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Influence of Industrial Effluents on the Population of *Azotobacter* sp. and Nitrifiers in the Agricultural Soils of Anantapuramu District in Andhra Pradesh

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ABSTRACT: The study was designed to evaluate the microbial population of soil environment contaminated with industrial pollution. Soil samples were collected from the surrounding areas (1/4th km) of Saptagir Camphor industry, Anantapuramu, Andhra Pradesh, India. Soil samples without effluent discharges served as control were collected from adjacent site (1 km) away from the same camphor industry. Triplicate soil samples of each polluted and non-polluted soil samples were withdrawn at periodic intervals such as 0, 10, 20, 30 and 40 days for the enumeration of microbial population. The estimation of population of *Azotobacter* sp. and nitrifiers was done by most-probable number (MPN) method. Population of *Azotobacter* sp. and nitrifiers were significantly enhanced in polluted soil than in non-polluted soil and were increased with increase in the incubation period up to 20 days compared to 0 day, however, population count was decreased after 20 days of incubation Camphor industry. The population count decreases with increase in the dilution factor. Gradual decrease in the population of microbes after 20 days may be due to the depletion of nutrients. In spite of the variations in the population in polluted and non polluted soil, there is no much impact in the agriculture comparatively. This may be due to the resistant developed by the microbes present in the polluted soil of Saptagir Camphor industry. Studies have shown that the ability of microbes to tolerate a definite level of pollutants under natural conditions might be owing to the complex nature of the soil environment.

KEYWORDS: Saptagir Camphor industrial effluents, *Azotobacter* sp. and nitrifiers, Polluted and Non- polluted soil.

I. INTRODUCTION

Soil is the key component of natural ecosystem [26] and as such environmental sustainability depends largely on a sustainable soil ecosystems. Soil productivity is considered as important factor for success of agricultural production rather than soil fertility [7]. It is a vibrant habitat for huge diversity of life forms. Microbial diversity constitutes the most extraordinary reservoir of life in the biosphere. Over millennia microbes have adapted to extremely diverse environment and developed an extensive range of new metabolic pathways or library of catabolic enzymes [10]. The expedited advent of urbanization and industrialization for economic growth has adversely affected the biological diversity, which is one of the major concerns of developing countries.

Industrial effluents entering the soil is one of major sources of environmental toxicity. It has deleterious impact on the soil microflora. The effluents of sugar and textile industry and their deleterious effects on the soil microflora. The microbial flora too is affected by it as compared to the control water sample due to the high BOD and COD values [3]. Cassava mill effluent has negatively affected the microbial populations and physicochemical parameters at various depths [25]. Above certain concentrations and over a narrow range, the heavy metals turn into toxins [5]. Moreover, these metals adversely affect natural microbial populations leading to disruption of vital ecological processes [9]. Many industrial effluents had heavy metals such as Pb, Cd, Cr, Ni and Hg as effluent constituents. These heavy metals increased their concentrations and thereby reduced soil microbial process and microbial biomass [19]. [24] reported

that several chemical and biochemical properties of the investigated soil changed in response to the application of POME (palm oil mill effluents). Organic amendment produced a decrease in pH and increase in electrical conductivity. Industrial pollutants affect the growth, morphology and metabolism of soil microorganisms, through functional disturbance, protein denaturation or the destruction of the integrity of cell membranes. Soil microorganisms are essential in the decomposition of soil organic matter, any decrease in the microbial diversity or abundance may adversely affect nutrient absorption from the soil.

II. REVIEW OF LITERATURE

Population of *Azotobacter* sp.

Azotobacter sp. is a genus of free-living diazotrophic bacteria whose resting stage is a cyst. It is primarily found in neutral to alkaline soils, in aquatic environments, and on some plants. It has several metabolic capabilities, including atmospheric nitrogen fixation by conversion to ammonia. *Azotobacter* sp. are heterotrophic rhizobacteria enable to fix N₂ between 2-15 mg N/g carbon source [31]. This extracellular structure plays an important role in improving soil porosity and transport of heavy metal pollutants in the soil. *Azotobacter* resistance to 20 mg/L of HgCl₂ has been isolated from tailings in Buru Island although lower cell viability was evident [12]. Organic matter maintains viability of heterotrophic *Azotobacter* sp.

The presence of Cd in the soil resulted in the maximum inhibition (84.9%) in the number of free living *Azotobacter* sp. *chroococcum* cells over the control. The phytotoxicity was apparently due to the susceptibility of the free living *Azotobacter chroococcum* cells to the toxic doses of heavy metals. Protein content increased from 19.0-71.4% in metal exposed plants at metal concentrations equivalent to those found in polluted soil [27]. A total of 57 [36 and 21] *Azotobacter chroococcum* were isolated from wheat (*Triticum aestivum*) rhizospheric soil irrigated with industrial wastewater (about a decade) and ground water (uncontaminated) and characterized on the basis of morphological, cultural and biochemical characteristics. Rhizospheric soils were analyzed for metal concentrations by atomic absorption spectrophotometry and the test soil samples were contaminated with Fe, Zn, Cu, Cr, Ni and Pb. All the isolates of *A. chroococcum* were tested for their resistance against Hg²⁺, Cd²⁺, Cu²⁺, Cr³⁺, Cr⁶⁺, Zn²⁺, Ni²⁺ and Pb²⁺. Among 36 isolates of *Azotobacter* sp. from soil irrigated with industrial wastewater, 94.4% were resistant to Pb²⁺ and Hg²⁺ and 86.1%, 77.5% and 63.8% were resistant to Zn²⁺, Cr⁶⁺ and Cr³⁺ respectively [4]. However, the literature on the population of *Azotobacter* sp. influenced by the industrial pollutants is sparse.

Population of nitrifiers

Nitrifiers in soil occupy a key position in the oxidation of ammonia and ammonium ions and form nitrites and nitrates. Population of nitrifiers were influenced by a number of environmental factors [17]. The influence of plow, sub tillage, and no-tillage fallow management on nitrifier and denitrifier populations and available N in surface soil (300 mm) was evaluated at two sites in western Nebraska during spring growth of winter wheat (*Triticum aestivum* L.). At both sites, nitrifier populations in the surface 150 mm of no-tillage and sub tillage soils were 22 to 56% and 16 to 35% lower, respectively, than those in plowed soil. Surface applications of 44 kg N/ha as NH₄NO₃ increased soil nitrifier populations six to nine fold in the surface 0 to 150 mm layer; this effect was not influenced by tillage practice. Lower populations of nitrifiers were paralleled by lower NO₃-N levels in no-tilled compared with plowed soils [8]

Nitrifying bacteria persisted in soil contaminated with hydrocarbon containing a limited amount of ammonium for several years. These conditions reduced the number of nitrifying bacteria but probably selected an ammonia-oxidizing community with a higher ammonium affinity and a higher activity than the community in the agricultural soil. The ammonia-oxidizing bacterial community in contaminated soil was not directly affected by a hydrocarbon addition, possibly because of its high affinity for ammonium [16]. Soil leaching experiments were conducted to assess the impact of discharging wastewater, of two salinities, on bacterial population sizes and microbial activities in mangrove soils. The total counts of both aerobic and anaerobic heterotrophic bacteria, nitrifiers and denitrifiers in mangrove soils receiving wastewater were significantly higher than those found in the control soils [23]. Microorganisms play vital role in circulation and transformation of mercury compounds in the environment. The concentration of <10 ppm Hg may have toxic effects on nitrifiers in soils. Increased concentrations of lead in surface soil layers negatively affect soil microflora [2]. Ammonia, thiocyanate, free cyanide, ferric cyanide, phenol and *p*-cresol inhibits the process of nitrification in an activated sludge system, and then correlated their threshold concentrations with the full-scale pre-denitrification process for treating cokes wastewater [35]. Nitrifying bacteria were most adversely affected in both the dry and rainy seasons in soil polluted with cassava mill effluents. Nitrifying bacteria were negatively affected by the alkaline environment [13]. However, inadequate literature is available on the population of nitrifiers influenced by the industrial pollutants.



III. MATERIALS AND METHODS

Soils

Soil samples were collected from the surrounding areas (1/4th km) of Saptagir Camphor industry, Anantapuramu, Andhra Pradesh, India. Soil samples without effluent discharges served as control were collected from adjacent site [1 km) away from the same camphor industry. Soil samples were air dried and mixed thoroughly to increase homogeneity and shifted to < 2 mm sieves for estimation of physico-chemical properties by standard methods. Both polluted and non-polluted soil samples of camphor industry were used for studying various microbial populations and soil enzyme activities.

Saptagir Camphor Limited: Manufacture and supply of terpene chemical and associated products. Camphor is used in traditional pooja rituals as well as pharmaceuticals. Synthetic camphor is white crystalline solid with pharma grade 95% purity.

Population of *Azotobacter* sp. and nitrifiers

Five gram portions of both polluted and non-polluted soil samples were placed in (15 × 150) mm test tubes and were incubated at room temperature (28 ± 4°C) and moisture content was maintained at 60% water holding capacity (WHC) until the incubation period is completed. Triplicate soil samples of each polluted and non-polluted soil samples were withdrawn at periodic intervals such as 0, 10, 20, 30 and 40 days for the estimation of the population of *Azotobacter* sp. by most-probable number (MPN) method [1] and [15]. The estimation of the population of nitrifiers, by most-probable number (MPN) method [1] and [20].

MPN method for *Azotobacter* sp.

The population of *Azotobacter* sp. in incubated soil samples (polluted and non-polluted) were estimated by most probable number technique. Ten-fold serial dilutions of soil samples were made and the numbers were calculated using the probability tables [1]. 5 ml portions of sterile mannitol agar medium [30] was taken in five MPN tubes and inoculated with 0.5 ml aliquots of the suspensions from 10⁻⁵ to 10⁻⁸ soil dilutions. Inoculated soil samples were incubated at 30°C for 48 hrs. Microscopic examination of the *Azotobacter* sp. shown the off white to beige homogeneous free flowing powder.

Composition of mannitol agar medium

Dipotassium phosphate	:	1 g	
Magnesium sulphate	:	0.2 g	
Sodium chloride	:	0.2 g	
Ferrous sulphate	:	TRACE	
Soil extract	:	5 g	
Mannitol	:	20 g	
Agar	:	15 g	
Distilled water	:	1000 ml	
pH	:		8.3 ± 0.2

MPN method for nitrifiers

To estimate nitrifiers, MPN method was employed, following two-fold serial dilutions [32]. Five MPN tubes, each containing 5 ml of steam sterilized mineral salts medium [1] received from 1:500 to 1:32,000. Viable cells of nitrosifying and nitrifying bacteria were determined by estimating nitrite following diazotization method [6] MPN tubes were calculated by referring to the tables of [11].

IV. RESULTS AND DISCUSSION

Physico-chemical characteristics of polluted and non-polluted soil samples of Saptagir Camphor industry

Table 1: Physico chemical properties of polluted and non-polluted soil of Saptagir Camphor industry

S.No	Physico-Chemical Properties of soil	Saptagir Camphor polluted soil	Saptagir Camphor non-polluted soil
1	Color	Red	Red
2	Water holding capacity (ml g ⁻¹ soil)	42.39	37.97
3	Texture		
	Sand (%)	54.3	52.2
	Silt (%)	32.4	34.5
	Clay (%)	13.3	13.3
4	pH ^a	8.58	8.5
5	Electrical conductivity (m.mhos)	223	202
6	Organic carbon (%)	1.61	0.48
7	Organic matter (%) ^b	2.76	0.82
8	Total nitrogen (%) ^c	0.079	0.050
9	NH ₄ ⁺ -N (µg g ⁻¹ soil) ^d	7.10	6.22
10	NO ₂ ⁻ -N (µg g ⁻¹ soil) ^e	0.64	0.41
11	NO ₃ ⁻ -N (µg g ⁻¹ soil) ^f	0.99	0.80
12	Sulphur (%)	1.62	2.13

^a1:1.25 (soil:water) ;^bWalkey-Black method [14] ;^cMicro-Kjeldhal method [14] ;^dNesslerization method [14] ;^eDiazotization method [6] ;^fBrucine method [28]**The effect of Saptagir Camphor industrial effluents on soil microorganisms**

The world has entered a period of unprecedented environmental change as evidenced in the rapid growth of natural and man-made changes in the biosphere in recent times. Environmental pollution resulting in changes in environmental quality is one of the issues raising widespread alarm. An increase in socio-economic activities worldwide has been accompanied by an even faster growth in pollution stress. This is because the majority of human needs can only be met



with goods and services provided by industries. Industries have the capacity to improve as well as degrade the environment. Raw materials, which have been taken from the natural resource base, are converted into products by the industry. [18] reported that during the production process pollutants are often released into the environment. Soil is an efficient purifying medium with a great capacity to receive and decompose wastes matter by its micro flora yielding nutrients in the process [22]. However, if the input of the pollutants exceeds the soil purifying limit, the effectiveness of soil microbial activity is reduced considerably. As a result, there will be an adverse change in the soil physicochemical properties which consequently affect the growth and development of the crop plants. Microbial activity in POME amended soils was increased under laboratory-controlled conditions irrespective of dose and duration of incubation. However, microbial and biochemical properties examined indicated and initial stress on microbes especially at early stage of incubation, but was quickly stabilized due to buffering capacity of soil [24]. [34] found that the long-term effects of nearly 20 yrs of heavy metal contamination near a copper smelter could affect the activity and community composition of soil microorganisms. [21] reported that the functional potential of the microbial population measured as sole carbon source utilization by Ecoplates differed between the soils, but there was no change in the number of substrates utilized. The observed changes in the different soil microbial populations are probably a combination of both direct and indirect effects of the mercury contamination.

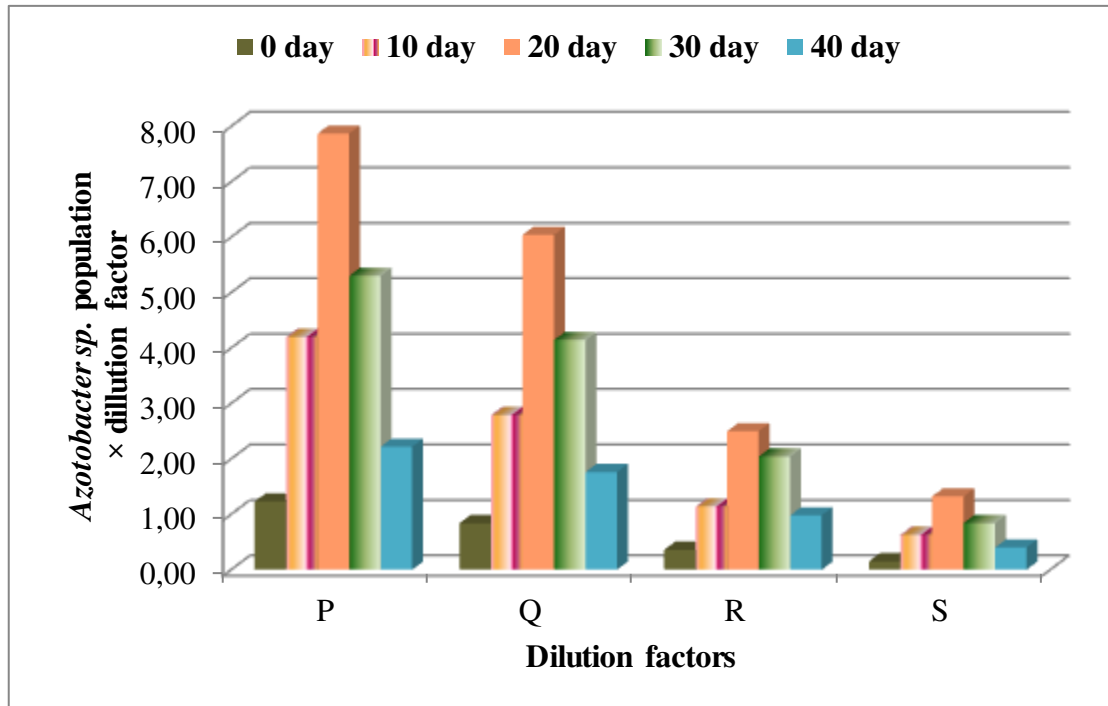
Population of *Azotobacter* sp.

Azotobacter sp. are heterotrophic, free-living diazotrophic, aerobic nitrogen fixing bacteria in soils [30]. It has the highest metabolic rate compared to any other microorganisms. *Azotobacter* sp. are heterotrophic rhizobacteria enable to fix N₂ between 2-15 mg N/g carbon source [31]. They produce growth regulators auxin, cytokinins and gibberelins that stimulate cell extension and division which promote plant growth.

The population *Azotobacter* sp. (MPN) of polluted and non-polluted soils of Saptagir Camphor industry were presented (Table 2 and 3) respectively. Population of *Azotobacter* sp. (MPN) has been decreasing with increasing dilution factor from 10⁻⁵ to 10⁻⁸. The population in the dilution factor 10⁻⁵ is higher than 10⁻⁸ on 0 day. The maximum population of *Azotobacter* sp. was observed on 20 day in the dilution factor 10⁻⁵ ie. 7.87 in polluted soils and 6.04 in non-polluted soil. The minimum population of *Azotobacter* sp. was observed on 40 day in the dilution factor 10⁻⁸ ie. 0.40 in polluted soils and 0.21 in non-polluted soil. However, the population of polluted soils is two-fold higher than non-polluted soil in all the dilutions. The same trend was observed on all the incubation days, in polluted and non-polluted soils of Saptagir Camphor industry. The population of *Azotobacter* sp. on 0 day is relatively low, as the days of incubation increased the population significantly increased upto 20 day whereas on further increasing the incubation (30 and 40 days) there was a significant decrease in population. The population of *Azotobacter* sp. on 40 day was subsided when compared to 20 day. The population of *Azotobacter* sp. in dilution factor 10⁻⁶ on 40 day (1.76 and 1.17) and on 20 day (6.04 and 3.89) in both polluted and non-polluted soils respectively. This is because, as incubation period increases the depletion of nutrients occurs and hence the population gradually retards on prolonged incubation.

Table 2: Population of *Azotobacter* sp. (MPN × 10² g⁻¹) in polluted soil samples of Saptagir Camphor industry

Dilution factors	Saptagir Camphor Polluted Soil sample				
	Incubation in days				
	0 day	10 days	20 days	30 days	40 days
P	1.23a (100)	4.20b (341)	7.87c (640)	5.30d (431)	2.22e (180)
Q	0.84a (100)	2.79b (332)	6.04c (719)	4.14d (493)	1.76e (209)
R	0.36a (100)	1.14b (317)	2.50c (694)	2.04d (567)	0.98e (272)
S	0.15a (100)	0.63b (420)	1.32c (880)	0.84d (560)	0.40e (267)



*Dilution factor P = 10⁻⁵; Q = 10⁻⁶; R = 10⁻⁷; S = 10⁻⁸

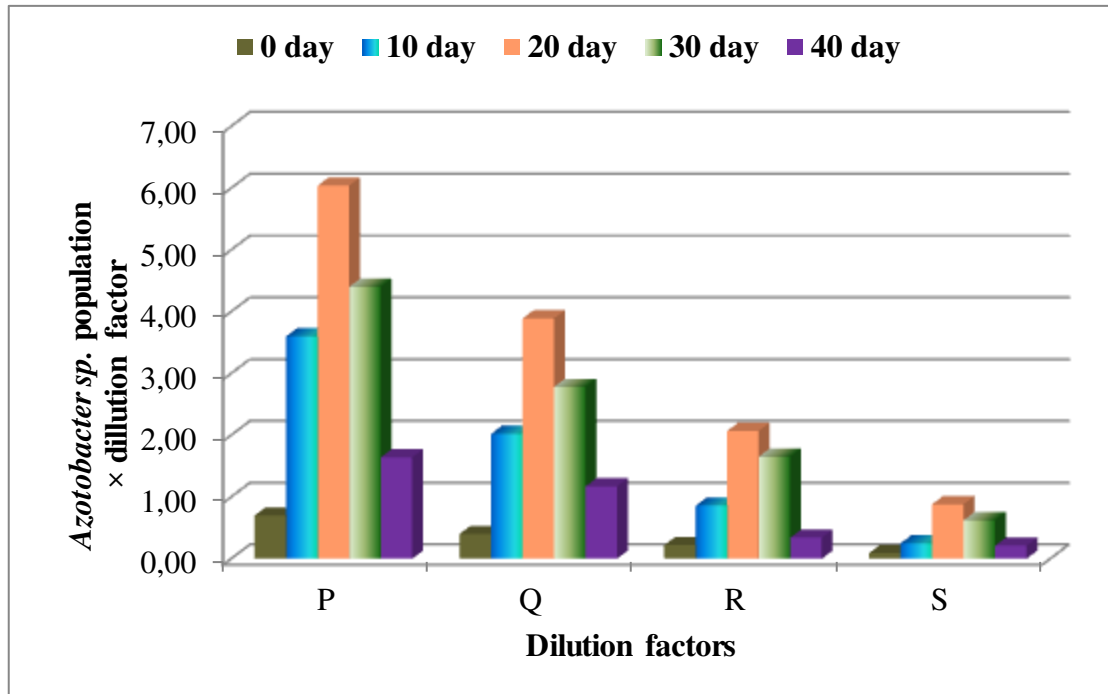
Figures, in parenthesis, indicate relative production percentages.

Values in the table and figure are means of triplicates.

Means, in each column, followed by the same letter or not significantly different ($P \leq 0.005$), from each other according to Duncan's Multiple Range (DMR) test.

Table 3: Population of Azotobacter sp. (MPN × 10² g⁻¹) in non polluted soil samples of Saptagir Camphor industry

Dilution factors	Saptagir Camphor non Polluted Soil sample				
	Incubation in days				
	0 day	10 days	20 days	30 days	40 days
P	0.70a (100)	3.60b (514)	6.04c (863)	4.40d (628)	1.64e (234)
Q	0.40a (100)	2.02b (505)	3.89c (972)	2.78d (695)	1.17e (292)
R	0.22a (100)	0.86b (391)	2.07c (941)	1.65d (750)	0.34e (154)
S	0.09a (100)	0.25b (278)	0.88c (978)	0.62d (689)	0.21e (233)



*Dilution factor P = 10⁻⁵; Q = 10⁻⁶; R = 10⁻⁷; S = 10⁻⁸

Figures, in parenthesis, indicate relative production percentages.

Values in the table and figure are means of triplicates.

Means, in each column, followed by the same letter or not significantly different ($P \leq 0.005$), from each other according to Duncan's Multiple Range (DMR) test.

The polluted soils of Saptagir Camphor industry shows higher population of *Azotobacter* sp. than in non-polluted soil. This may be due to the release of effluents in organic nature. The increased population of *Azotobacter* sp. is due to the presence of increased levels of total nitrogen content in polluted soil. Modest reports have been published on the influence of industrial effluents on population of *Azotobacter*.

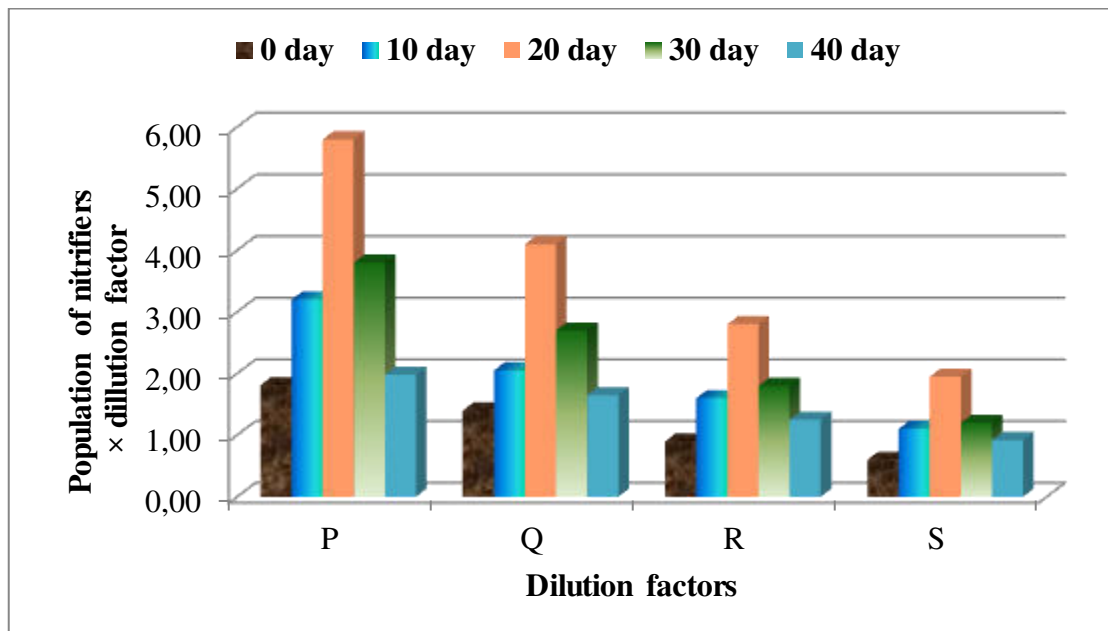
Population of nitrifiers

The population nitrifiers (MPN) of polluted and non-polluted soils of Saptagir Camphor industry were represented (Table 4 and 5) respectively. Population of nitrifiers (MPN) has been decreasing with increasing dilution factor from 10⁻⁵ to 10⁻⁸. The population in the dilution factor 10⁻⁵ is higher than 10⁻⁸ on 0 day. The maximum population of was observed on 20 day in the dilution factor 10⁻⁵ ie. 5.80 in polluted soil and 4.30 in non-polluted soil of Saptagir Camphor industry. The reduced population was observed on 40 day in the dilution factor 10⁻⁸ ie. 0.92 in polluted soil and 0.20 in non-polluted soil. However the population of nitrifiers in polluted soils is comparatively higher than non-polluted soil in all the dilutions. The population of nitrifiers on 0 day is relatively low, as the days of incubation increased the population significantly increased upto 20 day whereas on further increasing the incubation (30 and 40 days) there was a significant reduction in population. The population on 40 day was subsided compared to 20 day. The population of nitrifiers in dilution factor 10⁻⁶ on 40 day (1.65 and 0.98) and on 20 day (4.10 and 3.35) in both polluted and non-polluted soils respectively. This is because, as incubation period increases the depletion of nutrients occurs and hence the population decreases on prolonged incubation. The polluted soil of Saptagir Camphor industry shows enhanced population of nitrifiers than in non-polluted soil. This may be due to the release of effluents in organic nature. Polluted soil contains high nitrate contents than in non-polluted soil.



Table 4: Population of nitrifiers (MPN × 10² g⁻¹) in polluted soil samples of Saptagir Camphor industry

Dilution factors	Saptagir Camphor Polluted Soil sample				
	Incubation in days				
	0 day	10 days	20 days	30 days	40 days
P	1.81a (100)	3.20b (177)	5.80c (320)	3.80d (210)	1.98a (109)
Q	1.40a (100)	2.05b (146)	4.10c (293)	2.70d (193)	1.65a (118)
R	0.90a (100)	1.60b (178)	2.80c (311)	1.80d (200)	1.25e (139)
S	0.60a (100)	1.10b (183)	1.95c (325)	1.20 ^b (200)	0.92e (153)



*Dilution factor P = 10⁻⁵; Q = 10⁻⁶; R = 10⁻⁷; S = 10⁻⁸

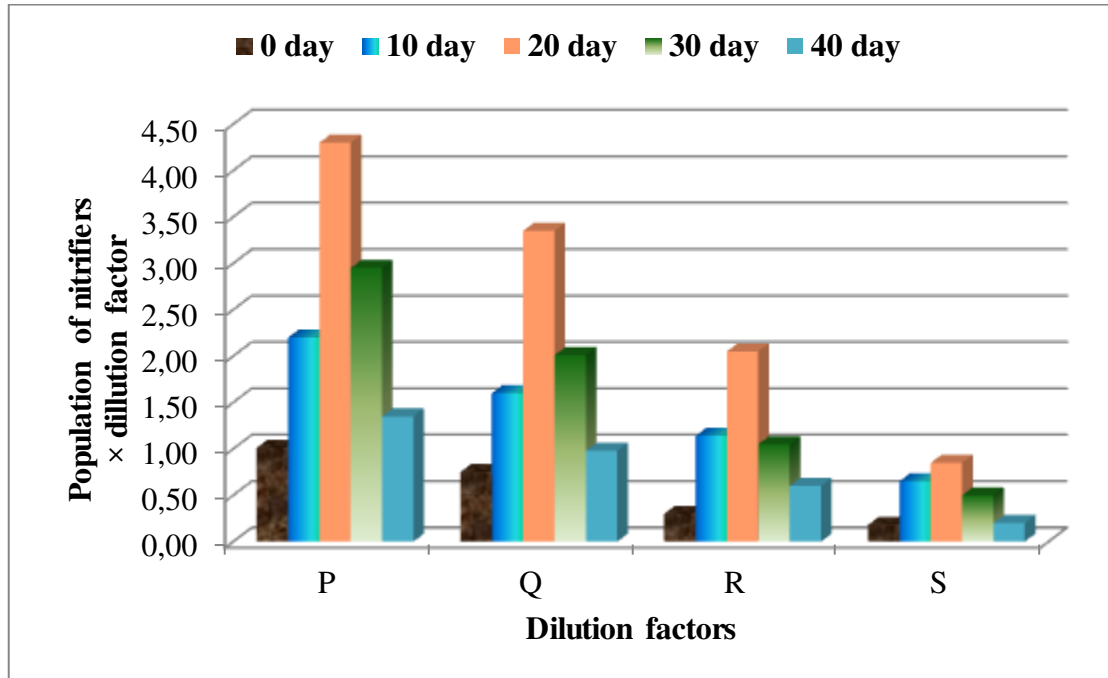
Figures, in parenthesis, indicate relative production percentages.

Values in the table and figure are means of triplicates.

Means, in each column, followed by the same letter or not significantly different ($P \leq 0.005$), from each other according to Duncan's Multiple Range (DMR) test.

Table 5: Population of nitrifiers (MPN × 10² g⁻¹) in non polluted soil samples of Saptagir Camphor industry

Dilution factors	Saptagir Camphor non Polluted Soil sample				
	Incubation in days				
	0 day	10 days	20 days	30 days	40 days
P	1.01a (100)	2.20b (218)	4.30c (503)	2.95d (292)	1.35a (134)
Q	0.75a (100)	1.60b (213)	3.35c (582)	2.01d (268)	0.98a (131)
R	0.30a (100)	1.14b (380)	2.05c (514)	1.05b (350)	0.60e (200)
S	0.18a (100)	0.65b (361)	0.85c (472)	0.50d (278)	0.20a (111)



*Dilution factor P = 10⁻⁵; Q = 10⁻⁶; R = 10⁻⁷; S = 10⁻⁸

Figures, in parenthesis, indicate relative production percentages.

Values in the table and figure are means of triplicates.

Means, in each column, followed by the same letter or not significantly different ($P \leq 0.005$), from each other according to Duncan's Multiple Range (DMR) test.

The contents of total nitrogen and sulphur are also higher in polluted soils than in non-polluted soils. As the period of effluent release increases the microorganisms present in the polluted soil tend to acquire resistance towards the effluents. Nitrifying bacteria persisted in contaminated soil containing a limited amount of ammonium for several years. These conditions reduced the number of nitrifying bacteria [16]. The presence of tolerant populations of nitrifying organisms in the contaminated soils was demonstrated. Tolerance was also eventually acquired after a longer lag phase, by the non-contaminated soil populations although the rate of activity was often reduced [29]. The autotrophic nitrifying bacteria are active at lower pH. Nitrifying bacteria had the least population ($0.6 \times 10^1 \pm 0.03\text{cfu/g}$) in soil polluted with cassava mill effluents. Nitrifying bacteria are very sensitive to environmental stress and had very low population [13].

V. CONCLUSION

Thus, the results revealed that the microbial density is high in polluted soil as compared to non-polluted soil. The current study of Saptagir Camphor effluent discharge suggested that the high inorganic as well as organic pollution load, serves as nutrients for microbial growth hence increasing their population in polluted soil around Saptagir Camphor industrial area as compared to the non-polluted soil taken in this study. The chemical nature of effluent was complex and contained large amounts of organic and inorganic compounds. The soil around the industry receives plenty of treated and non-treated effluent since decade serves as a broth for an enormous diversity of microbes. The microbial population able to use organic load like drug residues of effluent are reported in this study. These microbes are forced to live in the medium of high organic and toxic load in the soil thereby acclimatized to process them. The values of physico-chemical characteristics are slightly higher in polluted soil than in non-polluted soil. These effluents have deleterious effects on the soil sample collected from the site of effluent discharge. The effluents doesnot have a major impact on soil for cultivation purpose but changes the diversity of soil microflora and soil enzyme activities. Results suggest that the microbial population in the polluted site increased, indicating that the microbes have developed resistance towards the effluents discharged from the industry. The alkaline environment may have encouraged the

activity of the enzymes. In fact, studies have shown that the ability of microbes to tolerate a definite level of pollutants under natural conditions might be owing to the complex nature of the soil environment. Soil microbial populations such as *Azotobacter* sp. and nitrifiers were significantly higher in polluted soil samples than in non-polluted soil. Population of *Azotobacter* sp. and nitrifiers were significantly enhanced in polluted soil than in non-polluted soil and were increased with increase in the incubation period up to 20 days compared to 0 day, however, population count was decreased after 20 days of incubation. The population count decreases with increase in the dilution factor. This may be due to the depletion of nutrients in the soil. The population of *Azotobacter* sp. in polluted soil is two-fold higher than in non-polluted soil.

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