Microbial activity and diversity in the late Pleistocene palaeosols of alluvial Mahi River basin, Gujarat, western India

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The present study was aimed at establishing microbial activity and diversity in two palaeosols (P1 and P2) intercalated within the late Quaternary continental sequences of Mahi River basin, western India. The two palaeosols have been dated back to ~45 and 30 ka. High microbial biomass carbon, soil respiration, microbial quotient, metabolic quotient and dehydrogenase activity revealed that the organic matter decomposition and carbon flow to the atmosphere were much higher in P1. Soil *in situ* enzymes, specific enzymatic quotients and specific enzyme indices revealed that P1 has been more productive than P2. The order of biogeochemical cycling in P1 was N > P ≥ C, whereas P2 showed N > P > C. 16S rRNA gene profiling using denaturing gradient gel electrophoresis indicated greater heterotrophic bacterial diversity and higher carrying capacity of P1 compared to P2. The contrasting microbial activities and diversity in these palaeosols point to different environments during palaeosol formation, and are in general agreement with the climatic inferences drawn earlier.

Keywords. Palaeosol, metabolic quotient, microbial activity and diversity, specific enzymatic quotient.

PALAEOSOLS (fossil soils) are preserved throughout the geologic record and provide signatures of past climatic and environmental changes[1] as well as landscape evolution, including allogenic processes[1–5]. Several studies pertaining to palaeosols from various parts of the Indian subcontinent have been carried out[6–8]. However, studies on microbial activity and biogeochemical cycling which control ecosystem functions and shape the landscape are rare, especially from the Indian subcontinent, though elsewhere such studies have been made in great detail[9–16]. Demkina *et al.*[12] reported that spatial variability in the number of microorganisms in some of the Russian palaeosols is approximately the same or rather high compared to the surface soils. Further, they have shown that spatial variability in the respiration activity of the buried palaeosols is two to four times higher than the surface soils. It has been shown that the microbial species active at the time of formation of the palaeosols have survived till date[9]. This may be due to various adaptation mechanisms of soil microbial communities (anabiosis, transformation of bacteria into nanoforms, etc.). The microbial DNA extracted from the palaeosol correlates strongly ($r^2 = 0.97$) with the total microbial biomass[13]. The microbial communities have also been used to infer palaeoclimate for the last 5 ka (refs 11 and 16).

Most of the palaeosol microbial studies have been restricted to total microbial biomass and active microbial biomass measurements[9–16]. However microbial ecology in relation to palaeoenvironments is not yet fully understood. Very little is known about microbial diversity and the biogeochemical cycling of elements of the palaeosols. The present study was therefore aimed at the evaluation of microbial parameters of the palaeosols of the alluvial Mahi River basin, Gujarat, with a view to understand ecosystem function in association with biogeochemistry by a polyphasic approach.

Materials and methods

Study site

The site Rayka under study is about 22 km NE of Vadodara located on the left bank of the semi-arid Mahi River basin, Gujarat, western India (Figure 1 *a*). The mean annual rainfall in the area is about 600–650 mm. The site lies within the alluvial zone of the Mahi River basin[17]. About 38 m exposed section on the left bank of the Mahi River shows alluvial deposits and intercalated palaeosols (Figures 1 *b* and *c*) of late Pleistocene age[8,18,19]. A summary of the chronology (Figure 1 *b*) and inferred palaeomonsoon variability based on the fluvial and aeolian record from the Mahi basin has been well documented[20,21]. P1 has been dated to ~45 ka, whereas P2 to ~30 ka. Information on palaeoenvironments in which these palaeosols have formed is well documented[8,20,21]. The base of the sediment sequences along these cliffs is dated back to 125 ka (refs 20, 21).

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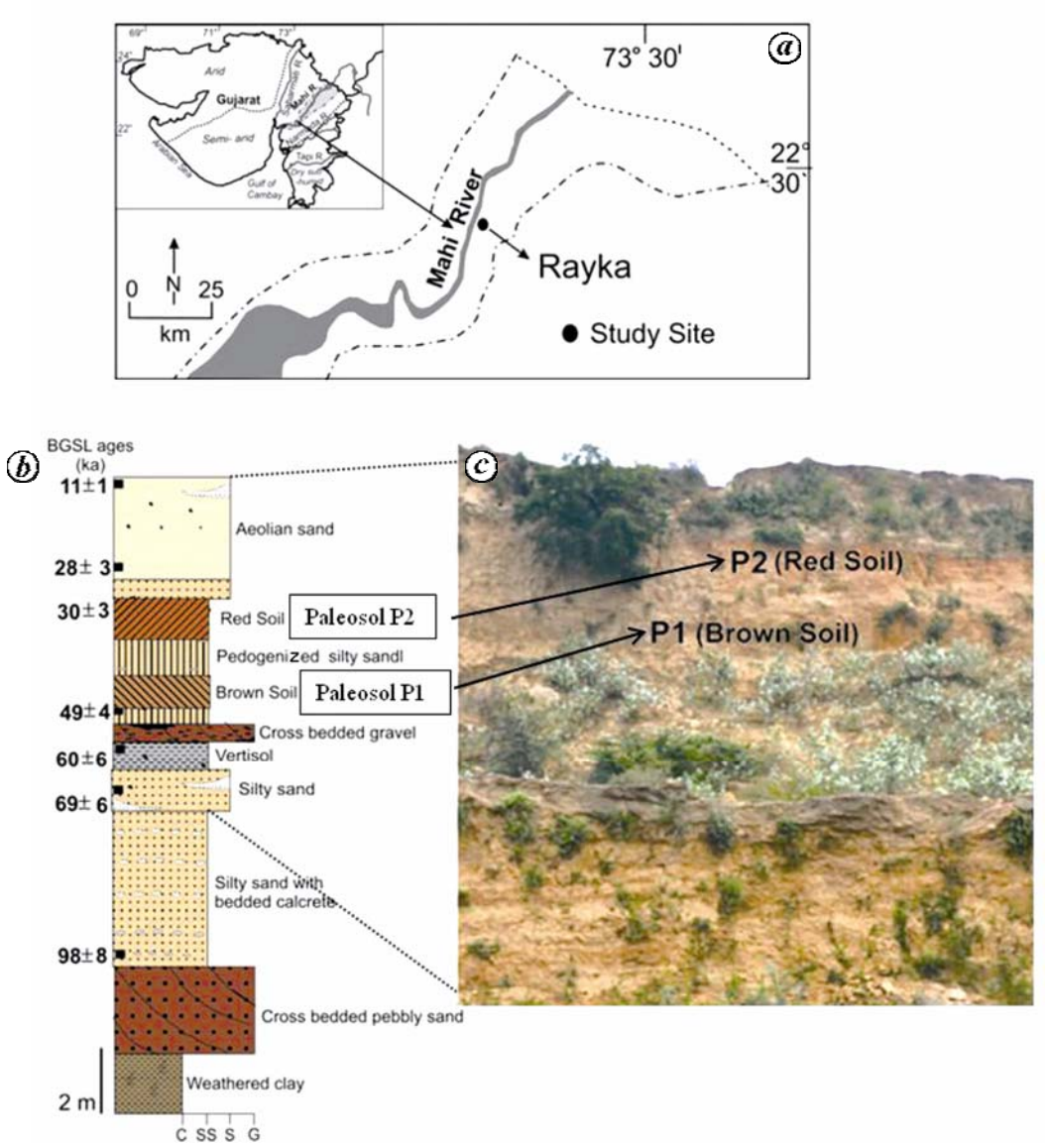

Figure 1. ***a***, Map showing location of Mahi River basin and study site; ***b***, Stratigraphic log at Rayka (after Juyal *et al.*[20,21]) and ***c***, Exposed section at Rayka showing locations of P1 and P2.

Sample collection

Triplicate composite samples of two different buried palaeosols P1 and P2 were collected from the exposed section at Rayka. Prior to the collection of soil samples, the exposed surface of the palaeosol was scraped clean. Autoclaved polythene bags were used for collecting the samples which were immediately kept in a box containing ice packs before being transported to the laboratory. All the samples were sieved (< 4 mm), cleaned of visible roots and plant residues, and stored at 4°C.

Palaeosol physico-chemical analysis

Palaeosol samples were analysed for soil moisture content, pH, soil texture and water-holding capacity following the standard protocols[22]. Soil pH was determined potentiometrically in 0.01 M $CaCl_2$ solution using glass electrode. Soil moisture was determined by oven-drying the soil samples at 105°C for 24 h. Palaeosol macro and micro elements, including soil organic C (C_{org}) were analysed at the laboratory of Gujarat State Fertilizers Corporation (GSFC), Vadodara.

Soil enzyme assays

Soil enzymes such as β-glucosidase (GSA), alkaline phosphatase (APA), and protease (PSA) involved in C, P and N cycling respectively, were estimated. APA and GSA activities were determined by incubating 0.5 g of fresh soil with substrate (*p*-nitrophenyl phosphate for APA, and *p*-nitrophenyl β-D-glucopyranoside for GSA) at

pH 8 (0.1 M maleate buffer) and 37°C. After 1 h, 0.5 ml of 0.5 M $CaCl_2$, 2 ml of 0.5 M NaOH and 5 ml of distilled water were added, the samples were filtered (Whatman No. 42), and *p*-nitrophenol in the filtrate was measured spectrophotometrically at 398 nm (refs 23 and 24). Units for APA and GSA are expressed as μg *p*-nitrophenol (pNP) g^{-1} oven-dried palaeosol h^{-1}. PSA activity was determined using the method of Ladd and Butler[25] with sodium caseinate as the substrate. Soil sample (2 g) was added to 5 ml of a 1% solution of sodium caseinate in Tris-HCl buffer (pH 8.1) and incubated for 2 h at 50°C, after which 2 ml of a 17.5% aqueous solution of trichloroacetate was added to the tubes to stop the enzymatic reaction, and then filtered. Then 2 ml of the filtrate was mixed with 3 ml of a 1.4 M aqueous solution of sodium carbonate and 1 ml of Folin–Ciocalteu phenol reagent (FC reagent), and assayed spectrophotometrically at a wavelength of 578 nm against Tris-HCl buffer (pH 8.1). PSA activity is expressed as μg tyrosine g^{-1} oven-dried palaeosol 2 h^{-1}. Soil dehydrogenase (DHA) activity actually represents instant metabolic activities of soil microorganisms[26]. DHA activity in palaeosols was measured by reduction of soluble tetrazolium salt 2,3,5-triphenyltetrazolium chloride, as artificial electron acceptor to red-coloured formazans, extracted and estimated colorimetrically[27]. The DHA activity is expressed as μg triphenyl tetrazolium formazan (TPF) g^{-1} oven-dried palaeosol 24 h^{-1}. All determinations of enzyme activities were performed in triplicates and values reported as means with standard deviation.

Palaeosol microbiological analysis

Palaeosol microbial biomass C (C_{mic}) was determined by the chloroform fumigation extraction method[28]. Palaeosol basal soil respiration (BSR) was determined by measuring CO_2 evolution[29]. In brief, field-moisture soil samples (equivalent to 20 g oven-dried soil) were placed in gauze and incubated in 500 ml air-tight glass vessels at 30°C for 48 h. The CO_2 evolved from the soil was absorbed in 15 ml 0.1 M NaOH and the unconsumed base titrated with 0.1 M HCl following additions of $BaCl_2$. Glucose at final concentration of 10 mg/g of palaeosol sample was used in substrate-induced respiration (SIR) and the procedure followed as above.

In order to evaluate the palaeosol microbial ecophysiology, the ratios between microbiological parameters have been used in the present study. Ecophysiological indices such as microbial quotient (C_{mic}/C_{org}) and metabolic quotient (q-CO_2) were measured[30]. Microbial quotient was calculated by expressing C_{mic} as a percentage of total soil C_{org} (ref. 31). The microbial q-CO_2 was determined by dividing basal respiration (mg CO_2–C g^{-1} dry soil h^{-1}) by microbial C (g C_{mic} g^{-1} dry soil)[32]. For evaluating the significance of microbial populations and microbial activity in the cycling of elements, specific enzymatic quotients (SEQs) were determined in the present study. SEQs for DHA, APA, PSA and GSA were calculated by dividing enzymatic activities per unit time by C_{mic} (enzyme activity g^{-1} palaeosol per unit time g C_{mic} g^{-1} dry soil). Specific enzyme activities for unit total C_{org} were also determined. All determinations of enzymatic activities were performed in triplicate and values are reported as means with standard deviation. Total viable heterotrophic bacteria of palaeosols were enumerated on R_2A agar[33]. In brief, 10 g of moist sieved (4 mm) palaeosol was homogenized in 90 ml of 0.85% (wt/vol) saline and serially diluted (10 fold) in the same. Aliquots (100 μl) were spread on triplicate plates of R_2A plates, incubated at 30°C for 2 days, and the colonies were counted.

Microbial diversity studies of palaeosols by denaturing gradient gel electrophoresis

Cultivable heterotrophic bacterial diversity was established in palaeosols of Mahi River basin by PCR–16S rRNA-based denaturing gradient gel electrophoresis (DGGE)[34]. In brief, 10 g of palaeosol was taken in 90 ml of R_2A broth and allowed to incubate for 2 days at room temperature. The community DNA of heterotrophic bacteria was extracted[35] and checked for purity and molecular size using conventional agarose gel electrophoresis. Universal eubacterial primers[34] GC-341F and 534R were used for the amplification of 16S rRNA gene fragments suitable for DGGE. Conditions for PCR were 95°C for 10 min, followed by 18 cycles of 95°C for 1 min, 59°C (reduced 0.5°C during each cycle up to 55°C) for 45 s, and 72°C for 45 s followed by 20 cycles of 95°C for 1 min, 55°C for 45 s, and 72°C for 45 s, and a final extension at 72°C for 5 min. The resulting PCR products were directly subjected to DGGE. The DCode system for DGGE (Bio-Rad Laboratories Ltd, UK) was used. PCR-amplified samples were loaded onto 8% polyacrylamide–bisacrylamide gels with denaturing gradient from 40% to 60% where 100% denaturant was 7 M urea and 40% (vol/vol) deionized formamide with 1× TAE as electrophoresis buffer. Electrophoresis conditions were 60 V for 12 h at 60°C. Gels were stained with 0.5 mg/ml silver nitrate and visualized. Band patterns were analysed with AlphaEase FC software (Alpha Innotech, CA). The diversity of the microbial community was expressed as the Shannon index of general diversity (*H*). *H* was calculated on the basis of the bands on the gel tracks, using the densitometric curves wherein the intensity of the bands was reflected as peak heights. The equation for the Shannon index is as follows: $H = -\sum(ni/N)\log(ni/N)$, where *ni* is the height of the peak and *N* the sum of all peak heights of the densitometric curve. The range-weighted richness (*Rr*), reflecting the carrying capacity of the palaeosols

were determined[36]. *Rr* is the total number of bands multiplied by the percentage of denaturing gradient needed to describe the total diversity of the sample analysed, according to the formula $Rr = (N2 \times Dg)$, where *N* represents the total number of bands in the pattern, and *Dg* denaturing gradient between the first and the last band of the pattern[36].

Results and discussion

Juyal *et al.*[20] suggested that during the last 125 ka, the fluvial sedimentation pattern in the Mahi basin changed frequently. The variation in the nature of sediments provides a clue to understand changes in the precipitation regime and fluctuating water budget in the basin that receives the southwest monsoon. The palaeosols P1 and P2 are estimated to have an age difference of about 15,000 years. P1 is the older, well-pedogenized, silty brown soil, whereas P2 is a red alfisol.

Palaeosol physico-chemical analysis

Physico-chemical characteristics of palaeosols are important to understand the soil productivity[1–3]. Table 1 gives the physico-chemical properties of P1 and P2. The lower palaeosol (P1) dating back to ~45 ka has high soil organic C, clay content and soil water-holding capacity compared to the upper palaeosol (P2). The Fe content is found to be more in P2 rendering red colour to the palaeosol[20]. The physico-chemical characteristics of the two palaeosols provide insight into the differences in quality of the past landscapes during 45 and 30 ka respectively.

Palaeosol microbial analysis

Soil organic matter (SOM) decomposition rates can be understood as a function of three parameters: microbial biomass, microbial respiration rate and microbial enzymes[28,37]. Soil C_{mic} is the living component of SOM[38], and it generally constitutes about 1–5% of the total organic matter[39]. Relatively high C_{mic} (Figure 2 *a*) was found in P1 than P2, which indicates higher bacterial distribution per gram of P1 palaeosol. This is corroborated by total viable bacterial counts (Table 2). Microbial quotient is the ratio of biomass C to soil C_{org}, which reflects the contribution of microbial biomass to soil C_{org}[31]. A slightly higher microbial quotient was found in P2 (Table 2).

Microbial activity measurements include respiration, measurement of metabolic CO_2 and general evaluation of soil enzymatic activity involved in transformations of elements. It is known that the soil CO_2 emission rate is determined by microbial activity and is considered as the basic portion of the total carbon flow to the atmosphere[40]. The microbial activity related to the mineralization of palaeosol organic matter was estimated on the basis of CO_2 evolution rate (Figure 2 *b* and *c*). Results show that P1 had a greater BSR activity than P2, suggesting that the soil microorganisms in P1 have higher availability of SOM for respiration and also the capability of SOM breakdown compared to those in P2.

In general, soils receive exogenous substrates at regular intervals during the course of landscape evolution. The organic substrates introduced into the soil may enhance or reduce the microbial mineralization of SOM, that is defined by positive or negative priming effect (PE). Positive PE is considered as one of the factors

Table 1. Physical and chemical parameters of Rayka palaeosols

Physical parameters				Soil texture		
Palaeosol	Soil moisture content (%)	Soil dry mass (%)	Soil water-holding capacity (%)	Sand (%)	Silt (%)	Clay (%)
P1	12	88	12	40.5	51	8.5
P2	6	94	13	44	50.2	5.8

Chemical parameters								
Palaeosol	Organic carbon (%)	pH	Electrical conductivity	S (ppm)	Zn (ppm)	Fe (ppm)	Mn (ppm)	Cu (ppm)
P1	0.61	8.1	0.41	26.40	1.10	4.8	3.82	3.66
P2	0.47	7.6	0.44	16.9	0.58	6.2	2.32	0.46

Table 2. Total viable count, empirical indices such as microbial quotient, metabolic quotient and diversity indices like species richness and evenness, and range-weighted richness of Rayka palaeosols

Palaeosol	Total viable count (10^5 cells/g^{-1} of soil)	Microbial quotient (C_{mic}/C_{org}) (%)	Metabolic quotient (q-CO_2) (μg C mg C^{-1}_{mim} h^{-1})	Species richness (*H*)	Species evenness (*E*)	Range weighted richness (*Rr*)
P1	6.6 ± 1.52	4.43 ± 0.15	11.54 ± 0.484	2.66	2.20	20.2
P2	4.2 ± 2.08	4.30 ± 0.21	7.49 ± 0.81	2.28	2.13	10.12

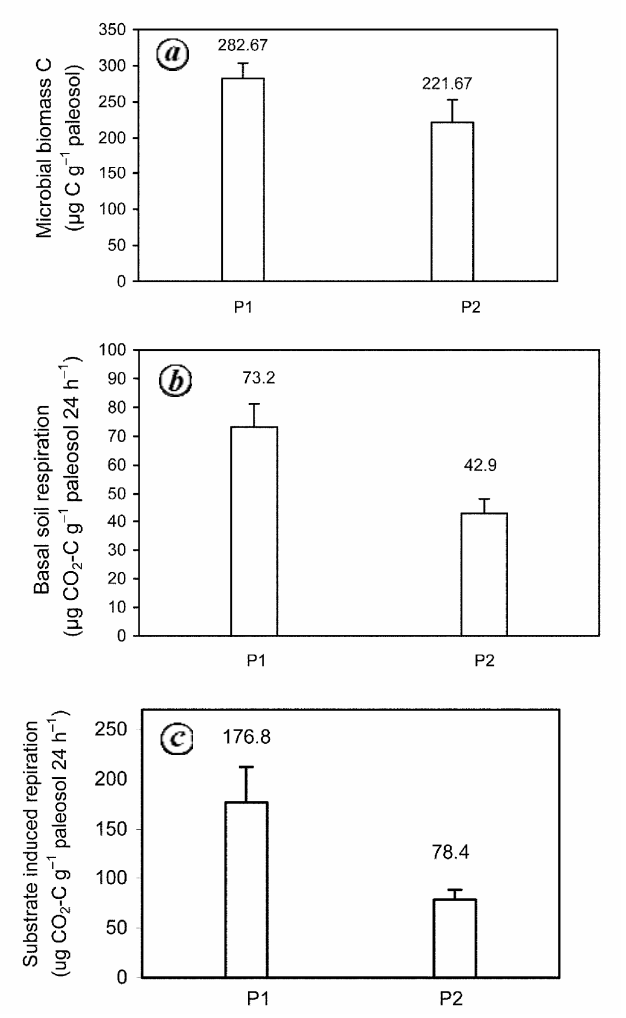

Figure 2. Soil microbial activity in the Rayka palaeosols P1 and P2. ***a***, Microbial biomass C; ***b***, Basal soil respiration, and ***c***, Substrate-induced respiration.

escalating the atmospheric CO_2 concentration[41]. In order to evaluate the microbial response and the concurrent PE as a result of addition of exogenous substance, SIR was monitored with readily the utilizable substrate, glucose. Although carbon mineralization rates increased in both soils, the increase was relatively higher (by 141.53%) in P1 compared to that in P2 (by 82.8%). Overall, P1 had high SIR activity (Figure 2 *c*) than P2. Based on the values of both BSR and SIR, it can be inferred that the microbes in P1 have greater potential in SOM decomposition and carbon flow to the atmosphere[40]. The composition of the microbial communities and in particular the competition between K-strategists and r-strategists is used for determining the priming effects[40]. The input of an easily metabolized substrate may favour the growth of r-strategists in comparison to K-strategists, since the r-strategists start to grow within hours and utilize the major portion of such substrates. Hence, the greater SIR activity associated with P1 is a likely response of r-strategists[40]. Activity of r-strategists can reflect the nutritional status of the environment; such activity was lower in P2 due to oligotrophic nature. The microbial potential in decomposition of organic matter was elucidated by metabolic quotient (Table 2). Based on the respiration rates as well as q-CO_2, we infer that the SOM decomposition is relatively higher in P1 than P2.

Soil microbial enzymes play a key role in the decomposition of organic residues and are considered fundamentally good indicators of soil quality[42]. Several enzymes are known to be involved in the cycling of nutrients and can be used as potential indicators of nutrient cycling processes[43]. DHA is associated with living cells, linked with microbial oxidoreduction processes and is the principal agent in the degradation of SOM[22,44]. Remarkably high amount of DHA activity was found in P1 (Figure 3 *a*) and is in general agreement with high microbial respiration activity, indicating that organic matter decomposition is much active in P1.

To evaluate the fluxes of elements in palaeosols, hydrolytic enzymes such as GSA, PSA and APA involved in C, N, P cycles respectively[45,46] were determined for the palaeosols (Figure 3). Hydrolase-enzyme activities indicate the soil potential to carry out specific biochemical reactions and also maintain soil fertility[47]. It was found that all the three enzymes were relatively high in P1 (Figure 3 *b–d*), indicating that the microbially mediated C, N and P cycle fluxes are higher in P1 dated to ~45 ka. SEQ give an idea about the microbial role in biogeochemical cycles. The order of biogeochemical cycling in P1 is $N > P \geq C$, whereas for P2 it is $N > P > C$. The same trend was observed with specific enzymatic indices (SPIs) calculated by unit C_{org} (Table 3). Both SEQ and SPI were relatively high in P1, indicating good soil quality and health.

Recently, we reported microbial activity in relation to soil physical and chemical properties in the present-day (surface) soils of the Mahi River basin[48]. DHA activity of the surface soils of Rayka showed strong correlation with SOM and soil moisture content; however PSA activity did not correlate with any of the soil parameters studied. The studies reported here show similar trend in the buried palaeosols of Rayka, where higher organic matter and moisture content (in P1) was concurrent with higher enzyme activity. The results indicate that the physicochemical properties of the soils remain the main determinants of microbial activity regardless of the age of the soil.

Molecular diversity fingerprinting of palaeosols by DGGE

DGGE is a well-established molecular tool in microbial ecology[49]. This fingerprinting technique provides a molecular profile on polyacrylamide gel representing the genetic

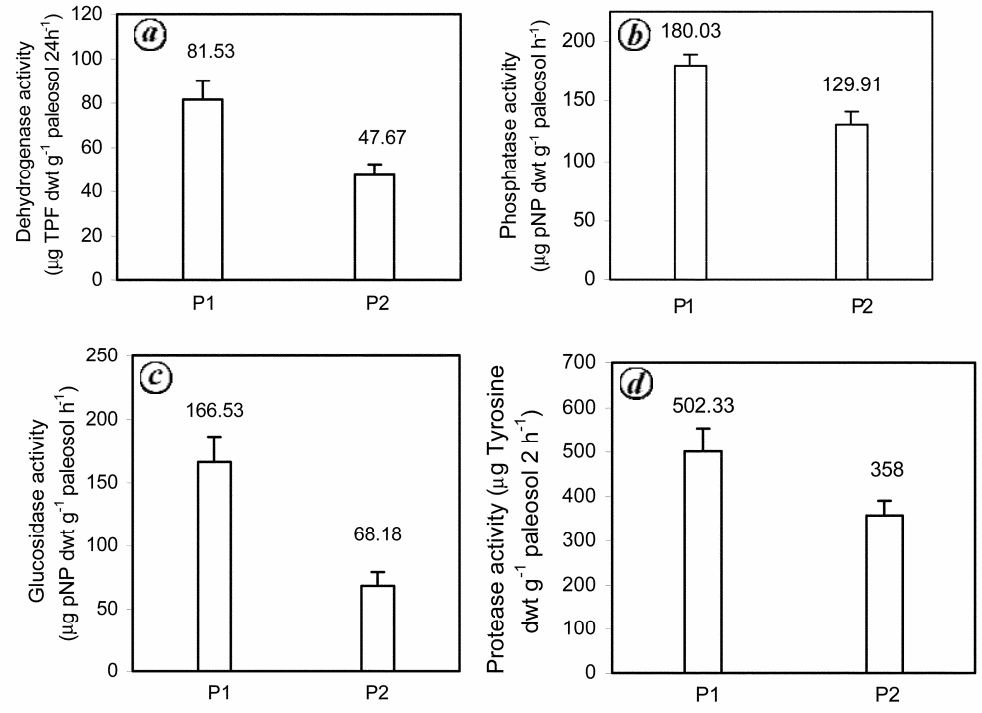

Figure 3. Soil microbial enzyme activity in P1 and P2. ***a***, Dehydrogenase; ***b***, Phosphatase; ***c***, Glucosidase, and ***d***, Protease.

Table 3. Specific enzymatic quotients (SEQs) and specific enzymatic indices (SPIs) for the Rayka palaeosols. SEQ was calculated per unit microbial biomass (C_{mic}), whereas SPI was calculated per unit soil organic carbon (C_{org})

Palaeosol	SEQ–DHA	SEQ–APA	SEQ–GSA	SEQ–PSA	SPI–DHA (%)	SPI–APA (%)	SPI–GSA (%)	SPI–PSA (%)
P1	0.288 ± 0.02	0.635 ± 0.03	0.59 ± 0.07	0.885 ± 0.08	1.33 ± 0.13	2.94 ± 0.15	2.72 ± 0.32	3.74 ± 0.34
P2	0.211 ± 0.02	0.583 ± 0.05	0.308 ± 0.04	0.80 ± 0.06	0.89 ± 1.01	2.76 ± 0.25	1.44 ± 0.22	3.80 ± 0.30

SEQ–DHA, µg TPF 24 h^{-1} µg C_{mic} g^{-1} dry wt palaeosol; SEQ–APA, µg pNP h^{-1} µg C_{mic} g^{-1} dry wt palaeosol; SEQ–GSA, µg pNP h^{-1} µg C_{mic} g^{-1} dry wt palaeosol; SEQ–PSA, µg Tyrosine 2 h^{-1} µg C_{mic} g^{-1} dry wt palaeosol; SPI–DHA, µg TPF 24 h^{-1} g^{-1} C_{org} g^{-1} dry wt palaeosol; SPI–APA, µg pNP h^{-1} g^{-1} C_{org} g^{-1} dry wt palaeosol; SPI–GSA, µg pNP h^{-1} g^{-1} C_{org} g^{-1} dry wt palaeosol; SPI–PSA, µg Tyrosine 2 h^{-1} g^{-1} C_{org} g^{-1} dry wt palaeosol. DHA, Dehydrogenase; APA, Alkaline phosphatase; GSA, β-Glucosidase, and PSA, Protease.

structure and diversity of a microbial community from a specific environment, with a high versatility, reliability and reproducibility[36]. Each band on the denaturing gradient gel represents a specific 16S rDNA sequence from a specific microbial species or ribotypes, which can be enumerated or identified by the band positions on the gel and their abundance estimated from the band intensities. In the present study DGGE of the 16S rRNA gene was used to compare the heterotrophic bacterial communities of palaeosols (Figure 4). Relatively more number of bands were observed in P1 than in P2, indicating high heterotrophic bacterial diversity in P1. Diversity indices *H* and *E* were relatively high in P1 than in P2 (Table 2). Range weighted richness (*Rr*)[36] value for P1 was found to be 20.22, whereas for P2 it was found to be 10.12, allowing the inference that P1 has a broad carrying capacity so that the system supports the growth of a diverse group of bacteria[36], about two times greater than P2. Bands numbered 3, 4, 6–9 were common in both the palaeosols (Figure 4), indicating distribution of these ribotypes in both. Bands numbered 1, 2 and 5 (Figure 4) are highly specific to P2 and these bands were virtually absent in P1. Bands denoted as a–g are specific to P1 (Figure 4) and are absent in P2, indicating development and maintenance of distinct species in the two soils during the course of time.

Overall the microbial distribution and activity in these palaeosols seem to have depended on the soil physico--chemistry[1–3]. The changes in the climate from arid to humid are reflected in the structure of the microbial communities[50]. The data are consistent with monsoonal data from the area[20]. P1 has been productive and points to high monsoonal conditions[20]. An increase in the active biomass of the microorganisms, total microbial biomass and C_{org} is observed in humid climatic conditions[50]. Microorganisms consuming plant residues show an increased ecological–trophic structure in the microbial community and a decrease in the index of oligotrophy[49]. Microbial activities such as C_{mic}, C_{org}, q-CO_2 and lower oligotrophy

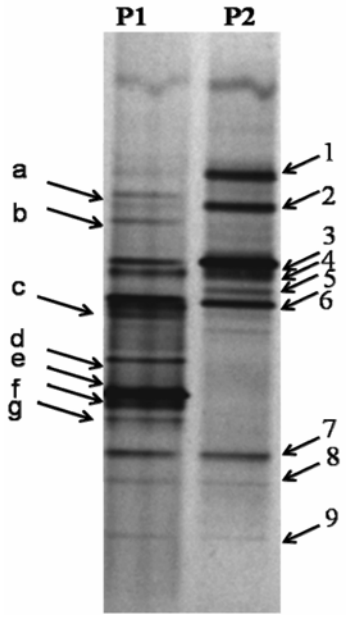

Figure 4. 16S rRNA molecular microbial diversity profiling of P1 and P2 using denaturing gradient gel electrophoresis.

of microorganisms suggest an increasing input of plant material to the soil. In arid regions, such as the Lower Mahi River basin, increase in the plant biomass could be explained by higher precipitation during ~45 ka (ref. 20). P2, however, shows lower values of microbial quantitative indices and an increase in the microbial oligotrophy, and could be explained by lower precipitation during ~30 ka (ref. 20).

Conclusion

The following conclusions may be drawn from this study:

1. Organic matter decomposition and carbon flow to the atmosphere are much higher in the P1 brown palaeosol dated to ~45 ka.
2. Soil *in situ* enzymes, SEQ and SPI reveal greater productivity and quality of P1 (~45 ka) than P2 (~30 ka).
3. The order of microbial activity in biogeochemical cycling in P1 was found to be $N > P \geq C$, whereas in P2 it was $N > P > C$.
4. P1 showed high heterotrophic bacterial diversity and broader carrying capacity compared to P2.
5. Contrasting microbial activity and diversity in these palaeosols indicate two different environments and are in agreement with climatic inferences drawn by earlier workers[20,21].

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