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Degradation of malathion by *Pseudomonas* during activated sludge treatment system using principal component analysis (PCA)

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Abstract: Popular descriptive multivariate statistical method currently employed is the principal component analyses (PCA) method. PCA is used to develop linear combinations that successively maximize the total variance of a sample where there is no known group structure. This study aimed at demonstrating the performance evaluation of pilot activated sludge treatment system by inoculating a strain of *Pseudomonas* capable of degrading malathion which was isolated by enrichment technique. An intensive analytical program was followed for evaluating the efficiency of biosimulator by maintaining the dissolved oxygen (DO) concentration at 4.0 mg/L. Analyses by high performance liquid chromatographic technique revealed that 90% of malathion removal was achieved within 29 h of treatment whereas COD got reduced considerably during the treatment process and mean removal efficiency was found to be 78%. The mean pH values increased gradually during the treatment process ranging from 7.36—8.54. Similarly the mean ammonia-nitrogen (NH_3-N) values were found to be fluctuating between 19.425—28.488 mg/L, mean nitrite-nitrogen (NO_3-N) ranging between 1.301—2.940 mg/L and mean nitrate-nitrogen (NO_3-N) ranging between 0.0071—0.0711 mg/L. The study revealed that inoculation of bacterial culture under laboratory conditions could be used in bioremediation of environmental pollution caused by xenobiotics. The PCA analyses showed that pH, COD, organic load and total malathion concentration were highly correlated and emerged as the variables controlling the first component, whereas dissolved oxygen, NO_3-N and NH_3-N governed the second component. The third component repeated the trend exhibited by the first two components.

Keywords: activated sludge system; malathion, principal component analyses (PCA); raw wastewater; removal efficiency

Introduction

Principal component analyses (PCA) is a classical ubiquitous statistical technique for data analysis and processing, it is a simple well known geometrical tool of orthogonalizing data which is very easy to understand. The starting point of any statistical analysis is to plot the data. This geometrical approach allows one to investigate patterns associated with the variables in the study. Moreover, PCA often generates components that have valuable biological meanings (Lukman *et al.*, 1999).

Recently, the significance of the mode of growth of microorganisms has received considerable attention due to its potential biotechnological application in bioremediation of environmental pollution through bioreactors (Lauwer *et al.*, 1990). It has been reported that these microorganisms perform their activity efficiently in the activated sludge system (Barker and Dold, 1995). The treatment of domestic wastewater with the activated sludge treatment system is rather easier as it contains readily biodegradable organic matter which is mainly composed of volatile fatty acids and low molecular weight carbohydrates that can be metabolized readily by the bacteria (Henz *et al.*, 1987). Presently, environmental engineers are designing and operating the treatment facilities that utilize living organisms to bring about the destruction or transformation of organic and inorganic waste material. Bioremediation technology to treat the hazardous waste has gained considerable attention as it is ecologically sound and economical as compared to other technologies and has been attempted successfully in many countries of the world (Ritmann *et al.*, 1988; Enrica, 1994). Numerous reports are available indicating the use of microorganisms for pollution control, in studying bioremediation, many researchers have tried to isolate and identify microorganisms from soil or water to obtain pure cultures and then examine their biodegradation capacities. However, some reports describe the use of indigenous microorganisms rather than introducing special microorganisms for bioremediation (US-EPA, 1991; Christodoulatos *et al.*, 1997; Bitton, 1998; Robert, 1998).

Pesticides have played an important part in dramatic increases in agricultural productivity, which have been achieved in the developed world over the last few decades. Groundwater and surface water pollution by pesticides has been reported by many authors (Getenga *et al.*, 2000). While pesticides uses have improved world food supply and have been responsible for better growth and yield, their irresponsible and indiscriminate uses have greatly increased environmental problems. The removal of pesticides from the wastewater by activated sludge system has given much more attention in past few years. Along with many benefits these pesticides have rendered their use had also caused many serious problems in terms of harmful effects on non target

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organisms (Gossel and Bricker, 1994; Hoskin and Walker, 1997). Their presence even in minute quantities results in an adverse effect on ecological systems. The model pesticide used in the present study is malathion which belongs to organophosphorous class of pesticide. Malathion was selected on the basis of its worldwide application. It is a non-systemic, one of the world's most widespread general purpose organophosphate insecticide with high selective toxicity that is mostly used for the control of sucking and chewing insects on fruits and vegetables and for controlling mosquitoes and flies(Gutmann and Besser, 1990). Its usage ranges from household to acreage application to control insects foraging on grains, cotton, tobacco and fruits. Malathion is also used in veterinary medicine (Osweiler *et al.* 1984), and in public health practices as an anti-infective agent (Wester and Cashman, 1989) to control insect vector borne diseases such as malaria (*Anopheles* mosquito), dengue (*Aedes aegypti*), and yellow fever (*Aedes*) (Caglioti, 1983). Some studies reported the degradation of low concentration of malathion by heterogeneous bacterial population. It was also reported that several bacterial and fungal isolates obtained from soil and wastewater, are also capable of degrading malathion (Paris *et al.*, 1975). The extent to which pesticide residues could be removed from treated produce by washing may be influenced by a variety of factors such as the chemical properties and formulation type of the pesticide, the nature of the commodity, the length of time that the residue has been on the commodity's surface, and the rinsing time and rinsing agents used (Leyva *et al.*, 1998). Most of the studies indicate that the major pathway of malathion disappearance in soil, water, sediments and salt marsh environments is biologically mediated (Kumari *et al.*, 1998; Guha *et al.*, 1997; Beyers and Myers, 1996; Ashok and Seth, 1989; Walker, 1976; Bourquin, 1975; Mostafa *et al.*, 1972).

Statistical analysis involves the risk of finding strong relationships that exist as a result of an artifact in the data set. Even though further mechanistic understanding is required, this statistical analysis can be used efficiently to predict the real situation. The statistical software package used was STATISTICA for Windows release 5 for statistical analysis in the present study. PCA describes the variation of a set of multivariate data as a reduced set of uncorrelated variables, each of which is a particular linear combination of the original variables. PCA is useful when a relatively large part of the total variance is explained by only a few components (Vink and VanDer, 1997).

1 Materials and methods

1.1 Pesticide used

The organophosphate pesticide used in the present study is commercially available as malathion, chemical name S-1, 2-bis (ethoxycarbonyl) ethylo, o-dimethyl phosphorodithioate. Table 1 represents the different physical and chemical properties of malathion, whereas the chemical structure is presented in Fig. 1.

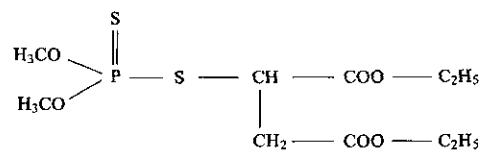

Fig.1 Chemical structure of malathion

Table 1 Physical and chemical properties of malathion (Edmundson, 1988)

Property	Value	Property	Value
Nature	Amber liquid	Molecular formula	$C_{10}H_{19}O_6PS_2$
Melting point	2.85℃	Molecular weight	330.36 mg/mol
Boiling point	156—157℃ (10.7 mm Hg)	Half life in soil	24 h to 6 d
Vapor pressure	4×10^{-5} mmHg(130℃)	Half life in water	1.5 d to 21 weeks
Specific gravity	1.23 (25℃)	log octanol/water partition coefficient	2.36
Refractive index	1.4985	Solubility in water	145 mg/L (20℃)
Organic solvent	Miscible		
Aquatic toxicity	High		

1.2 Wastewater collection

Grab samples of mixed liquor (raw domestic wastewater) were collected during the present study. The wastewater must be homogenous and smooth in consistency before it is processed.

1.3 Isolation, identification and characterization of bacteria culture

The bacterial culture capable of degrading malathion was isolated from the evenly mixed soil, obtained within the campus premises using enrichment technique. The optimum concentration of malathion for bacterial growth was determined by the inoculation of the bacterial culture on nutrient agar plates containing 2.85, 5.7 and 8.55 mg/ml

concentration of malathion. Morphological characteristics of the culture was studied by using Gram's staining method whereas for cultural characterization, the dehydrated medium (Acumedia) was used to prepare nutrient agar and broth in sterile distilled water which were autoclaved at 15 psi for 15 min and pH was adjusted at 7.2. Similarly, for determining fermentation of sugars, peptone water with 1% sugar containing Andrade's indicator was used. Glucose phosphate broth was used for determining methyl red test and for the study of the production of acetyl methyl carbinol. Indole production was determined by using peptone tryptone. Similarly, catalase activity was also determined by adding a few drops of 3% hydrogen peroxide on 24 h grown agar slopes and also in broth cultures and tubes were examined for the evolution of oxygen.

1.4 Malathion degradation studies in biosimulator

The performance efficiency of *Pseudomonas* for malathion degradation was determined in the biosimulator (NBS; New Brunswick Scientific Company) and was evaluated. The technical details and the general layout of the biosimulator for malathion degradation is shown in Table 2 and Fig.2. Approximately 8.0 L of sample was transferred carefully into the heavy wall, borosilicate glass jar of a compact bench scale stainless steel biosimulator (model MF-114). The sample was strongly agitated by impeller with flat stirring paddles and by the four vertical baffles. Agitation was continuously monitored on a calibrated electrical tachometer, which provides accurate speed indication. Air was metered through a pressure regulator, needle valve flow meter and a stainless steel filter.

1.5 Processing of samples

The samples were collected from the biosimulator vessel after every 2 h and were analyzed for

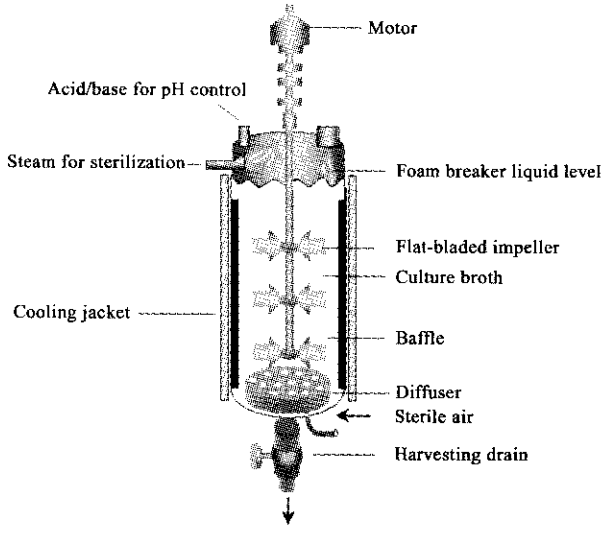

Fig.2 General layout of a biosimulator (activated sludge treatment system)

Table 2 Technical data of biosimulator

Biosimulator (NBS)	Model MF-114
(1) DO-controller	Model DO-81
(2) pH-controller	Model pH-22
Vessel capacity	14 L
Total load* (RWW; 7.5 L and culture inoculum; 500 ml)	Approximately 8 L
Retention time (treatment period)	29 h
Operating temperature (ambient)	25—28℃
DO-concentration	4.0 mg/L
Total malathion concentration (TMC)	1010.80 mg/L
Total organic load (in terms of COD)	12640 mg/L
Sample collection	2 h
Size of inoculum**	3.0×10^9 bacteria/ml

Notes: * RWW (KUC): raw domestic wastewater collected from wastewater treatment plant Karachi University Campus, Pakistan; ** size of inoculum: 24 h old grown culture of *Pseudomonas* (culture was streaked on nutrient-agar slants and incubated for 24—48 h, several washings were taken from the nutrient broth and matched with the McFarland's turbidometric index)

chemical oxygen demand (COD), ammonia-nitrogen (NH_3-N), nitrite-nitrogen (NO_2-N) and nitrate-nitrogen (NO_3-N) as the methods described in the Standard Methods (1998) and US-EPA (1974). pH and DO was measured using pH and DO controller of New Brunswick Scientific Company. Thin layer chromatographic (TLC) procedures described by Kadoum (1970) were employed to identify malathion. The following solvent systems were used for chromatographic identification: benzene-hexane-acetic acid (40:40:20), hexane-acetic acid-ether (75:15:10), benzene-acetic acid (4:1).

The spots of separated components were visualized by using freshly prepared DCO (2,6-dibromo-N-chloro-*p*-quinoneimine diluted to 0.5 % in acetone) as described by Menn *et al.* (1957) and Jaglan and Gunther (1970), whereas for HPLC (Shimadzu, Japan) chromatographic system consisted of a solvent delivery pump LC-10 AS, connected with an autoinjector model SIL-6A and a rheodyne injection valve fitted with a sample loop (20 μl). A guard column filled with μBondapak C_{18} analytical waters μBondapak reversed-phase column, effluents was monitored by UV-detector (visible spectrophotometer detector SPD-10A; λ=220 nm). The output of the detector was connected to a chromatopack (CR6A). Mobile phase consisted of methanol (Merck analytical grade) since malathion is miscible with alcohols (Montgomery, 1993). The flow rate was adjusted at 1.5 ml/min with total elution time of 12 min for each run.

1.6 Principal component analyses

The data obtained was subjected to principal component analyses which is a variance oriented technique where the component score is directly derived by a linear transformation. The use of PCA permits an objective summarization of the variables in the data matrix by extracting a new set of variables called principle components. The first principal component is the combination of variables that accounts for the largest part of variance in the sample. It is described as the linear combination, y_1, of the original variables, of which the total variance is maximized for all vectors a_{11} ... a_{1p}.

$$y_1 = a_{11}\, x_1 + a_{12}x_2 + \ldots\, a_{1p}x_p \qquad (1)$$

The second component accounts for the next largest amount of variance and is uncorrelated with the first, etc. To decide how many components are needed to represent data, the percentage of total variance explained by each component is examined (Feoli, 1977; Nichols, 1977). Only components that account for variances greater than the variances of all variables are included. Hence, the sum of the vectors that represent the variables (Eigenvalue) should be larger than 1.

We excluded variables from the data set that: (1) display very little variation over the locations and were therefore expected to be non-discriminating; and (2) revealed concentrations too small (< d) to inhibit microbial activity and affect biotransformation processes. A selection of 9 variables (10 observations) was used in PCA. The program package developed by Orloci and Kenkel (1985) was used to accomplish PCA ordination in the present study.

For a useful interpretation of the component matrix, one should attempt to minimize the number of variables that have high loadings onto one component. To achieve this we carried out an orthogonal rotation of the component matrix, so that the variables score high on the individual, non-correlated components. Since rotation does not influence the Eigenvalue, it does not affect the goodness of fit of a component solution (Hair, 1992).

A component score F_{jk} was estimated by multiplying the standardized value of each variable with its corresponding component loading:

$$F_{jk} = \sum_{i=1}^{n} X_{ij}\, F_{jk} = X_{1j}\, F_{1k} + X_{2j}\, F_{2k} + \cdots X_{nj}\, F_{nk} \qquad (2)$$

in which X_{ij} is the standardized value of the i-th variable for case j, and is the F_{jk} component loading for the k-th component and the j-th variable.

2 Results and discussion

2.1 Isolation and study of bacterial culture for malathion degradation

On the basis of morphological, cultural and biochemical characteristics, the bacterium isolated was found to belong to the genus *Pseudomonas*, according to the Bergeys Manual of Determinative Bacteriology (1994). Bacteria belonging to the genus *Pseudomonas* are highly oxidative, aerobic and metabolically versatile and have been reported to degrade aromatic hydrocarbons, oil, petroleum products and pesticides which are being used for *in situ* biorestoration (Christodoulatos, 1997). In the present study the DO concentration was maintained at 4.0 mg/L, at this level of DO the performance of the system especially with reference to COD removal, changes in nitrogenous compounds and malathion degradation was evaluated. Very slight variation in DO concentration was observed and it was more or less the same as adjusted prior to the treatment.

2.2 Trend of pH on malathion degradation

The pH has a significant effect on the growth rate of both *Nitrosomonas* and *Nitrobacter* (Wong-Chong and Loehr, 1975). The pH of the samples fluctuated in a narrow range of 7.36 to 8.54. The results showed a trend towards alkaline side. There would be two sources of malathion removal in the system; one is the microorganisms and the second is the pH itself. The organisms do bring about the biodegradation of malathion but a part of them may also become unstable at pH above neutral (Ruzicka *et al.*, 1967).

2.3 Trend of organic load on malathion degradation

The organic matter content of wastewater was gradually reduced as reported in Table 3. Ahmed *et al.* (1988) also reported that COD removal increased with the retention time. The mean removal efficiency was found to be 78% after 29 h treatment. The COD removal efficiency was linear throughout the study period as reported by Polprasert *et al.* (1992). Retention time has a slight positive effect on COD removal efficiency, which could possibly be due to the DO concentration. However, Toprak (1995) reported that COD removal is a function of retention time.

2.4 Nutrients availability during malathion degradation

NH_3-N, NO_2-N and NO_3-N removal efficiency was not significant and fluctuated between 19.425—28.488, 1.301—2.940 and 0.0071—0.0711 mg/L respectively. It is difficult to account for this behavior in view of lack of more data. This situation has been particularly observed in wastewater treatment facilities with short retention time and high organic loading rate. The results of NH_3-N were not consistent as that of COD (Table 3). However, it may be that heterotrophic bacterial activity was more pronounced as compared to autotrophic bacteria which are responsible for NH_3-N conversion into nitrate through nitrification processes. It is suggested to study heterotrophic and autotrophic bacterial activity

relationship in the activated sludge treatment process especially in the presence of different sources of organic carbon (Barnes and Bliss, 1983).

The mean results of NO_2-N were consistent throughout the study period (1.301— 2.940 mg/L). The mean NO_3-N shows the trend similar to NH_3-N and ranged between 0.0071 to 0.0711 mg/L. The results of these three forms of nitrogen indicate that the microbial activity converts the organic nitrogen to ammonia. This reaction is highly dependent on pH. Alkaline pH favors the molecular forms whereas the neutral or acidic pH favors the ionic forms. However, the onward conversion of ammonia to nitrite and nitrate was restricted due to lower concentration of DO (Idelovitch and Michail, 1981).

2.5 Estimation of malathion degradation by HPLC

The mean percentage degradation of malathion by *Pseudomonas* at 4.0 mg/L DO, after 29 h treatment was found to be 90% shown in Table 3 and illustrated in Fig.3. A previous study showed that numerous bacteria isolated from salt marsh environment capable of degrading malathion up to 90% when supplied with additional nutrients as energy and carbon sources (Bourquin, 1977). The wastewater represents heterogeneous population of microorganisms, some of which may utilize malathion as a carbon source. Paris *et al.* (1975) also reported the degradation of low concentration of malathion by heterogeneous bacterial population. It was also reported that several bacterial and fungal isolates obtained from soil and wastewater are also capable of degrading malathion (Chakrabarty, 1982). However, it also becomes clear that addition of *Pseudomonas* into raw wastewater did enhance malathion degradation. The maximum degradation in this condition may be due to the fact that *Pseudomonas* had adapted itself to the highest concentration of malathion. Results of the present study are in accordance with the findings of Matsumura and Boush (1966) who found that malathion was rapidly

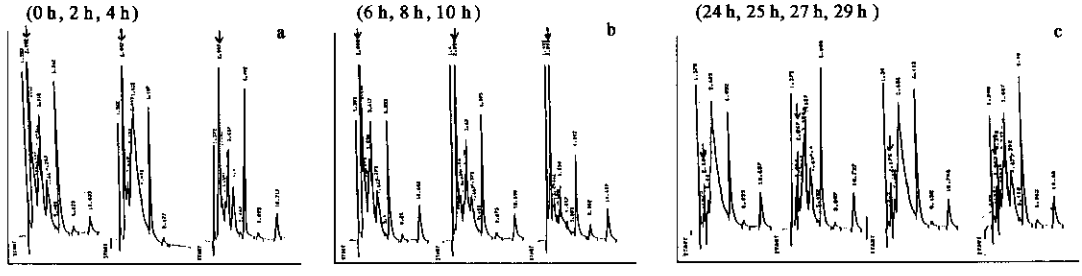

Fig.3 High pressure liquid chromatogram of malathion
Mobile phase: methanol; flow rate: 1.5 ml/min; column: μBondapak C_{18}(250×4.6 I.D); detection: 220 nm; temperature: ambient

Table 3 Basic data of biosimulator used for principal component analyses

No.	Rt, h	Peak elution, min *	pH	Concentration, mg/L						
				Dissolved oxygen	Organic load	Total malathion	COD	NH_3-N	NO_2-N	NO_3-N
1	0	2.082	7.36	4.14	12640	1010.80	1580	20.225	2.940	0.0711
2	2	2.097	7.42	4.12	11360	989.977(2)	1420(10)	19.425	2.794	0.0196
3	4	2.097	7.46	3.95	10400	954.902(5)	1300(18)	22.957	2.825	0.0269
4	6	2.088	7.53	3.98	9440	945.401(6)	1180(25)	24.423	2.710	0.0071
5	8	2.088	8.02	3.86	8480	764.46(24)	1060(33)	25.356	1.819	0.0103
6	10	2.095	8.16	3.75	7520	435.654(57)	940(41)	28.488	1.988	0.0227
7	24	2.160	8.40	4.11	4960	109.975(89)	620(61)	27.205	1.301	0.0279
8	25	2.167	8.53	4.21	4800	109.772(89)	600(62)	26.556	1.532	0.0261
9	27	2.175	8.51	4.10	3840	108.964(89)	480(70)	27.992	2.239	0.0481
10	29	2.063	8.54	3.96	2720	98.957(90)	340(78)	28.488	1.534	0.0556

Notes: Based on mean results of five experiments; * not used for PCA analysis; although DO meter was fixed at 4.0 mg/L level but DO was monitored in the biosimulator at different intervals of time; figures in brackets indicate percentage removal

metabolized by the *Pseudomonas*.

2.6 Principal component analyses

For the purpose of present study, the biosimulator performance efficiency in relation to inoculation of a strain of *Pseudomonas* with a continued supply of approximately 4.0 mg/L of DO and a retention time of 29 h was subjected to PCA. The data set consists of 10 observations and 9 variables related to biosimulator performance, including retention time, pH, DO, organic load in terms of COD and total malathion concentration (Table 3). The data was standardized before carrying out a PCA to develop a linear combination that could be used for prediction.

Fig.4 is based on the principal components I, II and III, which explain 94% of the total variability (Table 4). The first component I explaining 69% is primarily a function of pH, COD, organic load and TMC as indicated by eigenvector coefficient (Table 4).

Table 4 Results of PCA

Principal component	Eigenvalue, %	Cumulative variance	Ranked eigenvector element	Associated variable
I	68.95064	68.95064	-0.970099	pH
			0.965892	COD
			0.965891	Organic load
			0.958905	Total mal. conc.
			-0.945362	Retention time
			-0.888099	NH_3-N
			0.877328	NO_2-N
			0.163304	NO_3-N
			-0.016647	Dissolved oxygen
II	15.05570	84.00634	0.850231	Dissolved oxygen
			0.678756	NO_3-N
			-0.236609	NH_3-N
			0.236135	Retention time
			0.185514	NO_2-N
			-0.115229	Total mal. conc.
			-0.076830	COD
			-0.076829	Organic load
			-0.012952	pH
III	10.04505	94.05140	-0.701492	NO_3-N
			0.513643	Dissolved oxygen
			-0.311235	NH_3-N
			-0.187036	NO_2-N
			0.069967	COD
			0.069966	Organic load
			-0.061956	Total mal. conc.
			0.047519	pH
			0.019950	Retention time

Note: Eigenvalues and eigenvector elements together with associated variables for the first three principal components

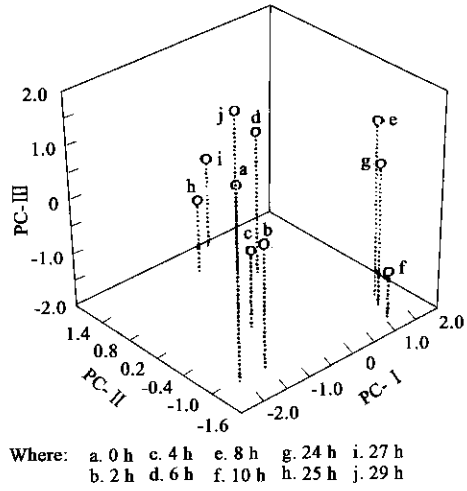

Fig.4 Three-dimensional principal components ordination of the biosimulator using 9 descriptors (see text for explanation)

The principal component II explains 15% variability which is mainly governed by DO, NO_3-N, NH_3-N and retention time (Table 4). The principal component III accounts for 10 % variability of the total variance and it is also mainly governed by NO_3-N, DO, NH_3-N and NO_2-N (Table 4). Table 5 provides the correlation coefficients between the 9 variables and the first three principal components.

3 Conclusions and recommendations

Experimental results obtained from the biosimulator revealed that the activated sludge treatment system for malathion degradation using *Pseudomonas* is more pronounced within the initial 24 h, hence making the system more economical. However, at this stage only 61% COD was removed. A critical evaluation of Table 3 would tend to favor the idea that bacterial viable density correlates to some extent with the degradation rate in the first 10 h. When the cells entered log phase and multiplying geometrically a corresponding increase in biodegradability was lacking (24—29 h). High malathion degradation rate in the initial stages (first 24 h) of bacterial growth is difficult to explain. Perhaps molecular oxygen is playing some role in oxidizing malathion thereby adding to the biological breakdown of malathion. The percentage degradation of malathion in the biosimulator indicates that the malathion degrading bacteria were also present in the system but they first adapted themselves to the malathion containing environment. However, once the culture established in the new environment rapid degradation of the malathion was observed.

The multivariate statistical method, principal component analyses exposed the groups of correlated variables and their importance in the data structure. pH, COD, organic load and TMC were highly

Table 5 Correlation coefficients between principal component and the variables

Component	Variable								
	Retention time	Dissolved oxygen	pH	Organic load	Total mal. conc.	COD	NH_3-N	NO_2-N	NO_3-N
PC-I	0.9537	0.44984	-0.9672	0.9581	0.958	0.9652	0.9352	0.9267	0.89313
PC-II	0.2582	0.5724	-0.14519	0.1719	0.2581	0.181	0.3134	0.2581	0.3928
PC-III	0.1310	-0.294	0.1321	0.176	-0.1761	0.2068	-0.2745	0.2193	0.8342

correlated with each other and emerged as the variables controlling the first component, DO, NO_3-N and NH_3-N governed the second component. The third component of PCA essentially repeated the trend exhibited by the second component. The PCA can be easily updated as new data become available. Further research is going on in this aspect to upgrade the performance efficiency of biosimulator.

(1) This research will provide new scientific dimensions for further research on remediation of pesticides by microbiological transformations.

(2) The scientific investigations and their possible practical implementation during this study would encourage the researchers for the development of new ideas related to the treatment of hazardous wastes.

(3) Further research on the role of DO on the growth of activated sludge microorganisms as well as on specific pesticide degrading pure culture would help in the better application of activated sludge system for the removal of hazardous wastes.

(4) The interacting chemical reactions taking place in the activated sludge system needs further research in order to elucidate the role of microorganisms in the chemical reactions.

(5) As the biochemical reaction for pesticide degradation depends upon extra cellular enzymes further research with cell-free extract would be worthwhile.

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