacterial population were recorded. But, significantly high atrazine resistant heterotrophic bacterial load ($10^1$ - $10^4$ cfu ml$^{-1}$) was recorded in the water samples drawn from the outlet of the irrigation channel. This is suggestive of the enrichment of heterotrophs from the crop field soil in the irrigation water. In fact, such enrichment of irrigation channel water is clearly evident in the case of THBP, atrazine resistant *Azotobacter* sp. and PSB with higher load in the outlet water samples, when compared to inlet samples (Table 7).

Identification of THBP revealed the predominance of *Pseudomonas* sp. (57%) followed by *Bacillus* sp. (38%) and *Micrococcus* sp. (3%). Other bacteria belonging to various genera *viz.* *Proteus* sp. *Flavobacterium* sp. *Enterococcus* sp. *etc.*, have also occurred in only one sampling cycle and hence, they were not included while computing the data for percentage distribution analysis (Fig. 2). A similar pattern in the generic distribution of atrazine resistant heterotrophic bacteria was recorded with the predominance of *Pseudomonas* sp. (44.88%) followed by *Bacillus* sp. (29.92%) (Fig. 3). PSB population was composed exclusively of *Bacillus* sp.
Table 4. Incidence of heterotrophic bacteria. *Azotobacter* sp., *Azospirillum* sp. and Phosphate solubilizing bacteria in the soil samples of sugarcane field.

<table>
<thead>
<tr>
<th>Sampling Cycle</th>
<th>Heterotrophic bacterial population (cfu gm⁻¹)</th>
<th><em>Azotobacter</em> sp. load (cfu gm⁻¹)</th>
<th><em>Azospirillum</em> sp. load (cfu gm⁻¹)</th>
<th>Phosphate solubilizing bacterial population (cfu gm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>$91 \times 10^6$</td>
<td>$50 \times 10^3$</td>
<td>$40 \times 10^2$</td>
<td>$40 \times 10^3$</td>
</tr>
<tr>
<td>II</td>
<td>$100 \times 10^6$</td>
<td>$70 \times 10^3$</td>
<td>$80 \times 10^2$</td>
<td>$270 \times 10^2$</td>
</tr>
<tr>
<td>III</td>
<td>$80 \times 10^6$</td>
<td>$35 \times 10^3$</td>
<td>$70 \times 10^2$</td>
<td>$130 \times 10^2$</td>
</tr>
<tr>
<td>IV</td>
<td>$60 \times 10^4$</td>
<td>-</td>
<td>$45$</td>
<td>$85 \times 10^2$</td>
</tr>
<tr>
<td>V</td>
<td>$87 \times 10^4$</td>
<td>-</td>
<td>$35 \times 10^2$</td>
<td>$205 \times 10^2$</td>
</tr>
<tr>
<td>VI</td>
<td>$90 \times 10^4$</td>
<td>$52 \times 10^3$</td>
<td>$162 \times 10^2$</td>
<td>$41 \times 10^2$</td>
</tr>
<tr>
<td>VII</td>
<td>$51 \times 10^4$</td>
<td>$45 \times 10^3$</td>
<td>$30 \times 10^2$</td>
<td>$70 \times 10^1$</td>
</tr>
<tr>
<td>VIII</td>
<td>$72 \times 10^4$</td>
<td>$70 \times 10^3$</td>
<td>$90 \times 10^2$</td>
<td>$58 \times 10^2$</td>
</tr>
<tr>
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<td>$120 \times 10^3$</td>
<td>$50 \times 10^2$</td>
<td>$52 \times 10^2$</td>
</tr>
<tr>
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<td>$48 \times 10^4$</td>
<td>$85 \times 10^3$</td>
<td>$65 \times 10^2$</td>
<td>$90 \times 10^2$</td>
</tr>
</tbody>
</table>

- = NO growth was recorded

Sugarcane field soil samples were enumerated for various bacterial groups as per the methodology cited in Chart 4. For THBP enumeration NA was used and incubated at 37°C for 24 - 48 hrs. *Azotobacter* sp. was enumerated with Ashby’s medium (RT for 7 - 10 days). For *Azospirillum* sp. enumeration, MPN technique was employed with NFSM medium (RT 5 - 7 days) and from EPA manual, cfu gm⁻¹ was calculated. Pikovskaya’s medium was employed in PSB enumeration (RT 3 - 5 days) and colonies with halo zone around their outgrowth were enumerated.
Table 5. Incidence of heterotrophic bacteria, *Azotobacter* sp., *Azospirillum* sp. and phosphate solubilizing bacteria in the irrigation channel water samples of sugarcane field.

<table>
<thead>
<tr>
<th>Sampling Cycle</th>
<th>Heterotrophic bacterial population (cfu ml⁻¹)</th>
<th><em>Azotobacter</em> sp. (cfu ml⁻¹)</th>
<th><em>Azospirillum</em> sp. (cfu ml⁻¹)</th>
<th>Phosphate solubilizing bacterial population (cfu ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inlet</td>
<td>Outlet</td>
<td>Inlet</td>
<td>Outlet</td>
</tr>
<tr>
<td>I</td>
<td>220 x 10⁵</td>
<td>39 x 10⁵</td>
<td>40 x 10⁵</td>
<td>-</td>
</tr>
<tr>
<td>II</td>
<td>40 x 10⁵</td>
<td>51 x 10⁵</td>
<td>60 x 10⁵</td>
<td>35 x 10³</td>
</tr>
<tr>
<td>III</td>
<td>80 x 10⁵</td>
<td>71 x 10⁵</td>
<td>50 x 10¹</td>
<td>90 x 10³</td>
</tr>
<tr>
<td>IV</td>
<td>50 x 10⁵</td>
<td>56 x 10⁵</td>
<td>280 x 10⁵</td>
<td>120 x 10¹</td>
</tr>
<tr>
<td>V</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>VI</td>
<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>VII</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>VIII</td>
<td>78 x 10⁵</td>
<td>38 x 10⁵</td>
<td>78 x 10¹</td>
<td>80 x 10³</td>
</tr>
<tr>
<td>IX</td>
<td>47 x 10⁵</td>
<td>50 x 10⁵</td>
<td>45 x 10¹</td>
<td>35 x 10³</td>
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<tr>
<td>X</td>
<td>38 x 10⁵</td>
<td>56 x 10⁵</td>
<td>10 x 10¹</td>
<td>120 x 10³</td>
</tr>
</tbody>
</table>

-= No growth was recorded

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Table 6. Incidence of atrazine resistant heterotrophic bacteria. *Azotobacter* sp., *Azospirillum* sp. and phosphate solubilizing bacteria in the soil samples of sugarcane field.

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<th><em>Azotobacter</em> sp. load (cfu gm⁻¹)</th>
<th><em>Azospirillum</em> sp. load (cfu gm⁻¹)</th>
<th>Phosphate solubilizing bacterial population (cfu gm⁻¹)</th>
</tr>
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<tbody>
<tr>
<td>I</td>
<td>32 × 10²</td>
<td>90 × 10¹</td>
<td>-</td>
<td>90 × 10²</td>
</tr>
<tr>
<td>II</td>
<td>280 × 10²</td>
<td>42 × 10¹</td>
<td>45 × 10¹</td>
<td>120 × 10²</td>
</tr>
<tr>
<td>III</td>
<td>37 × 10³</td>
<td>110 × 10¹</td>
<td>70 × 10¹</td>
<td>30 × 10³</td>
</tr>
<tr>
<td>IV</td>
<td>45 × 10³</td>
<td>-</td>
<td>70 × 10¹</td>
<td>142 × 10²</td>
</tr>
<tr>
<td>V</td>
<td>72 × 10³</td>
<td>-</td>
<td>-</td>
<td>150 × 10²</td>
</tr>
<tr>
<td>VI</td>
<td>72 × 10³</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>VII</td>
<td>86 × 10³</td>
<td>32 × 10¹</td>
<td>68 × 10¹</td>
<td>76 × 10²</td>
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<td>VIII</td>
<td>96 × 10³</td>
<td>22 × 10¹</td>
<td>90 × 10¹</td>
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<td>32 × 10¹</td>
<td>88 × 10³</td>
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<td>66 × 10³</td>
<td>45 × 10¹</td>
<td>38 × 10¹</td>
<td>40 × 10³</td>
</tr>
</tbody>
</table>

- = No growth was recorded

Sugarcane field soil samples were enumerated for various atrazine resistant bacterial groups as per the methodology cited in Chart 4. For ARHBP enumeration atrazine (0.1%) incorporated NA was used and incubated at 37°C for 24 - 48 hrs. *AR Azotobacter* sp. was enumerated with atrazine (0.1%) incorporated Ashby's medium (RT for 7-10 days). For AR*Azospirillum* sp. enumeration, MPN technique was employed with atrazine (0.1%) incorporated NFSM medium (RT - 5 - 7 days) and from EPA manual, cfu gm⁻¹ was calculated. Atrazine incorporated Pikovskaya's medium was employed in ARPSB enumeration (RT - 3 - 5 days) and colonies with halozone around their outgrowth were enumerated.

AR = Atrazine Resistant.
Table 7. Incidence of atrazine resistant heterotrophic bacteria. *Azobacter* sp. *Azospirillum* sp. and phosphate solubilizing bacteria in the irrigation channel water samples of sugarcane field.

<table>
<thead>
<tr>
<th>Sampling Cycle</th>
<th>Heterotrophic bacterial population (cfu ml⁻¹)</th>
<th><em>Azobacter</em> sp. (cfu ml⁻¹)</th>
<th><em>Azospirillum</em> sp. (cfu ml⁻¹)</th>
<th>Phosphate solubilizing bacterial population (cfu ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inlet</td>
<td>Outlet</td>
<td>Inlet</td>
<td>Outlet</td>
</tr>
<tr>
<td>I</td>
<td>-</td>
<td>120 × 10^3</td>
<td>-</td>
<td>42 × 10^4</td>
</tr>
<tr>
<td>II</td>
<td>-</td>
<td>80 × 10^3</td>
<td>-</td>
<td>86 × 10^3</td>
</tr>
<tr>
<td>III</td>
<td>30 × 10^4</td>
<td>50 × 10^3</td>
<td>-</td>
<td>102 × 10^4</td>
</tr>
<tr>
<td>IV</td>
<td>48 × 10^4</td>
<td>70 × 10^4</td>
<td>-</td>
<td>30 × 10^4</td>
</tr>
<tr>
<td>V</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>VI</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>VII</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>VIII</td>
<td>30 × 10^4</td>
<td>90 × 10^3</td>
<td>-</td>
<td>35 × 10^4</td>
</tr>
<tr>
<td>IX</td>
<td>120 × 10^4</td>
<td>78 × 10^4</td>
<td>-</td>
<td>75 × 10^4</td>
</tr>
<tr>
<td>X</td>
<td>87 × 10^4</td>
<td>101 × 10^4</td>
<td>-</td>
<td>58 × 10^4</td>
</tr>
</tbody>
</table>

- = No growth was recorded.

Sugarcane field water samples were enumerated for various atrazine resistant bacterial groups as per the methodology cited in Chart 4. For ARHBP enumeration atrazine (0.1%) incorporated NA was used and incubated at 37°C for 24–48 hrs. AR *Azobacter* sp. was enumerated with atrazine (0.1%) incorporated Ashby's medium (RT for 7–10 days). For AR *Azospirillum* sp. enumeration MPN technique was employed with atrazine (0.1%) incorporated NFPM medium (RT-5–7 days) and from EPA manual, cfu ml⁻¹ was calculated. Atrazine incorporated Pikovsky's medium was employed in ARPSB enumeration (RT-3–5 days) and colonies with halo zone around their outgrowth were enumerated. AR = Atrazine Resistant.
Heterotrophic bacteria isolated from sugarcane field soil samples were identified up to generic level on the basis of microscopic, biochemical and physiological characteristics (refer Chart. 1.).
Atrazine resistant heterotrophic bacteria isolated from sugarcane field soil samples were identified up to generic level on the basis of microscopic, biochemical and physiological characteristics (refer Chart 1.).
3.4. Discussion

Distribution of different groups of bacteria in a cultivated sugarcane field was analysed in the present investigation. For this purpose, soil sediment and water samples were drawn from the crop field and the irrigation channel respectively. The sampling period spanned over 15 months and samples were taken in ten cycles. In the field soil samples, the Total Heterotrophic Bacterial Population (THBP) was recorded to be in the range of $10^4 - 10^6$ cfu g$^{-1}$. In the initial samples THBP load was in the order of $10^6$ cfu g$^{-1}$. A slow recession in this load was observed as the sampling schedule progressed (Table 4 and 6). In this juncture, it has to be noted that the field from where samples were taken was actively cultivated for sugarcane with constant application of atrazine (5 - 10 lbs/acre). While comparing the incidence of THBP with that of the distribution of atrazine resistant heterotrophic bacteria, a slow but a steady enrichment of atrazine resistant population were noticed in the present study (Table 4 and 6). Walker and Welch (1991) had obtained a similar data and suggested on the enrichment of pesticide degrading organisms. Their field experiments, involving repeated application of different herbicides included simazine, propyzamide, linuron, alachlor and napropamide, recorded an enhanced degradation of these chemicals, which was interpreted as a consequence of slow enrichment of respective microbial degraders. Findings of Entry et al. (1995) also revealed such a pattern of enrichment in the population of microorganisms capable of degrading atrazine and 2, 4 - D in riparian forest. Wherein they recorded a steady increase in total fungal and bacterial biomass that were capable of mineralizing atrazine and 2, 4 - D.
A slow reduction in THBP with concomitant rise in the atrazine degrading population (Tables. 4 and 6) is suggestive of the probable onset of the process of accumulation of atrazine and its metabolites in the soil that could have resulted in the enrichment of degraders. Analysing these data statistically (linear correlation analysis) revealed a negative correlation among these groups (-0.732) that reiterates this suggestion. Percich and Lockwood (1978) reported changes in microbial population with the accumulation of atrazine in a loamy soil treated with atrazine at different rates. An increase in the microbial population that listed actinomycetes, bacteria and fungi was demonstrated in their experiments. Similar inferences on the inhibitory effects of pesticides on THBP has been observed by Burnet and Hodgson (1991) in their experiments involving sulfonylurea herbicides, chlorosulfuron and sulfonyluron methyl. Informations suggestive of the impact of agricultural chemicals on soil microorganisms can be found in the works of Arthur and Wang (1999); Pennanen et al. (1999) and Staley (1999). Cole (1976) reported sustained soil microbial population of degraders with long term atrazine application.

Fluctuations in heterotrophic bacterial population were also recorded in the water samples drawn from the irrigation channel in the present investigation. Only in few sample cycles, atrazine resistant bacterial population was recorded in the inlet water samples that too at an extremely very low population density. Increase in bacterial densities in the outlet water samples might be due to their entry from crop field soil. Kuwatsuka and Miwa (1989) have remarked such changes in the population of 2, 4 - D degraders. Similar informations on the changes in bacterial population in water samples were
reported by Miwa and Kuwatsuka (1990); Taraban et al. (1993) and Vandepitte et al. (1994). Changes in microbial biomass, activity and community structure at different regions in the cultivated field was recorded by Federle et al. (1990) which amply support the data of the present study.

Contrary to THBP, the results of the present investigation on the distribution of beneficial microorganisms such as Azotobacter sp. Azospirillum sp. and PSB were recorded to be fairly consistent throughout the experimental period. This was true in the case of atrazine resistant population of Azotobacter sp. and Azospirillum sp. in spite of they being in very low load. An interesting inference was drawn on the occurrence of total and atrazine resistant PSB, wherein the data in this study suggested that the PSB population seemed to be composed exclusively of atrazine degraders. Support for such changes and selective enrichment of microbial degraders are also noticed from the works of Soulas (1993); Bezbaruah et al. (1995) and El - Fantroussi et al. (1999). Jalali and Sharma (1993) had also recorded such interactions between the beneficial microflora and herbicide in their study involving the distribution of mycorrhizal fungi in pesticide exposed tea plantation.

Among the bacterial communities in an ecosystem, those belonging to the genera of Pseudomonas and Bacillus were often recorded to be highly persistent and also resist a variety of xenobiotics. Both these organisms were recognized for their metabolic versatility. Examining the Table. I. would provide ample support for this feature of these organisms. Pseudomonas sp. owing to the presence of a range of accessory genetic materials (plasmids) was recorded to have remarkable potential for survival in diverse ecosystems. On
the other hand. *Bacillus* sp. due to its ability to withstand adverse environmental conditions through sporulation is often reported to be persistent even in hostile physico-chemical conditions (Mashetty *et al.*, 1995; Gowrisankar *et al.*, 2000). Deo *et al.* (1994) in their exhaustive review article had remarked that *Pseudomonas* strains were often dominant members of populations selected from natural sources such as soil, polluted waters and sediments. Occurrence of pesticide resistant *Pseudomonas* sp. and *Bacillus* sp. were reported by Gowrisankar *et al.* (1997) in their experiments involving the distribution analysis of dimethoate metabolizing bacterial population in rice soil. Oyamada and Kuwatsuka (1990) have reported naproanilide degrading *Pseudomonas* sp. in the soil ecosystem. *Pseudomonas* sp. isolated from the rhizosphere of sugarcane was recorded to posses the potential to degrade hexachlorocyclohexane (Sahu *et al.*, 1993). Reports of McGhee and Burns (1995) also indicate the predominance of *Pseudomonas* sp. *Xanthomonas* sp. and *Rhodococcus* sp. in 2, 4 - D and MCPA contaminated soil systems. The metabolic potentials of *Pseudomonas* sp. are also well illustrated from the observations of Sahasrabudhe and Modi (1991); Ramos *et al.* (1995) and Vilchez *et al.* (2000).

Incidence of atrazine resistant and degrading *Bacillus* sp. was reported by Korpraditskul *et al.* (1993b). Roane and Kellogg (1996) had recovered bacteria belonging to the genera *Bacillus* and *Pseudomonas* with inherent ability to decontaminate heavy metal pollution in a soil system. Mashetty *et al.* (1995) had reported the ability of *Bacillus* sp. a native isolate to degrade aromatic compounds such as 4 - hydroxy benzoic acid. Predominance of heterotrophic
and pesticide resistant *Bacillus* sp. in soil ecosystem was reported by Roberts *et al.* (1993) and Gowrisankar *et al.* (1997). Presence of other heterotrophs, even though at lower concentration with atrazine resistant phenotype is not surprising, as soil due to its diverse nutrient availability would encourage the growth of different bacterial community.

Many researchers have reported the presence of different groups of microorganisms capable of resisting and degrading a wide variety of farm chemicals. Levanon (1993) had noticed the presence of a variety of bacteria and fungi that are capable of mineralizing atrazine, arachlor, malathion and carbofuran. Bevinakatti and Ninnekar (1992) had observed the occurrence of xenobiotic degrading *Micrococcus* sp. in soil. The incidence of 4-chlorobiphenyl degrading *Micrococcus* sp. in soil ecosystem was reported by Bevinakatti and Ninnekar (1993). *Flavobacterium* sp. as an atrazine resistant bacterial population was reported by Gowrisankar *et al.* (2000). Topp *et al.* (2000a) reported the occurrence of atrazine degrading *Pseudaminobacter* sp. in agricultural soils. Occurrence of herbicide resistant *Azotobacter* sp. was noticed by El-Hoseiny *et al.* (1995). Hickey *et al.* (1994) were able to observe atrazine transformation potentials in *Phanerochaete chrysosporium* drawn from soil ecosystem. El-Ayouty and Ezzat (1991) had recorded the herbicide metabolizing potentials of *Nostoc* sp.

In view of the potentials and adapted nature, this section of work in this study was carried out to understand the distribution relationships of total and atrazine resistant microbial population and the resistant bacterial strains were selected for further experiments. Many researchers have reinforced the
necessity of employing native microorganisms for a particular bioremediation programmes. as they are often found suitable to survive and overcome the competition of other organisms thereby ensuring the desired bioremediation effects. This includes. Javanjal and Deopurkar (1994); Radosievich et al. (1995); Radosievich et al. (1996); Fuller et al. (1998) and Tanghe et al. (1999). Shapir et al. (2000) reported the preference of native soil microorganisms to mineralize atrazine. Apart from bacteria. Shelton et al. (1996) remarked the relevance of native microorganisms belonging to the genera Streptomyces in metabolizing 12 different herbicides. Lloyd - Jones and Trudgill (1989) and Saraswat and Gaur (1995) laid emphasis on the necessity of selecting native microbes when required for bioremedial purposes.

In this background. indigenous atrazine resistant bacterial strains that were found predominant in the sugarcane field (Pseudomonas sp. and Bacillus sp.) were selected and proceeded for further investigation.