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Nitrification in the Intertidal Zone: Influence of Effluent Type and Effect of Tannin on Nitrifiers

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Nitrification by intertidal sediments was measured by using a tide simulator that approximated the cycle of seawater on tidal flats. Sediments were chosen from sites affected by industrial and municipal effluents and pastoral seepage and runoff. The ability of sediments from different sites to nitrify endogenous nitrogen varied markedly. All sites exhibited an initial lag before activity commenced. The duration of this lag and the rate of nitrate production were different at each site. The sediments were also capable of oxidizing NH_3-N supplied to them in seawater. This "nitrification potential" was highest at sites receiving nitrogenous effluents (slaughterhouse and sewage), but was also substantial in sediments affected by bark extract effluent and pasture runoff. The lowest potential and the longest lag were exhibited by sediments in an apple cannery effluent area. Enrichment cultures of nitrifying microorganisms were obtained from all sites using NH_4^+ as a source of energy, but enrichments for nitrite oxidizers were unsuccessful. Concentrated pine bark tannins, similar in origin to those in effluents at the well-nitrifying chipmill site, were tested for toxicity to pure cultures of nitrifying bacteria. Two *Nitrobacter* strains and one *Nitrosomonas* strain were unaffected by tannins even at 5 mg/ml. A *Nitrosolobus* and a *Nitrosospira* strain were inhibited partially at 5 mg/ml and only slightly or not at all at 1 mg/ml.

Nitrification is the process by which reduced forms of nitrogen, such as ammonia and amino acids, are oxidized to nitrite and nitrate. It is carried out exclusively by microbiological agents, the most important of which are the chemolithotrophic nitrifying bacteria typified by the ammonia-oxidizing genus *Nitrosomonas* and the nitrite-oxidizing genus *Nitrobacter*.

The ecological significance of nitrification to the food web in natural aquatic systems lies in the fact that most aquatic organisms excrete ammonia, which, even in low concentrations, is highly toxic to animal life. Moreover, nitrogen trapped in intertidal and estuarine sediments, either as ammonium cation adsorbed on colloidal surfaces or as organic nitrogen, is released as soluble nitrogen when converted to nitrate. For a general review of the literature on nitrification, refer to Painter (5).

The present study was undertaken to examine the nitrification potential of intertidal sediments and to assess the impact of organic effluent composition on nitrification. The study area was that described by Bohlool (N. Z. J. Mar. Freshwater Res., in press), where several organic effluents containing different carbon and nitrogen constituents have been discharged for over 5 years.

MATERIALS AND METHODS

Study area. The area chosen for this study was the intertidal zone of the Waimea Estuary (Inlet), Nelson District, New Zealand (41° 18'S, 173° 10'E).

Stations were established near major nutrient inputs into the intertidal zone. These consisted of: B1, pasture, pasture runoff only; B2, chipmill, effluent from hydraulic debarker; B3, slaughterhouse, animal waste; B4, sewage, untreated sewage effluent; and B5, apple cannery, cannery effluent. A more detailed description of these stations and the chemical composition of the effluents has been presented elsewhere (Bohlool, in press). An ecological survey of the waters and sediments of the Waimea Estuary has recently been completed (D. M. Updegraff, D. J. Stanton, and M. J. Spencer, N. Z. J. Mar. Freshwater Res., in press).

Experimental assembly. A tide simulator was designed to approximate the cycle of seawater on intertidal sediments. The assembly (see Fig. 1) consisted of columns (40 cm long and 7 cm in diameter) containing sediments to a depth of 15 cm. A perforated irrigation loop, made out of polyvinyl chloride tubing, was rested firmly on the surface of each sediment and was connected to a 2-liter water reservoir with polyvinyl chloride tubing. The reservoirs, one for each

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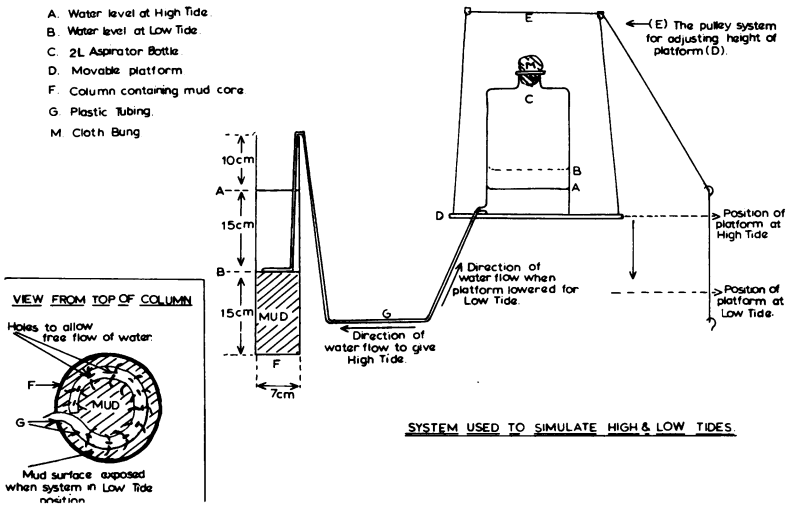

FIG. 1. *Tide simulator assembly. The position indicates high tide. Arrows show the direction of the flow of water at the changing of the tide.*

column, were supported on a movable platform that could be adjusted vertically through two side pulleys.

Incubations were carried out in a below-ambient-temperature (15 ± 2°C) incubation room. Both the columns and the reservoirs were wrapped in black cloth to prevent complications from algal photosynthesis and nitrogen uptake.

To simulate the tidal cycle on sediments, the water was circulated from the reservoirs to the columns daily. The platform bearing the reservoirs was raised to a position to allow water to flow into the columns and reach a height of 15 cm above the sediment surface. The reservoirs were fixed at this position for 8 h (high tide), after which time the platform was lowered to the ground, causing the water to drain out of the columns and the sediments to be exposed (low tide). This cycle of 8-h high tide and 15-h low tide was repeated every day for 70 days. Before the start of each experiment, the sediments were flushed with unamended seawater through two cycles, and the water was discarded. This was done to remove unbound nitrogenous compounds from the sediments. The water in the assemblies was periodically siphoned out, discarded, and replenished.

Nitrification measurements. The rates of autochthonous nitrification of the constituents of the sediments were measured by irrigating a series of columns with unamended seawater from the open sea. To assess the maximum potential of the sediments for oxidizing supplied ammonium, a second series of columns was supplied with an excess of ammonium-N in seawater [15 to 20 mg of NH_3-N as $(NH_4)_2SO_4$ per liter]. Controls for these experiments consisted of containers devoid of sediments and circulated in like manner, with either seawater or seawater containing NH_3-N.

Nitrate analyses were done by the cadmium reduction method described in *Standard Methods* (1). Samples were taken from the reservoirs one to two times each week, immediately after the columns had drained. The results are expressed as the cumulative amount of NO_2^--N plus NO_3^--N produced by the sediments. The values are converted to per square meter of the sediment surface and represent net nitrification values.

In one experiment, the effect of a nitrification inhibitor, N-Serve [2-chloro-6-(trichloromethyl)-pyridine; Dow Chemical Co.], on the activity of the columns was tested. N-Serve was first dissolved in 5 ml of 95% ethanol and added to 10 liters of seawater containing NH_3-N (final concentation of N-Serve, 5 mg/liter). This was then circulated in the tide simulator on nitrifying sediments, and the amount of nitrate appearing in the water was measured.

Enrichment cultures of nitrifiers. The media described by Schmidt et al. (7) were used for enrichment of nitrifying organisms. Water samples (10 ml) from the 30-day tide assembly were added to 90 ml of media in 250-ml flasks. These were incubated (shaken) for 3 to 4 weeks at 15°C in the dark. With ammonia as a substrate, the color change of the indicator in the media and the presence of NO_2 and/or NO_3 were considered indicative of the presence of ammonia oxidizers. For nitrite oxidizers, the appearance of nitrate was used as an indication. Five to six transfers into fresh media were made before the samples were considered positive. The cultures were also monitored by phase-contrast microscopy.

Effect of tannins on nitrifiers. Pure cultures of *Nitrobacter winogradskyi* serotypes W-1 and agilis-1, *Nitrosomonas europaea, Nitrosolobus* sp. (Fargo strain), and *Nitrosospira* sp. (Sitzbergen 30 strain) were obtained from the culture collection at the University of Minnesota. The *Nitrosospira* culture was isolated by N. Walker, Rothamsted Experimental Station, Harpenden, Herts, England.

Media for *Nitrobacter, Nitrosolobus,* and *Nitrosospira* were those used by Schmidt et al. (7). *N. europaea* was grown in the medium of Clark and Schmidt (2). Nitrite was determined according to the method of Shinn (8). Tannin was provided by W. D. Grant, Cawthron Institute; the preparation was comprised of

low-molecular-weight condensed tannins extracted and purified from *Pinus radiata* bark (4). Tannin was added to sterile nitrifier media as filter-sterilized solutions. Data reported are the average of duplicates.

RESULTS

Nitrification with unamended seawater. The activities in sediments, collected in September (Southern Hemisphere spring), irrigated with unamended seawater are shown in Fig. 2. All sites exhibited an initial lag period before the onset of activity. The duration of the lag and the rate of nitrate production varied substantially at different sites. The controls remained free of NO_3^- for the duration of the experiment.

Nitrification with seawater supplemented with NH_3-N. In two separate experiments, the potential of sediments for oxidizing added ammonia was measured (Fig. 3 and 4).

The sediments in Fig. 3 were collected in June (Southern Hemisphere winter), and those in Fig. 4 were collected in September. The activities in both sets of samples followed nearly the same pattern, except that the spring sediments showed shorter lag periods and generally higher activities. Only very slight traces of nitrate appeared in the no-sediment controls after 30 to 40 days of incubation.

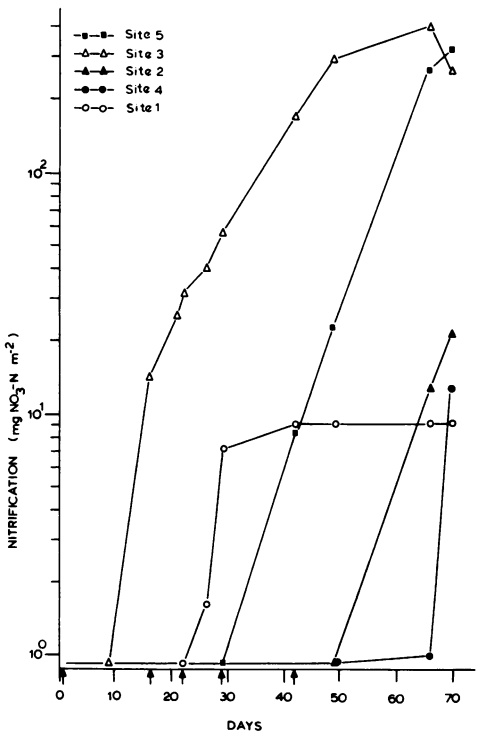

FIG. 2. *Nitrification activity of intertidal sediments, collected 16 September 1976: unamended seawater. The arrows indicate when the water in the assemblies was replenished.*

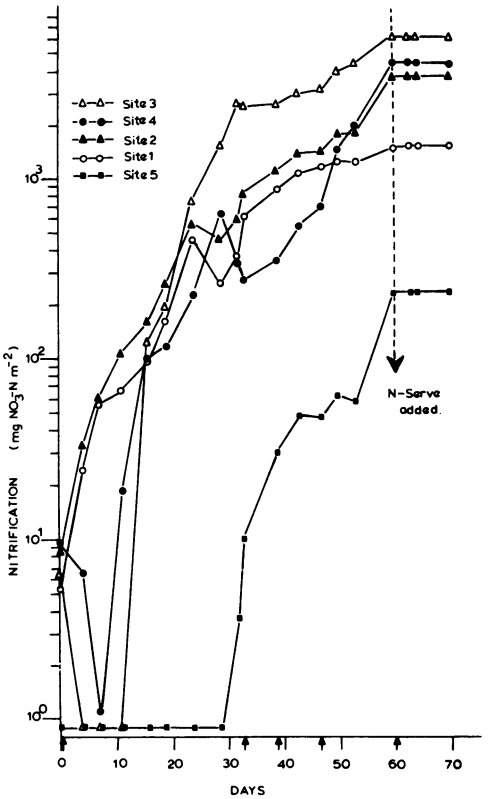

FIG. 3. *Nitrification potential of intertidal sediments, collected 17 June 1976: seawater amended with ammonium sulfate. The arrows indicate when the water in the assemblies was replenished.*

N-Serve, applied to the winter sediments after 60 days of nitrification, caused activity to stop in all the samples (Fig. 3).

Presence of nitrifiers. The results of enrichment cultures for ammonium- and nitrite-oxidizing microorganisms are presented in Table 1. When ammonium was used as a substrate, both nitrite and nitrate could be detected in the enrichment cultures from all five samples. No enrichments could be made from any of the sites when nitrite was used as a substrate.

Sensitivity of nitrifiers to pine bark tannin. Nitrification at site B2 was of particular interest in view of the tannin-enriched effluent discharged there by a *P. radiata* chipmill operation and in view of the reported sensitivity of nitrifiers to tannins (6). The effects of pine bark tannins on several genera of nitrifying bacteria under pure culture conditions are shown in Fig.

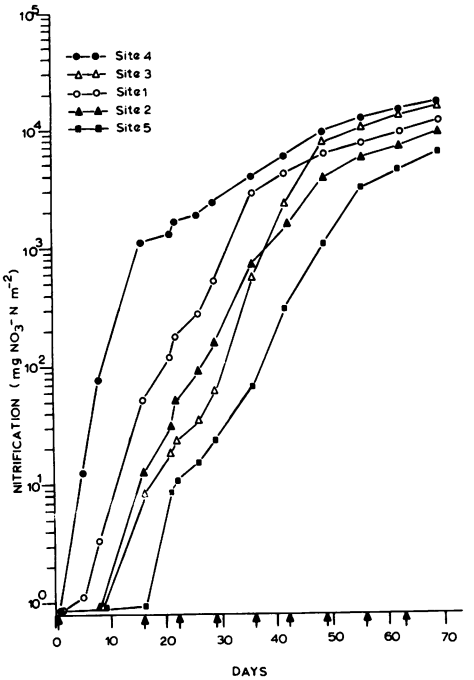

FIG. 4. *Nitrification potential of sediments, collected 16 September 1976: seawater amended with ammonium sulfate. The arrows indicate when the water in the assemblies was replenished.*

TABLE 1. *Enrichment of nitrifying microorganisms from intertidal sediments*

Site	NH_3[a]		NO_2^-
	NO_2^-[b]	NO_3^-	NO_3^-
B1, pasture	+	+	−
B2, chipmill	+	+	−
B3, slaughterhouse	+	+	−
B4, sewage	+	+	−
B5, apple cannery	+	+	−

[a] Substrate supplied.
[b] Product.

5. All genera proved to be surprisingly tolerant of the additions despite the high concentrations and absence of other bacteria to adsorb or inactivate the tannins. *Nitrosomonas europaea*, usually assumed to be a predominant ammonia oxidizer in natural environments, was unaffected by tannins even at 5 mg/ml. The two other ammonia oxidizers tested, *Nitrosolobus* sp. and *Nitrosospira* sp., were inhibited partially at 5 mg/ml and only slightly or not at all at 1 mg/ml. Neither of the nitrite-oxidizing (*Nitrobacter*) strains evidenced any inhibitory effects due to tannins.

DISCUSSION

Nitrification by intertidal sediments was shown to be notably affected by the types of effluents entering the intertidal zone.

The results with unamended seawater (Fig. 2) indicated that the nitrogen that is bound in the sediments could be released into solution in the form of nitrate. The onset of activity was preceded by an initial lag period, the duration of which varied from site to site. The shortest lag period and the highest activity were measured with sediments affected by the slaughterhouse effluent (site B3). Considering the high content of reduced nitrogen in this effluent (2), this is not surprising. The populations of nitrifiers probably were stabilized at a relatively high level due to continuous enrichment by this effluent.

The activity at site B5 (apple cannery) commenced much later than at site B3 (slaughterhouse), but reached approximately the same intensity. Cannery effluent is highly enriched in sugars but relatively low in nitrogen. It is interesting to note that this is the site that was reported to have the highest nitrogen fixation activity (Bohlool, in press).

The somewhat lower activities at site B1 (pasture) and B2 (chipmill) in Fig. 2 were expected because of the low nitrogen content of seepage water and bark extract (Bohlool, in press). The poor activity at site B4 (sewage), however, was unexpected, especially since the same sediments nitrified actively when presented with exogenous nitrogen (Fig. 4).

The results obtained with sediments exposed to an excess of ammonium nitrogen (Fig. 3 and 4) indicate the potential of the sites for handling discharged nitrogen in the effluents. The maximum amount of nitrate produced at each site could then be taken as an expression of the loading capacity of that site or the maximum equimolar amount of ammonium-N that could be processed at that site. The nitrification potential varied greatly at the five sites tested (Fig. 3 and 4). The initial lag before the onset of nitrification was also different for each site.

Samples collected in the spring (Fig. 4) showed greatly reduced lag periods and generally had higher activities than the winter samples (Fig. 3). This was probably due to the presence of higher populations of nitrifying bacteria.

N-Serve has been reported to be inhibitory to chemolithotrophic nitrification by blocking the action of the ammonia-oxidizing bacterium *Nitrosomonas* (4). In our studies (Fig. 3), the application of N-Serve caused an abrupt halt in

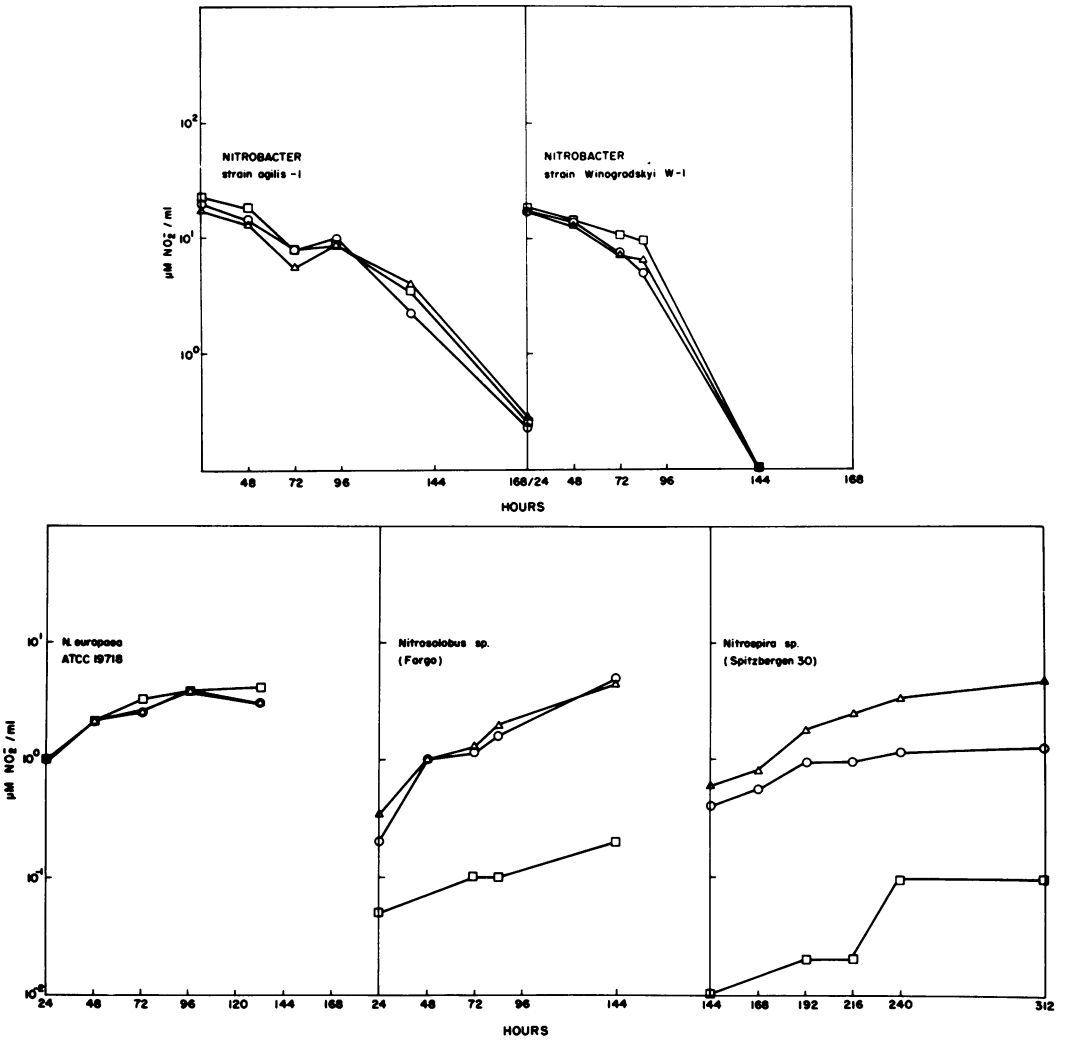

FIG. 5. *Effect of extracted and purified pine bark tannins on pure cultures of ammonia-oxidizing (lower) and nitrite-oxidizing (upper) bacteria. Tannin added at 5 mg/ml (□), 1 mg/ml (○), and 0 mg/ml (△).*

nitrate production by sediments. This indicates the chemolithotrophic nature of nitrification on intertidal sediments.

Enrichment cultures of nitrifiers were obtained from all sites. When ammonia was used as a source of energy, both nitrite and nitrate were present in the cultures. With nitrite as the oxidizable substrate, however, enrichment cultures failed to develop. The reason for this is not clear, but it may have been inhibition due to too high an initial concentration of nitrite in the medium.

Tannins have been reported by Rice and Pancholy (6) to be inhibitory to nitrification, and these authors postulate such inhibition to be the key to the conservation of nitrogen in the soils of climax forests. No support for this postulate was observed in the present investigations. Sediments at the chipmill site (B2) received large quantities of pine bark extract highly enriched in tannins and other phenolics. This situation has obtained for more than 5 years, yet sediments so exposed were clearly capable of nitrification at rates equivalent to those of the pastoral control site (Fig. 3 and 4). The ecological data are in accord with the pure culture studies summarized in Fig. 5, in which a range of chemoautotrophic nitrifying bacteria were shown to be relatively unaffected by high concentrations of pine bark tannins. The highest concentration used in the pure culture studies was 5 mg/ml; this is estimated to be the approx-

imate tannin content of the outflow from debarker machinery (W. D. Grant, Cawthron Institute, personal communication). Dilution of the outflow occurs during periods of tidal flushing, but it is nevertheless likely that these intertidal sediments encounter tannins at concentrations substantially greater than those occurring in forest soils. There is need to look more critically at nitrification potentials in forest soils, using improved methodology for study of the nitrifiers and less drastic techniques for the extraction of tannins from soil.

ACKNOWLEDGEMENT

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