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ANTIBIOTIC-RESISTANCE PATTERNS AND RELATIVE CONCENTRATIONS OF BACTERIA (GRAM-NEGATIVE RODS) FROM ASH DEPOSITS OF VARIOUS AGES ON THE KRAKATAU ISLANDS

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The Krakatau islands Rakata, Sertung and Panjang, have been colonized by plants, animals and microorganisms over about a century, since the area was probably sterilized by the eruptions of August 1883.

In 1930 the island of Anak Krakatau appeared and has since grown subaerially by periodic volcanic eruptions. Parts of this island may have been sterilized by ash eruptions in 1952 and 1953, and since 1962 lava flows have added new land surfaces to the island, the most recent being in 1980. At the northern end of Sertung Island, a long, narrow, sand spit built of eroded volcanic debris provides a land surface that is only a few decades old.

These very new land habitats on Anak Krakatau and the Sertung spit, when

examined for antibiotic-resistance patterns (resistotypes) of soil bacteria (Gram-negative rods, GNR), were shown to contain GNR much less antibiotic-resistant than those from the older habitats of Sertung on which over 100 years of post-eruptive colonization and succession has been possible. The concentration of soil micro-organisms was also considerably less in these very young land habitats; only where vegetation had become established were soil GNR significantly resistant to antibiotics and soil microbial concentrations similar to those in the older habitats of the archipelago.

1. Introduction

The volcanic eruptions of the Indonesian island of Krakatau in 1883 culminated in several cataclysmic explosions on August 26 and 27 that resulted in the loss of over two thirds of the island (Thornton & Rosengren 1988). The surviving remnant of Krakatau (now known as Rakata) and the adjacent islands of Panjang and Sertung (figure 1) were buried beneath tens of metres of hot volcanic ejecta consisting of rock fragments, pumice and fine volcanic ash. Scientists visiting the islands two months later experienced difficulty in walking on the new surface because of the heat it retained, and it is likely that the islands were sterilized by this enormous volcanic event. In the 103 years that have elapsed since that eruption, the islands have been recolonized by plants and animals (Thornton 1984).

The islands are uninhabited but landings are made frequently by fishermen who take timber, water, pumice and turtle eggs. Occasional parties of tourists may stay for 1 or 2 days and groups of scientists may camp on the islands for periods of up to several weeks.

Many landform changes have taken place in the Krakataus since 1883 and the volcanic ash

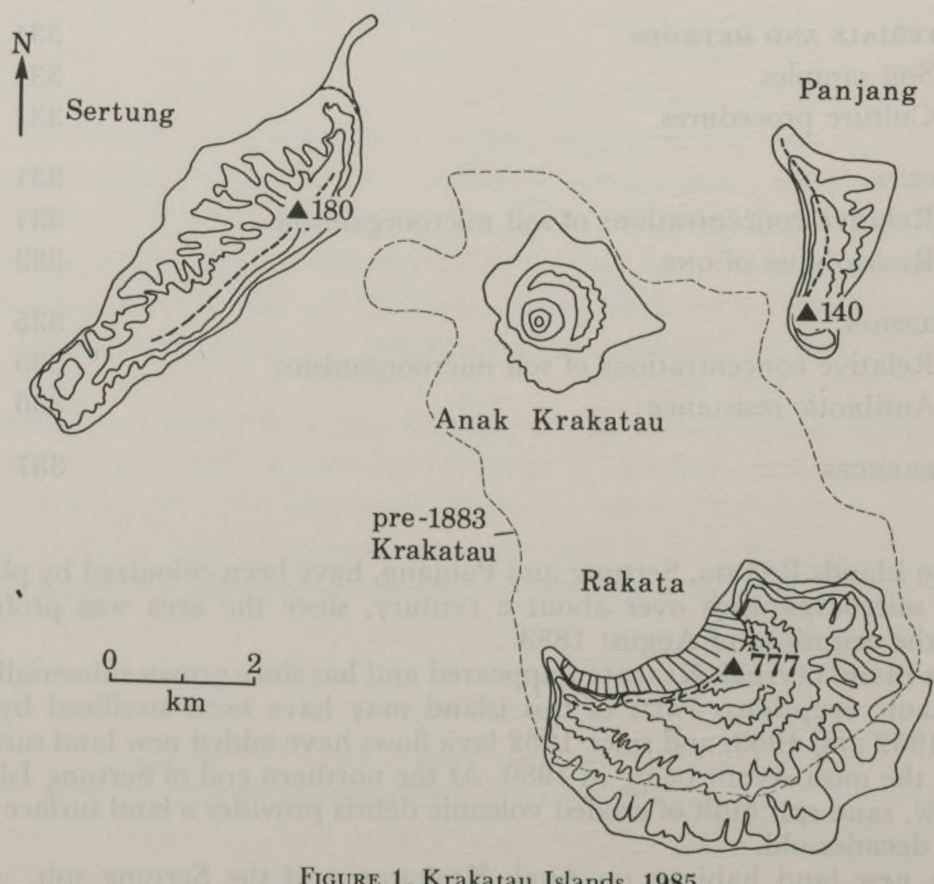

Figure 1. Krakatau Islands, 1985.

deposited by the 1883 eruption has begun to break down to form soils. Marine erosion and deposition has reshaped the coastline of the islands, one of the most notable changes being the growth of a long, tapering, sand spit at the northern end of Sertung (figure 2). This unstable feature has been breached by storm-wave action (and thereby shortened) at least once over the past 35 years. Wave erosion causes the exposed western coastline to retreat steadily, but accretion of sand at roughly the same rate along the eastern coastline leads to a steady migration of the spit eastwards: it is now about 600 m east of its position in 1946. Hence the terrain and vegetation of the spit is very young, with no part being older than 15–20 years (Rosengren 1985), and contrasts markedly with the main body of the island, which is covered in secondary forest (Thornton & Rosengren 1988). Soil samples from both the 'old' (hinterland) and 'new' (spit) areas of Sertung were compared to determine the antibiotic-resistance patterns of soil bacteria (Gram-negative rods, GNR) and concentrations of soil microorganisms.

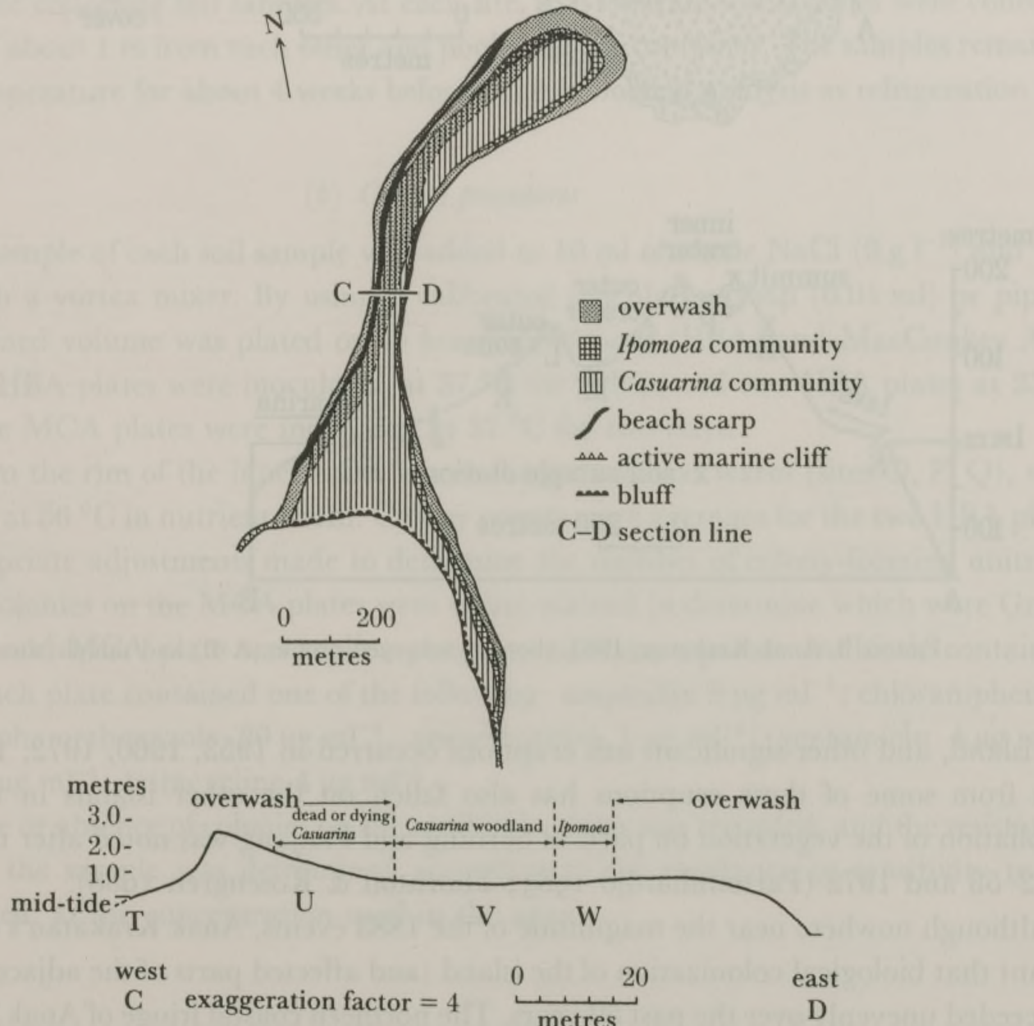

FIGURE 2. North Sertung, 1985, showing surveyed profile (C–D) and sample sites T–Z.

The volcanic island of Anak Krakatau first emerged in late 1927 as a result of submarine eruptions near the site of one of the destroyed vents of the former island of Krakatau. Since 1930 frequent eruptions have built a cone of ash and lava almost 200 m high, and several lava flows form a broad rampart on the western and southern coasts of the island (figure 3). The ash eruptions produce varied deposits, the commonest being angular fragments less than 4 mm in diameter. Eruptions in October 1952 deposited up to 3 m of this type of ash over much of

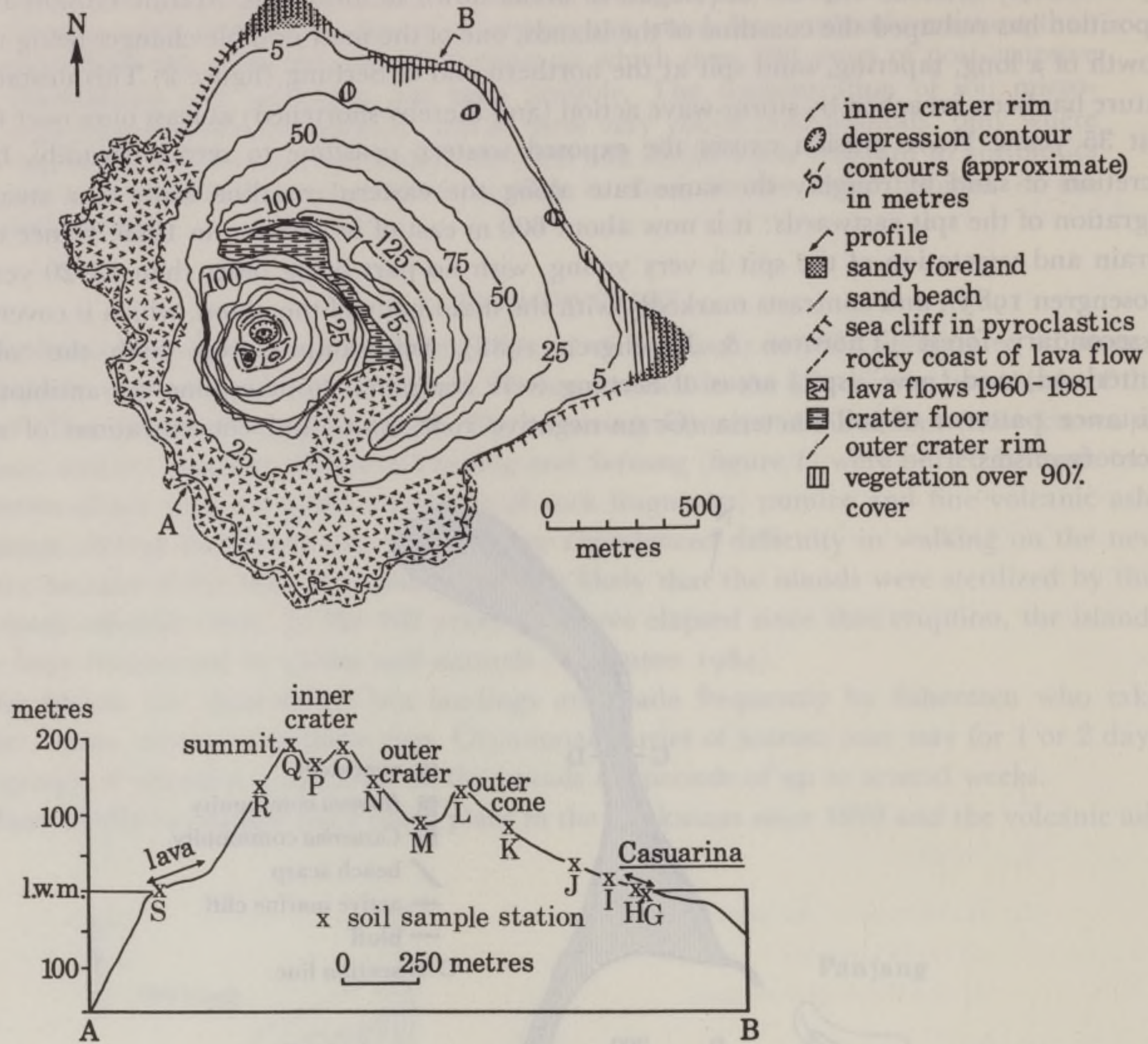

Figure 3. Anak Krakatau, 1985, showing surveyed profile (A–B) and sample sites G–S.

the island, and other significant ash eruptions occurred in 1953, 1960, 1972, 1975 and 1981. Ash from some of these eruptions has also fallen on the other islands in the group and defoliation of the vegetation on parts of Sertung and Panjang was noted after the eruptions of 1952–53 and 1972 (Partomihardjo 1983; Thornton & Rosengren 1988).

Although nowhere near the magnitude of the 1883 events, Anak Krakatau's eruptions have meant that biological colonization of the island (and affected parts of the adjacent islands) has proceeded unevenly over the past 50 years. The northern coastal fringe of Anak Krakatau may have changed little in outline since 1953, but the surface material here, as over most of the island, is no older than 10–20 years. The slopes adjacent to the active vents are built of ejecta that is only 1–5 years old. The development of soil bacteria (GNR) with respect to both the concentration and antibiotic resistance of soil bacteria (GNR) of 'new' and 'very new' areas of Anak Krakatau was compared.

2. Materials and methods

(a) *Soil samples*

Soil samples were collected in August 1985 when three of the authors (S.R.G., N.J.R. & I.W.B.T.) visited the islands as members of the La Trobe University Zoological Expedition. Soil sampling was done on Anak Krakatau along a surveyed transect that extended from the lower, older, vegetated slopes of the northeast, crossed the highest and youngest ash slopes of the active crater and descended to the 1979 lava flows on the southwest flank of the island. Elevation and distance were measured along the transect by theodolite and the resulting profile and position of 13 sample stations is shown in figure 3. Soil samples were collected at seven sites on Sertung (T to Z on figure 2), five on the spit and two in the mixed secondary forest of the main part of the island. Brief descriptions of site characteristics are given in tables 2 and 3.

Sterile plastic screwtop containers with a built-in sterile spatula attached to the inside of the lid were used for collecting soil samples. At each site, five separate soil samples were collected at a distance of about 1 m from each other and pooled in one container. The samples remained at ambient temperature for about 4 weeks before microbiological analysis as refrigeration was not available.

(b) *Culture procedures*

A 0.2 g subsample of each soil sample was added to 10 ml of sterile NaCl (9 g l^{-1}) and well suspended with a vortex mixer. By using a calibrated inoculating loop (0.01 ml) or pipette (0.1 ml) a defined volume was plated on to horse blood agar (HBA) and MacConkey Agar (MCA). Two HBA plates were inoculated at 37 °C for 2 days and two HBA plates at 25 °C for 5 days. The MCA plates were incubated at 37 °C for two days.

Samples from the rim of the inner cone, where the ground was warm (sites O, P, Q), were also incubated at 56 °C in nutrient broth. Colony counts were averages for the two HBA plates and the appropriate adjustments made to determine the number of colony-forming units per gram of soil. Colonies on the MCA plates were Gram-stained to determine which were Gram-negative rods, and MCA plate was replica-plated on to seven separate antibiotic-containing agar plates. Each plate contained one of the following: ampicillin 8 μg ml^{-1}; chloramphenicol 8 μg ml^{-1}; sulphamethoxazole 20 μg ml^{-1}; trimethoprim 1 μg ml^{-1}; gentamicin 4 μg ml^{-1}; cephalothin 8 μg ml^{-1}; tetracycline 4 μg ml^{-1}.

The presence or absence of colonies on the antibiotic plates was recorded, and the resistotype of the GNR in the sample was determined according to the resistance or sensitivity to the antibiotics tested, at the concentration used in the agar.

3. Results

(a) *Relative concentrations of soil microorganisms*

The relative concentration of soil microorganisms, as measured by growth on HBA at 25 °C and 37 °C, was related to the degree of vegetational cover.

On Anak Krakatau (table 1), the highest microbial soil densities were at sites H and I, which were well vegetated, whereas the lower densities were on the cone of the volcano (sites N, O, P, Q, R) where there was virtually no plant growth.

On Sertung also (table 2), the centre of the spit (site V) and the hinterland sites (Y, Z),

Table 1. Relative concentrations and antibiotic-resistance patterns (resistotypes) of soil bacteria (Gram-negative rods) from Anak Krakatau

Anak Krakatau site code	site description	elevation m	resistotypes of Gram-negative rods isolated[a]	number of antibiotic resistance factors per resistotype[a]	10^{-3} × concentration of microorganisms per gram of soil[b] incubated at 25 °C	incubated at 37 °C	comments
G	northeast coast, volcanic sand	LWM	c z	6 0	45	17	continuously washed by waves
H	northeast coast, pumice fragments and volcanic ash	HWM	xx z	1 0	250	260	considerable vegetation
I	*Casuarina* woodland with large trees up to 30 years old	13	a c	7 6	240	190	considerable vegetation
J	upper edge of coastal vegetation; mosses, ferns, *Saccharum spontaneum*, nearby casuarinas	35	d jj 11	6 5 4	39	27	some vegetation
K	slope of outer cone, sparse *S. spontaneum*	91	y	1	120	63	some vegetation
L	summit outer cone	135	p v	3 2	31	30	no vegetation
M	crater floor white ash and coarser black scoria	90	z	0	30	20	very few plants
N	slope of inner, active cone, very occasional ferns	129	z	0	7	1	vegetation absent or very sparse
O	north rim inner cone, ground warm to hot, tumarols	193	no Gram-negative rods detected		9	3[c]	no plants
P	crater floor of least active summit crater, hard mud	145	no Gram-negative rods detected		3	no growth[c]	no plants
Q	south rim inner cone, tumarols, ground very warm	196	z	0	2	1[c]	no plants
R	southwest slope inner cone	138	z	0	21	6	no plants
S	1979–80 lava flow with thin ash cover, occasional ferns	14	z	0	6	no growth	vegetation very sparse

[a] See table 4.
[b] Growth on horse blood agar.
[c] No growth at 56 °C.

which were heavily vegetated, had concentrations of soil microorganisms that were significantly higher than the less vegetated sites (T, U, W, X).

When sites are grouped into 'vegetated' or 'non-vegetated' (table 3) an approximate 10-fold difference in soil microbial concentrations is apparent; samples from vegetated sites contained, on average, 295×10^3 colony-forming units per gram of soil, and non-vegetated sites, on average, 26×10^3 colony-forming units per gram of soil.

No thermophilic bacteria (detectable by growth in nutrient broth at 56 °C) were detected

TABLE 2. RELATIVE CONCENTRATIONS AND ANTIBIOTIC-RESISTANCE PATTERNS (RESISTOTYPES) OF SOIL BACTERIA (GRAM-NEGATIVE RODS) FROM SERTUNG

Sertung site code	location of site	resistotypes of Gram-negative rods isolated[a]	number of antibiotic resistance factors per resistotype[a]	10^{-3} × concentration microorganisms per gram of soil[b] incubated at 25 °C	incubated at 37 °C	comments
T	LWM, west side spit	yy z	1 0	42	39	continuously washed by waves
U	HWM, west side spit, coarse pumice, beach litter	z	0	45	47	mainly dead or dying casuarinas
V	1–2 m, centre of spit, *Casuarina* woodland	r	3	400	300	considerable vegetation
W	HWM, east side spit, *Ipomoea* beach litter	z	0	60	60	some vegetation
X	LWM, east side spit, low wave energy beach	z	0	75	37	continuously washed by waves
Y	*ca.* 50 m, hinterland, weathered 1883 ash deposits, mixed secondary forest	f s	5 3	750	500	heavily vegetated
Z	*ca.* 80 m, as Y	ee	6	500	500	heavily vegetated

[a] See table 4.
[b] Growth on horse blood agar.

in soil at the summit of Anak Krakatau (sites O, P, Q) although ground surface heat was sufficient to penetrate boot soles if one remained stationary for a few minutes.

(*b*) *Resistotypes of* GNR

Overall, 15 different antibiotic-resistance patterns (defined as resistotypes) were detected (table 4). They ranged from 'a' (resistant to all seven antibiotics) to 'z' (sensitive to all). Resistotype 'z' was by far the most frequently detected, being isolated from samples from 12 out of 20 sites. Resistotype 'c' was detected at two sites; all other resistotypes were detected at one site only.

On Anak Krakatau the more resistant GNR (i.e. resistant to a number of antibiotics tested) were detected in soil close to growing plants (sites I and J) whereas the least-resistant GNR (especially resistotype 'z') were detected at sites devoid of plant growth (sites G, L, M, N, Q, R, S) (table 1). A similar phenomenon was observed on Sertung, where the well-vegetated sites (V, Y, Z) contained GNR that were more antibiotic resistant than those from the poorly vegetated sites (T, U, W, X) (table 2).

When all sites are grouped into 'vegetated' or 'non-vegetated' and the resistotypes of GNR compared (table 3), a correlation is seen between extent of vegetation and the range of antibiotic resistance of the soil GNR. Resistant GNR were associated with soil in which plants were growing. The average number of antibiotic-resistance factors per isolate of GNR from

Table 3. Comparison of vegetated and non-vegetated sites on Sertung and Anak Krakatau

vegetated sites: site	resistotype of Gram-negative rod[a]	number of antibiotic-resistance factors per resistotype[a]	10^{-3} × concentration of microorganism per gram soil[b] 25 °C	37 °C	non-vegetated sites: site	resistotype of Gram-negative rod[a]	number of antibiotic-resistance factors per resistotype[a]	10^{-3} × concentration of microorganism per gram soil[b] 25 °C	37 °C
		Anak Krakatau					Anak Krakatau		
H	xx	1	250	260	G	c	6	45	17
	z	0				z	0		
I	a	7	240	190	L	p	3	31	30
	c	6				v	2		
J	d	6	39	27	M	z	0	30	20
	jj	5							
	ll	4			N	z	0	7	1
	z	0							
K	y	1	120	63	O	no GNR isolated		9	3
					P	no GNR isolated		3	no growth
					Q	z	0	2	1
					R	z	0	21	6
					S	z	0	6	no growth
		Sertung spit					Sertung spit		
V	r	3	400	300	T	yy	1	42	39
						z	0		
W	z	0	60	60	U	z	0	45	47
		Sertung hinterland							
Y	f	5	750	500					
	s	3							
Z	ee	6	500	500					
average: z = 3/14 (21 %)		3.4	295	238	average: z = 9/13 (69 %)		0.9	26	17

[a] See table 4.
[b] Growth on horse blood agar.

vegetated sites was 3.4 compared with 0.9 for the isolates from non-vegetated sites. Moreover, only 3 out of 14 (21 %) isolates were resistotype 'z', compared with 9 out of 13 (69 %) in non-vegetated sites.

It is apparent that the extent of vegetational cover and the range of antibiotic resistance of the soil bacteria (GNR) are correlated.

Table 4. Resistotypes of Gram-negative rods as defined by their antibiotic-resistance patterns

resistotype	antibiotic to which resistotype is resistant (r) or sensitive (s): ampicillin 8 μg ml⁻¹	chloramphenicol 8 μg ml⁻¹	sulphamethoxazole 20 μg ml⁻¹	trimethoprim 1 μg ml⁻¹	gentamicin 4 μg ml⁻¹	cephalothin 8 μg ml⁻¹	tetracycline 4 μg ml⁻¹	number of antibiotics to which resistotype is resistant
a	r	r	r	r	r	r	r	7
c	r	r	r	r	s	r	r	
d	r	r	r	s	r	r	r	6
ee	r	s	r	r	r	r	r	
f	r	r	r	r	s	r	s	5
jj	s	r	s	r	r	r	r	
ll	r	r	s	s	s	r	r	4
p	r	s	r	r	s	s	s	
r	r	s	s	r	s	r	s	3
s	s	s	r	r	s	r	s	
v	s	s	r	r	s	s	s	2
xx	s	r	s	s	s	s	s	
y	s	s	r	s	s	s	s	1
yy	s	s	s	r	s	s	s	
z	s	s	s	s	s	s	s	0

4. Discussion

Anak Krakatau and the Sertung spit comprise young land habitats that have existed for only a few decades. Anak Krakatau, being an active volcano, also contains soil of varying ages and with very different stages of plant colonization and degrees of plant cover. The younger areas, with fewer or no plants, contain a lower concentration of soil microorganisms and their GNR have a low range of antibiotic resistance. The older areas, with denser and more varied vegetation cover, have much higher concentrations of soil microorganisms and the GNR are resistant to a greater variety of antibiotics. Similar correlations were found on Sertung when the older hinterland soil (sites Y and Z in secondary forest on the 1883 deposits) was compared with the 10–15 year old deposits on the spit.

(a) *Relative concentrations of soil microorganisms*

The lack of thermophilic bacteria in the hot ash near the volcanic craters of Anak Krakatau (sites O, P, Q) was surprising, and suggests that conditions are not yet suitable for the colonization or establishment of these bacteria.

Comparing the relatively older land surfaces, such as the main, forested part of Sertung (this study) with Rakata and West Java (Graves *et al.* 1988) the similarities in concentrations of soil microorganisms are noteworthy: Rakata (9.9×10^5 per gram of soil), west Java (10.1×10^5 per gram of soil) and Sertung sites Y and Z (5×10^5 per gram of soil). This uniformity exists despite the suboptimal storage conditions of the soil (unrefrigerated for several weeks before microbiological analysis) and the fact that they were collected on two separate expeditions one year apart.

In contrast, on the younger land habitats, most notably the active cone of Anak Krakatau, soil microorganism concentrations were extremely low, e.g. 10^3 per gram soil at sites N and Q, and samples from sites P and S grew no microorganisms at 37 °C. The more vegetated and stable northeast coastal fringe of Anak Krakatau (sites H, I) contained much higher concentrations of microorganisms (approximately 2.2×10^5 per gram of soil) but still only one half to one quarter those of the older land surfaces of the archipelago (the main forested part of Sertung and Rakata).

Assuming that the coastal fringe of Anak Krakatau is at the most 30 years old biologically, our findings demonstrate how quickly soil microorganisms may reach almost 'normal' concentrations once vegetation has become established. Ernst (1908) reported that De Kruyff examined soil from Rakata in 1906 (i.e. 23 years after the major 1883 eruption), and found high concentrations of bacteria in the soil, the average of four soil samples being 2.2×10^6 bacteria per gram of soil. The soil samples appear to have been taken from areas well vegetated with *Barringtonia* and *Casuarina*. The bacterial concentrations were similar to those of soil samples from Java that were examined at the same time. Jenny (1941) quotes work by Schuitemaker on Krakatau (Rakata) soil in 1928 (i.e. 45 years after the eruption) which suggested that the 'microbiological population compared favourably with that of garden soil', and of Hardy, who studied volcanic ash in the West Indies and stated that 'within 10 to 20 years sterile volcanic ash may give rise to fertile soil'. Webley *et al* (1952) found that the development of soil microflora is closely associated with vegetational succession and that soil microbial populations increased with the development of soil on sand dunes. Our findings, to a large extent, support these conclusions.

(*b*) *Antibiotic resistance*

In their study of Rakata, Graves *et al.* (1988) found that resistotype 'f' was the most frequently detected, being present at all four elevations sampled on the island and also at the west Java sites. Yet in the current study, resistotype 'f' was detected at only one site, the main forested part of Sertung (site Y). It appears that resistotype 'f' is present only in older soils and has not yet colonized Anak Krakatau. It will be of interest to see how many years elapse before this particular GNR establishes itself on Anak Krakatau and the Sertung spit. Conversely, in the current study the fully antibiotic-sensitive resistotype 'z' was much more frequently encountered on the younger land surfaces than on the older, and this may reflect the limited development of vegetation on Anak Krakatau and the Sertung spit.

Jones *et al.* (1986) reported that lake bacteria were more resistant to antibiotics when isolated from sites along shorelines and sheltered bays than when isolated from open water. Although they did not suggest it, their findings are consistent with the seeding of shoreline waters with antibiotic-resistant bacteria from plants growing on the shore.

There is no evidence from our studies that the presence of antibiotic-resistant soil bacteria is related to the use of antibiotics by man and their possible accidental release into the environment. This question has been discussed (Graves *et al.* 1988) in relation to public concern about antibiotic-resistant bacteria associated with fruit and vegetables. Antibiotic-resistant GNR have been isolated recently from potted plants growing in hospital wards (Siegman-Igra *et al.* 1986). However, none of the resistotypes isolated was involved in outbreaks of infections among patients, which suggests that the plant bacteria may have been naturally occurring, and unrelated to the use of antibiotics in the hospital. Our study of soil GNR on Rakata and west

Java (Graves *et al.* 1988), showed that the range of antibiotic resistance was similar in these two locations despite the fact that Rakata was probably sterilized by the 1883 eruption and has subsequently been exposed to only minimal human activities likely to lead to the release of antibiotics into the environment. We concluded that antibiotic-resistant GNR in the soil are part of the natural ecology of the soil and unrelated to human activity. Our current study supports this conclusion and implicates plants in the ecology of antibiotic-resistant GNR.

This study has recorded a stage in the colonization and development of bacteria (GNR) in tropical volcanic soils. We have shown that both concentration of soil microorganisms and the range of resistance to antibiotics of GNR are associated with the presence of growing plants and our results suggest that antibiotic resistance may not always be a result of human activity in the biosphere.

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