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Population Ecology of Nitrifiers in a Stream Receiving Geothermal Inputs of Ammonium

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The distribution, activity, and generic diversity of nitrifying bacteria in a stream receiving geothermal inputs of ammonium were studied. The high estimated rates of benthic nitrate flux (33 to 75 mg of $N \cdot m^{-2} \cdot h^{-1}$) were a result of the activity of nitrifiers located in the sediment. Nitrifying potentials and ammonium oxidizer most probable numbers in the sediments were at least one order of magnitude higher than those in the waters. Nitrifiers in the oxygenated surface (0 to 2 cm) sediments were limited by suboptimal temperature, pH, and substrate level. Nitrifiers in deep (nonsurface) oxygenated sediments did not contribute significantly to the changes measured in the levels of inorganic nitrogen species in the overlying waters and presumably derived their ammonium supply from ammonification within the sediment. Ammonium-oxidizing isolates obtained by a most-probable number nonenrichment procedure were species of either *Nitrosospira* or *Nitrosomonas*, whereas all those obtained by an enrichment procedure (i.e., selective culture) were *Nitrosomonas* spp. The efficiency of the most-probable-number method for enumerating ammonium oxidizers was calculated to be between 0.05 and 2.0%, suggesting that measurements of nitrifying potentials provide a better estimate of nitrifying populations.

Nitrification is the microbially mediated process by which ammonium is oxidized to nitrate via the intermediate nitrite. The process has two major consequences from a water quality standpoint. First, nitrification exerts an oxygen demand which may be a significant contributor to oxygen deficits observed in flowing waters (10, 14) and lake hypolimnia (9). Second, production of gaseous forms of nitrogen via coupled nitrification-denitrification processes may represent an important mechanism by which plant-available nitrogen is lost from aquatic systems (7).

An assessment of the significance of nitrification in a particular environment requires an understanding of the population ecology of nitrifying organisms, a subject that has received scant attention (2). Studies on nitrification in flowing waters are usually complicated by the high heterotrophic activity associated with the breakdown of the organic matter present in the effluent discharge supplying the reduced nitrogen. This high heterotrophic activity may result in other nitrogen transformations (ammonification, denitrification) masking the presence of nitrification (14). In this study, the ecology of nitrifying organisms in a stream receiving geothermal inputs of ammonium was investigated. The stream represented a model study system in which the complicating effects associated with high levels of organic matter were absent. It has been demonstrated, from longitudinal changes in the mass flow of nitrogen species, that nitrification is the predominant nitrogen transformation occurring in this stream (19).

MATERIALS AND METHODS

Study sites and survey dates. The Waiohewa Stream is formed by the confluence of the Tikitere and Ohuanui Streams and empties into the northeastern side of Lake Rotorua, North Island, New Zealand (Fig. 1). The catchment of the Ohuanui Stream is predominantly pasture, whereas that of the Tikitere Stream is dominated by the Hells Gate geothermal field. The Waiohewa Stream is 3 km in length and under base flow conditions has a travel time of 2.8 h, an average width of 4 m, and a depth of 0.25 m. The Tikitere and Ohuanui study sites (sites A and B, respectively) were 5 m above the confluence, and the Waiohewa study sites were 20 m below the confluence (site C), 500 m below the confluence (site D), and 2,400 m below the confluence (site E). Studies were performed on 29 October 1979, 10 December 1979, 7 February 1980, and 20 June 1980. All studies were conducted under base flow conditions, the 5-day antecedent rainfall being nil. Field measurements of dissolved oxygen were made with a model 57 dissolved oxygen meter (Yellow Springs Instrument Co., Yellow Springs, Ohio), and pH was measured with a pH meter (Radiometer, Copenhagen, Denmark). Temperature was also measured.

Chemical analysis. Samples taken for inorganic ni-

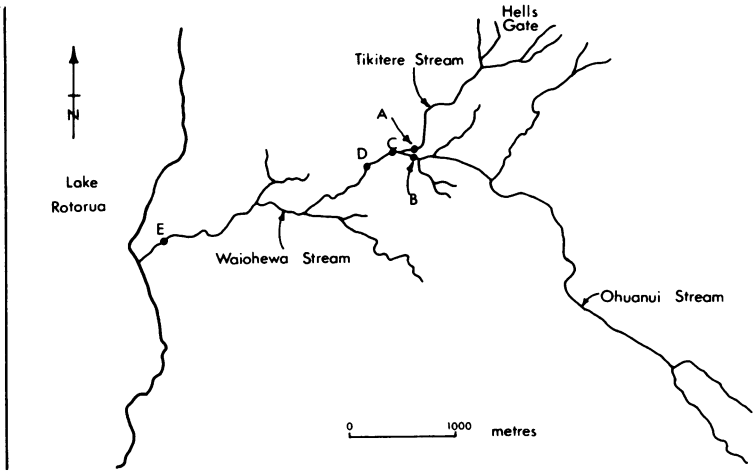

FIG. 1. Sampling sites.

trogen determinations were pressure filtered (0.45-μm cellulose acetate filters) in the field and kept on ice until return to the laboratory (<8 h), at which time they were frozen until analysis. All analyses were performed with an Auto-Analyser (Technicon Corp., Inc., Tarrytown, N.Y.). Nitrate-plus-nitrite content was determined by the hydrazine reduction method of Downes (6). Ammonium was determined by a salicylate-hypochlorite method (16). The levels of biologically oxidizable carbonaceous material in unfiltered samples were determined by the standard 5-day biological oxygen demand test (1), with the incorporation of nitrapyrin (final concentration, 10 mg · liter^{-1}) used to inhibit nitrification.

Sediment sampling. Sediment core samples were obtained by sinking longitudinally sliced and taped polyvinyl chloride tubing (25-mm diameter) into the sediment, placing a rubber bung on top, and carefully withdrawing. Overlying water was siphoned off, and the core was split for subsequent depth-interval sediment collection and storage on ice until return to the laboratory. Interstitial water was extracted from subsamples by centrifugation (5,000 × *g* for 20 min); supernatants were pressure filtered (0.45-μm cellulose acetate filters) and stored frozen before inorganic nitrogen analysis as described above. The standard deviations were always less than 5% of the means for triplicate samples. The sediment samples were also used in determinations of ammonium-oxidizing populations and nitrifying potentials.

Estimation of benthic nitrate flux. Benthic nitrate flux was estimated by measuring the rate of nitrate increase in the water above a section of isolated stream bed. Polyvinyl chloride tubing (7.5-cm diameter) was forced into the stream bed, thereby isolating an area of 44 cm^2 and an overlying water volume of 1 liter. Compressed air was constantly bubbled into the isolation tube just above the sediment to provide turbulence. Samples of the water in the tube were periodically withdrawn during 3 h of incubation and filtered in the field for subsequent inorganic nitrogen analyses as described above. The initial linear rates of nitrite-plus-nitrate nitrogen increase were converted to an areal expression to arrive at a benthic flux estimate expressed as milligrams of NO_3 N per square meter per hour.

Assays of nitrifying potentials. The nitrifying potentials of the indigenous population were assessed under optimal conditions in the laboratory by the procedure of Schmidt and Belser (14a), with incubation in an orbital incubator at 25°C and 120 rpm. Activities were calculated from the linear rates of NO_2-plus-NO_3 N increase observed for the initial 10 to 15 h of incubation and expressed as micrograms of nitrogen per gram on a wet weight basis. Assays were performed in triplicate, and the standard deviations were always less than 7% of the means. In some sediment samples, activities were so high that small sample inocula (1 to 2 g) were used. The standard conditions of the assay were varied to test the effects of ammonium concentration, pH, and temperature on observed activities.

Ammonium-oxidizing populations. Estimates of ammonium-oxidizing populations were made by a five-tube most-probable-number (MPN) technique. The medium used was that of Soriano and Walker (15) with the trace elements specified by Watson (17). Decimal dilutions were made in 1 mM phosphate buffer (pH 7.2) with the addition of 0.1% Tween 80 to the initial diluent used with sediment samples (4). Incubation was at 25°C for 5 weeks. Tubes were regarded as presumptively positive if acid production occurred (pink-to-yellow color change). The tubes diluted with the greatest dilution that resulted in one or more tubes showing acid production and the tubes diluted with the next greatest dilution were tested for the presence of NO_2-plus-NO_3 N by autoanalysis as described above to obtain a confirmed MPN.

Isolation of ammonium-oxidizing bacteria from the most dilute positive MPN tubes was performed by the MPN nonenrichment procedure of Belser and Schmidt (4), with two modifications. The number of all bacterial cells (i.e., both nitrifiers and contaminants) present in an MPN tube was estimated by an epifluorescence acridine orange direct count procedure (12). In addi-

tion, after the second transfer and incubation in the ammonium medium, a loopful of culture was streaked onto the mineral agar of MacDonald and Spokes (11), which was then incubated at 25°C for 5 weeks. Individual red colonies were subsequently examined by phase-contrast microscopy and tested with purity check medium for heterotrophic contamination (14a).

In addition, a successive enrichment procedure (i.e., selective culture) was used for ammonium oxidizer isolation. Samples of sediment (1 g) were inoculated into 100-ml aliquots of MPN ammonium medium in 250-ml Erlenmeyer flasks. The flasks were incubated in an orbital incubator at 25°C and 120 rpm and examined daily. When a color change was noted, 0.1 ml was transferred to fresh medium. This procedure was repeated seven times, after which a loopful of the enriched culture was streaked onto the agar of MacDonald and Spokes (11), which was incubated at 25°C for 5 weeks. Individual red colonies were examined microscopically and tested with purity check medium for heterotrophic contamination.

RESULTS

Physical and chemical data for the waters at the five experimental sites are summarized in Table 1. The geothermally derived Tikitere water (site A) is warm, acidic, low in dissolved oxygen, and rich in ammonium nitrogen. Mixing this water with that from Ohuanui Stream (site B), which contributes 82% of the flow to Waiohewa Stream (19) results in the waters of the upper Waiohewa Stream (sites C and D) being well oxygenated and possessing near-neutral pH and ammonium nitrogen levels of 2 to 3 mg · liter^{-1}. Nitrification-inhibited biochemical oxygen demands were low, indicating a low level of biodegradable organic matter.

Two distinct sediment types were observed at Waiohewa site D. The gravel sediment, which consisted principally of coarse particles (3 to 10 mm), was loose, and black reduced material was not observed until the following depths: >20 cm on 29 October 1979, 20 cm on 10 December 1979, and 12 cm on 7 February 1980. In contrast, the mud sediment, which consisted principally of fine silt particles, was compact, and black reduced material was observed nearer the surface (depths of 12, 7, and <1 cm on 29 October 1979, 10 December 1979, and 7 February 1980, respectively).

The depth profiles of ammonium nitrogen and nitrite-plus-nitrate nitrogen levels in the interstitial waters of the two sediment types are presented in Fig. 2. In surface sediments, levels of both inorganic nitrogen species approached those in stream water, whereas in deeper sediments, levels were higher or lower than those in stream water, depending on sediment type and sampling date. The highest ammonium nitrogen levels were obtained when anaerobic conditions were dominant (7 February 1980, mud). Accumulation of high nitrite-plus-nitrate nitrogen levels occurred when aerobic conditions were dominant (29 October 1979, gravel).

Estimates of benthic nitrate flux and nitrifying potentials at the experimental sites on 10 December 1979 are presented in Table 2. In Waiohewa Stream, in situ flux experiments demonstrated large ammonium nitrogen losses which were balanced by nitrite-plus-nitrate nitrogen gains, indicating that nitrification was the dominant transformation affecting levels of nitrogen species in the overlying water. No net changes in levels of inorganic nitrogen species were observed in bottle incubations of water alone, demonstrating that the observed nitrification activity was associated with the sediment. Nitrifying potentials were low in both the sediments and waters of Tikitere and Ohuanui Streams and in the waters of Waiohewa Stream. The nitrifying potentials observed in Waiohewa sediments were at least one order of magnitude higher than those found in any of the other samples. The addition of nitrapyrin (final concentration, 10 mg · liter^{-1}) to the nitrifying potential assay medium totally inhibited activity.

Depth profile analysis of sediments demonstrated that the potential for nitrification was highest and extended to the greatest depth in

TABLE 1. Physical and chemical characteristics of waters collected from the five experimental sites

Stream	Site	Nitrogen (mg · liter^{-1})		BOD (mg · liter^{-1})[b]	DO (mg · liter^{-1})[c]	Temp (°C)	pH
		NH_4	$NO_2 + NO_3$				
Tikitere	A	11.38	0.40	ND	4.9 (57)	23.3	3.5
Ohuanui	B	0.15	1.07	1.5	9.0 (86)	13.5	7.4
Waiohewa	C	2.57	0.92	1.4	8.5 (88)	16.8	6.8
Waiohewa	D	2.50	0.95	1.9	8.4 (87)	16.8	6.8
Waiohewa	E	1.45	1.83	1.8	7.3 (77)	17.6	6.8

[a] Means for four surveys on different days are shown.
[b] Five-day biochemical oxygen demand (BOD) with nitrification inhibition. ND, Not determined.
[c] DO, Dissolved oxygen. Numbers in parentheses are percent saturation values.

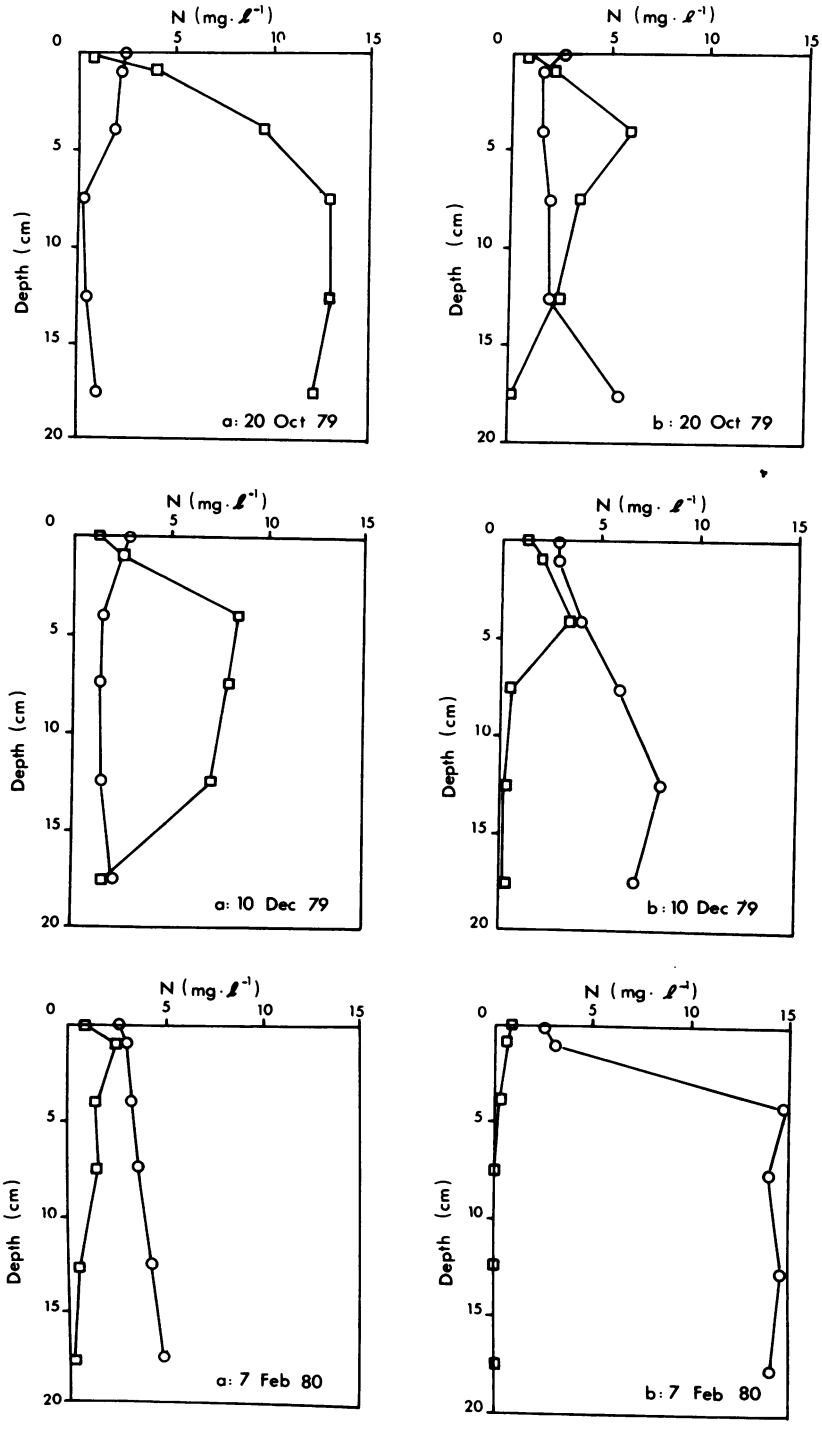

FIG. 2. Depth profiles of interstitial ammonium nitrogen (○) and nitrite-plus-nitrate nitrogen (□) in Waiohewa site D gravel (a) and mud (b) sediments.

TABLE 2. Estimates of benthic nitrate flux and nitrifying potentials at the five experimental sites on 10 December 1979

Stream	Site	Benthic nitrate flux (mg of $N \cdot m^{-2} \cdot h^{-1}$)	Nitrifying potential (μg of $N \cdot g^{-1} \cdot h^{-1}$)	
			Water	Sediment[a]
Tikitere	A	ND[b]	0	0.01
Ohuanui	B	ND	0.002	0.12
Waiohewa	C	72	0.003	5.21
Waiohewa	D	75	0.004	4.70
Waiohewa	E	33	0.009	2.50

[a] Data were obtained for surface (0 to 2 cm) samples.
[b] ND, Not determined.

samples in which oxidizing conditions prevailed (29 October 1979, gravel [Fig. 3]). In the mud sediment, in which reducing conditions were closer to the surface, the observed nitrifying potentials declined more rapidly with depth. Some activity was observed in samples taken from apparently anoxic sediments (e.g., mud sediment at a depth of >10 cm on 10 December 1979).

The level of ammonium nitrogen affected the nitrifying potentials of Waiohewa sediment samples. On 29 October 1979, the observed nitrifying potentials of surface (0 to 2 cm) sediment samples from site D were 20% higher when the standard medium of Schmidt and Belser (14a) was used, compared with levels observed when pH-adjusted Waiohewa water was used. The level of ammonium nitrogen in the standard medium was 14 $mg \cdot liter^{-1}$, whereas in the Waiohewa water sample, it was 2.59 $mg \cdot liter^{-1}$. Further studies on the effect of ammonium nitrogen level on nitrifier activities were performed on three later dates, using the standard assay medium with modified levels of ammonium nitrogen. All studies revealed that potential nitrifying activities (V) were dependent upon ammonium nitrogen level (S) in a manner consistent with the Michaelis-Menten kinetic equation, with Woolf plots ($[S \cdot V^{-1}]$ versus S) being linear (Table 3). The Michaelis-Menten kinetic parameters K_m (rate at 0.5 V_{max}) and V_{max} (theoretical maximum oxidation rate) were calculated from the Woolf plots and are presented in Table 3. The five determinations of the K_m for the indigenous ammonium-oxidizing population ranged from 0.85 to 1.91 mg of $N \cdot liter^{-1}$ (0.06 to 0.14 $mmol \cdot liter^{-1}$), with a mean value of 1.36 mg of $N \cdot liter^{-1}$ (0.10 $mmol \cdot liter^{-1}$), further demonstrating that nitrifiers in Waiohewa Stream were limited in activity by substrate level.

Sediment samples collected from Waiohewa site D on 29 October 1979 and 20 June 1980

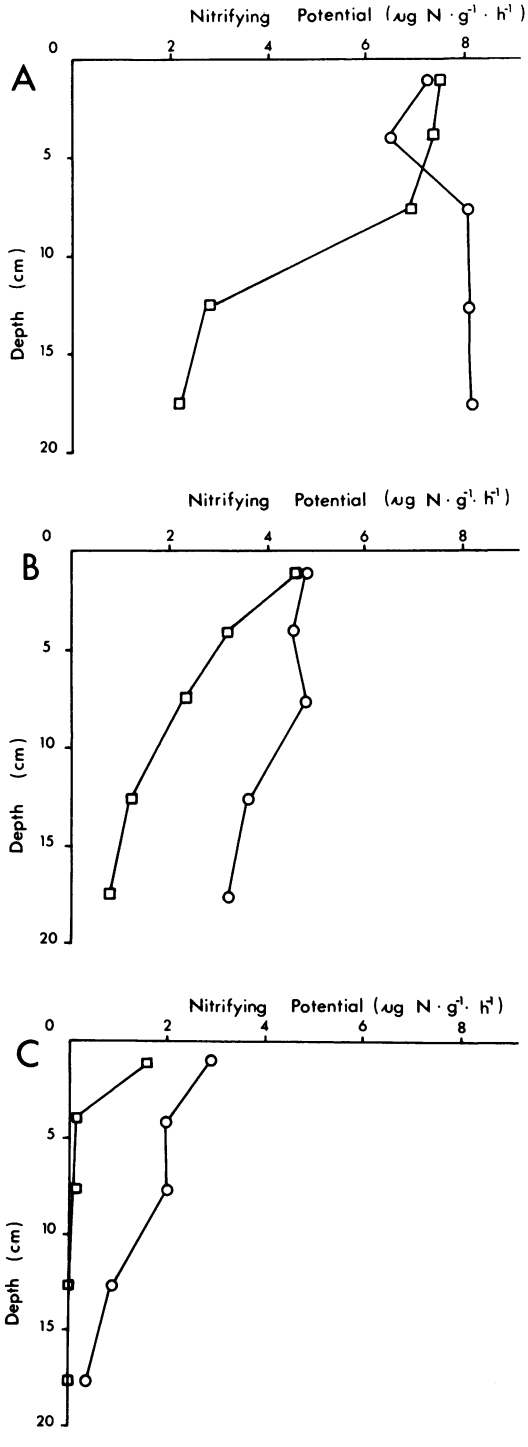

FIG. 3. Depth profiles of nitrifying potentials in Waiohewa site D gravel (○) and mud (□) sediments taken on 20 October 1979 (A), 10 December 1979 (B), and 7 February 1980 (C).

TABLE 3. Kinetic parameters and correlation coefficients obtained from Woolf plot transformations ([S · V^{-1}] versus S) of data obtained from assays of nitrifying potentials in Waiohewa sediment samples

Date	Site	V_{max} (μg of N · g^{-1} · h^{-1})	K_m (mg of N · $liter^{-1}$)	r [a]
12/10/79	D	5.10	1.27	0.97
12/10/79	E	2.73	1.61	0.97
2/7/80	D	3.15	1.15	0.94
2/7/80	E	1.73	0.85	0.93
6/20/80	D	3.50	1.91	0.93

[a] r, Linear correlation coefficient obtained from Woolf plot transformations of data (11 points per determination).

demonstrated the highest nitrifying potentials at pH 7.5 and 22 to 25°C. Under typical Waiohewa conditions, nitrifying potentials were inhibited 20% by suboptimal pH and 25% by suboptimal temperature.

MPNs of ammonium oxidizers in water and surface (0 to 2 cm) sediment samples from the five experimental sites are given in Table 4. Ammonium oxidizer MPNs were highest in Waiohewa sediments and usually one order of magnitude lower in other samples. Sediment depth profile estimations of ammonium oxidizers at site D followed the same general trends as those observed for nitrifying potentials with high MPNs, i.e., when aerobic conditions were prevalent (data not shown).

Ammonium oxidizers were isolated by MPN nonenrichment from the 30 most dilute positive MPN tubes. Tentative identification of isolates according to morphology (19) revealed 24 *Nitrosospira* isolates, 14 *Nitrosomonas* isolates, and 0 *Nitrosolobus* isolates (eight tubes contained both *Nitrosospira* and *Nitrosomonas* isolates). All six enrichment cultures (sites C, D, and E on two sampling dates) yielded *Nitrosomonas* isolates.

TABLE 4. MPNs of ammonium oxidizers in the waters and surface (0 to 2 cm) sediments of the five experimental sites

Site	Sample type	NH_4 oxidizer MPN on[a]:		
		10/29/79	12/10/79	2/7/80
A	Water	0	0	0
	Sediment	0	0	0
B	Water	4.3×10^1	5.0×10^1	7.5×10^1
	Sediment	7.5×10^3	2.4×10^2	2.4×10^2
C	Water	7.5×10^1	5.0×10^1	3.8×10^1
	Sediment	2.4×10^4	7.9×10^3	4.3×10^3
D	Water	9.3×10^1	5.0×10^1	2.1×10^1
	Sediment	1.5×10^5	7.9×10^4	4.3×10^3
E	Water	2.4×10^3	2.4×10^2	2.1×10^2
	Sediment	7.5×10^3	1.4×10^3	1.4×10^3

[a] Per gram of sediment or milliliter of water.

DISCUSSION

In a recent review, Belser (2) concluded that "in aquatic systems, nitrification appears to be associated with the sediments rather than the overlying water that is transporting the ammonium." Evidence from in situ nitrification studies, assays of nitrifying potentials, and ammonium oxidizer MPN determinations demonstrated that nitrification in Waiohewa Stream was principally sediment based. The travel time of Waiohewa Stream (2.8 h) is too short for planktonic nitrifier proliferation: minimum generation times for nitrifiers are at least 8 h, even under optimum laboratory conditions (5, 13). Therefore, the longitudinal increases in nitrifying potentials and ammonium oxidizer MPNs in the Waiohewa waters must have resulted from the sloughing of pregrown nitrifiers from the sediment, rather than planktonic growth.

The values for nitrifying potentials in the surface sediments of Waiohewa Stream ranged from 1.6 to 8.0 μg of N · g^{-1} · h^{-1} and were considerably higher than those reported by Belser and Mays (3) for intertidal sediments (0.006 to 0.105 μg of N · g^{-1} · h^{-1}). The estimates of benthic nitrate flux (33 to 75 mg of N · m^{-2} · h^{-1}) were also high, compared with marine sediment rates estimated by Goloway and Bender (8) (0.0075 to 0.059 of mg) N · m^{-2} · h^{-1}). The establishment of a highly active nitrifier population in the Waiohewa sediments reflects conditions favorable for nitrifier growth. Ammonium oxidizers in the Waiohewa surface sediments are supplied with a continuous flow of oxygenated water of near-neutral pH that contains substrate at levels approximately twice that of the observed half-saturation constant (K_m). By comparison, nitrifying potentials and ammonium oxidizer MPNs in the sediments of the two streams forming Waiohewa Stream were low, and the major limitations of nitrifier growth were probably acidity and low substrate level for Tikitere and Ohuanui, respectively.

Manipulating the conditions of the assays of nitrifying potentials demonstrated that ammonium oxidizers in the oxic surface sediments of Waiohewa Stream were probably limited in in situ activity by suboptimal temperature (35%), pH (20%), and substrate level (20%). Assuming that these effects are additive, the activities of ammonium oxidizers in situ may be calculated as 35% of their measured nitrifying potentials. For example, the nitrifying potential of 4.7 μg of N · g^{-1} · h^{-1} for the surface (0 to 2 cm) sediment

at site D on 10 December 1979 (Table 2) yields a calculated in situ activity of 1.65 μg of $N \cdot g^{-1} \cdot h^{-1}$ or, expressed on an areal basis, 59 mg of $N \cdot m^{-2} \cdot h^{-1}$ (wet density of sediment, 1.8 $g \cdot cm^{-3}$). The nitrate produced in the surface sediment would not have been significantly denitrified since oxic conditions prevailed, and it would not have been dissipated by downward diffusion since nitrate levels in deeper sediments were higher (Fig. 2). Assuming that steady-state conditions existed in the sediment at the time of sampling, all of the nitrate produced by nitrification activity in the surface sediment (59 mg of $N \cdot m^{-2} \cdot h^{-1}$) must have contributed to the observed benthic flux of 75 mg of $N \cdot m^{-2} \cdot h^{-1}$ (Table 2). These findings suggest that the activity of nitrifiers in the top few centimeters could effect the observed benthic nitrate flux. The large nitrifier population that existed in deeper oxic sediments (as evidenced by high nitrifying potentials [Fig. 3]) was apparently not involved in the sediment-water exchange of ammonium and nitrate. Presumably, nitrifiers in deep (nonsurface) sediments rely on ammonifiers to supply ammonium from organic nitrogen. During periods of sustained oxygen availability in the deep sediments, the size of the nitrifier population may well be limited by the rate of ammonification (2). However, temporal changes in the oxygen status of the sediments allow for periods of ammonium accumulation under anoxic conditions (e.g., Fig. 2, 7 February 1980), and the accumulation provides a high substrate level for the initial growth of nitrifiers during periods when the sediment becomes aerobic. The flush of nitrifier activity depletes ammonium to levels that are extremely limiting (nitrification rates greater than ammonification rates) and leads to an accumulation of nitrate (e.g., Fig. 2, 20 October 1979). The large nitrifying population established during the initial period of oxygen supply must subsequently survive in a state of diminished physiological activity and exist on the limited supply of ammonium from ammonification. The presence of nitrifying potentials in anoxic sediments (Fig. 3B, >10 cm, mud) suggests that nitrifiers produced during periods of oxygen supply were capable of surviving when oxidative activity was not possible. In summary, the nitrifying population that exists in the sediments may therefore be separated as follows on the basis of physiological states. (i) Nitrifiers in surface sediments are continuously active, obtaining ammonium from and releasing nitrate to the overlying water. (ii) Nitrifiers in nonsurface oxic sediments are presumably in a state of low physiological activity, relying on ammonification to supply the ammonium required for sustenance. (iii) Nitrifiers in anoxic sediments are inactive but presumably represent the initial population required for subsequent growth on the accumulated ammonium when oxic conditions subsequently occur.

In deep (nonsurface) sediments, gaseous loss of nitrogen via nitrification followed by denitrification seems to have occurred, the nitrate accumulated under oxic conditions (Fig. 2A) being lost as the sediments became anoxic (Fig. 2B and C).

That nitrapyrin completely inhibited activity in assays of nitrifying potentials demonstrates that nitrification in the Waiohewa sediments was mediated by chemoautotrophs and did not involve heterotrophs (2). Ammonium oxidizers obtained by an enrichment procedure (i.e., selective culture) were all *Nitrosomonas* spp., whereas isolates obtained by an MPN nonenrichment procedure were either *Nitrosomonas* spp. or *Nitrosospira* spp. All enrichment procedures entail the selective isolation of organisms capable of the most rapid growth under the conditions imposed by the investigator, rather than the isolation of those organisms most common in the original sample. For this reason, it is not surprising that *Nitrosomonas* spp. with laboratory culture generation times of 8 to 14 h (5) were selectively enriched over *Nitrosospira* spp., which possess a generation time of 20 h (5). The MPN nonenrichment procedure may better reflect the generic composition of the dominant or codominant nitrifiers in a natural population (4). The coexistence of multiple genera of ammonium oxidizers in the Waiohewa sediments suggests the presence of microsites differing in substrate concentrations, physical and chemical properties, or both (4). Since the dominant genus isolated was *Nitrosospira*, the majority of microsites must selectively favor its in situ growth.

An estimate of the efficiency of the MPN technique for counting ammonium-oxidizing bacteria can be made by using nitrifying potentials and the maximum oxidation rate per cell observed in pure cultures (3). In this study, counting efficiencies were estimated to be 0.3 to 2.0% using oxidation rates for *Nitrosomonas* spp. and 0.05 to 0.34% using oxidation rates for *Nitrosospira* spp. These extremely low counting efficiencies are similar to those observed by Belser and Mays (3) for intertidal sediments and suggest that nitrifying populations are better estimated by measurement of nitrifying potentials than by MPN counts.

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