logical bases of phytoplankton ecology. Can. Bull. Fish. Aquat. Sci. 210.
SOKAL, R. R., AND F. J. ROHLF. 1969. Biometry. Freeman.
WILKINSON, G. N. 1961. Statistical estimations in enzyme kinetics. Biochem. J. **30:** 324–332.
WILLIAMS, P. J. 1973. The validity of the application of simple kinetic analysis to heterogeneous microbial populations. Limnol. Oceanogr. **18:** 159–165.
WRIGHT, R. T., AND B. K. BURNISON. 1979. Heterotrophic activity measured with radiolabelled organic substrates, p. 140–155. *In* J. W. Costerton and R. R. Colwell [eds.], Native aquatic bacteria: Enumeration, activity and elogy. Am. Soc. Testing Mater.
———, AND J. E. HOBBIE. 1966. Use of glucose and acetate by bacteria and algae in aquatic ecosystems. Ecology **47:** 447–464.

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A method for determining exoproteolytic activity in natural waters

Abstract—A very sensitive method allowing determination of exoproteolytic activity in natural water is based on the use of aminoacyl-β-naphthylamide, which gives rise to a fluorescent product upon hydrolysis of its peptidelike bond by filtered or unfiltered water samples. Specificity of the method to exoproteases is demonstrated by a strong competitive inhibition effect of added proteins.

A good correlation is found between exoproteolytic activity and the rate of amino acid utilization by microheterotrophs in various aquatic environments.

Organic nitrogen supplied by phytoplanktonic production or decomposition in natural waters consists of about 85% proteins and peptides; only small amounts of free amino acids are directly produced (Billen 1982). Such high molecular weight material, either dissolved or particulate, cannot be directly taken up by bacteria but can only be absorbed after exoenzymatic hydrolysis (Rogers 1961).

Exoproteases can therefore be inferred to play an important role in the nitrogen cycle of aquatic ecosystems. Unfortunately, due to the lack of a convenient and sensitive method, little information is available concerning their occurrence and activity in natural waters. By the use of an insoluble synthetic protein-dye, releasing a soluble color upon enzymatic hydrolysis, some workers (Kim and ZoBell 1974; Little et al. 1979; Meyer-Riel 1981) have demonstrated free exoproteolytic activity in lake or seawater samples. This method, however, is not sensitive enough for rapid measurements and requires either very long incubation times (a few days) or preconcentration of the samples by dialysis or ultrafiltration. Here we report a very sensitive and reliable method of determining exoprotease activity in a few minutes, without any concentration of the sample even in oligotrophic waters.

The method is adapted from the procedure of Roth (1965) in clinical analysis. It is based on the use, as a substrate for proteolytic exoenzymes, of aminoacyl-β-naphthylamide, which gives rise to a fluorescent product after hydrolysis of the peptidelike bond (Fig. 1). The standard procedure is described below. Two milliliters of an unconcentrated water sample, either unfiltered of filtered through a cellulose acetate membrane, are transferred to a sterile quartz fluorimeter cell kept at about 20°C; 50μl of a sterile 40 mM solution of aminoacyl-β-naphthylamide solution is added, and the increase of fluorescence at 410 nm under 340 nm excitation is measured as a function of time over 10–100 min with a Perkin Elmer 2000 fluorimeter.

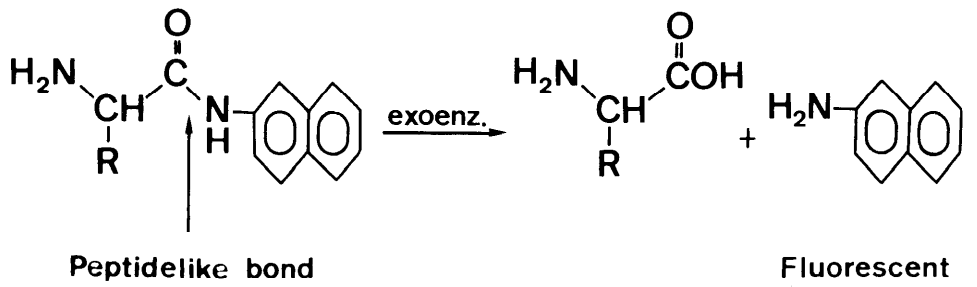

Fig. 1. Hydrolysis of aminoacyl-β-naphthylamide into amino acid and β-naphthylamine.

All results reported here have been obtained with L-leucyl-β-naphthylamide (LLβN: K&K Labs.), although other aminoacyl derivatives are commercially available.

Typical results are presented in Fig. 2. A measurable proteolytic activity was present in all freshly collected unfiltered samples tested, either from offshore seawater or polluted river water. In all cases, autoclaved samples lose their activity. The increase of fluorescence is linear for at least 100 min (Fig. 2). The potential protease activity is expressed as the amount of β-naphthylamine produced per minute of incubation.

The choice of the final LLβN concentration (1,000 μM) in the standard procedure was based on the results of preliminary tests at varying substrate concentrations (Fig. 3). The hydrolysis of LLβN in natural water samples approximately obeyed Michaelis-Menten kinetics, with an apparent K_m close to 100 μM. The concentration used in the standard assay is therefore designed to saturate the exoenzymes present.

Only an apparent K_m value can be giv-

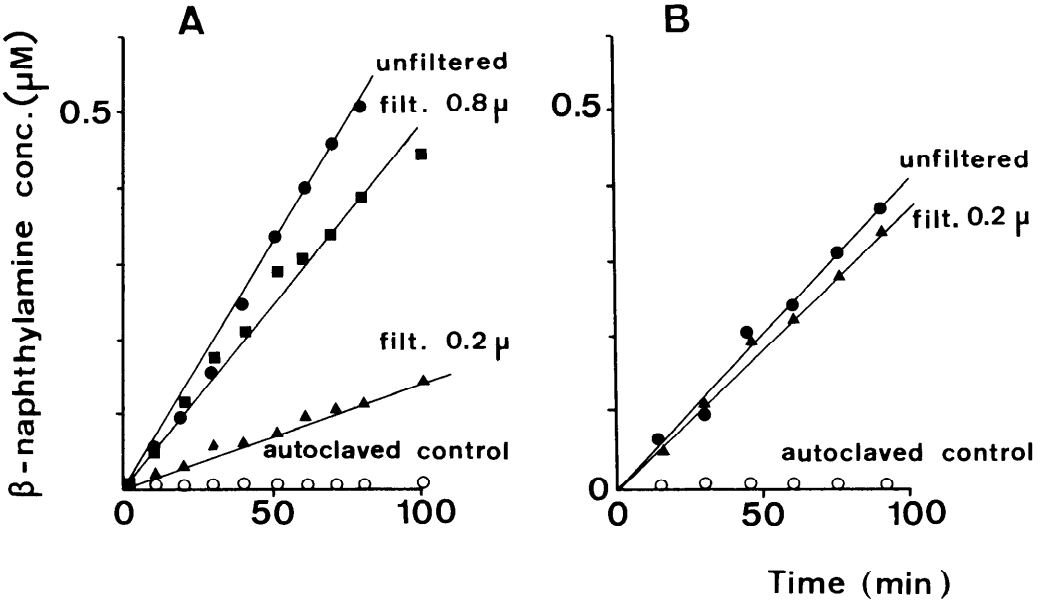

Fig. 2. Kinetics of β-naphthylamine release in filtered and unfiltered water samples after addition of 1 mM L-leucyl-β-naphthylamide. Evolution of β-naphthylamine in autoclaved control is also shown. A—Sample collected in English Channel, off Boulogne, June 1981; B—sample collected in Belgian coastal zone, light vessel *West Hinder*, June 1981.

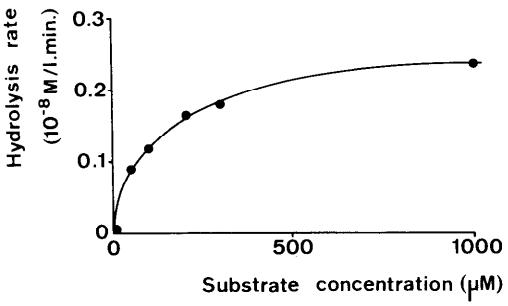

Fig. 3. Effect of varying L-leucyl-β-naphthylamide concentration on its hydrolysis rate in a seawater sample.

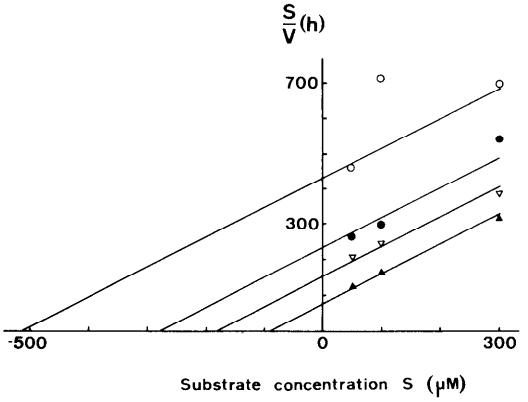

Fig. 4. Inhibitory effect of serum albumin (○—200 μM; ●—100 μM; ▽—50 μM; ▲—0 μM) on the hydrolysis of L-leucyl-β-naphthylamide (reciprocal plot).

en here, as the measurements were made in the presence of another presumably competitive substrate (the natural substrate in the water samples). This possible competition effect by proteins was studied further by adding varying concentrations of serum albumin to the sample. A clear competitive inhibition effect was observed (Fig. 4). The inhibition constant of albumin (which, in the case of competitive inhibition, is equal to its own K_m for the enzymes) is about 50 μM. This indicates that the enzymes responsible for LLβN hydrolysis have a higher but similar affinity for proteins. On the other hand, free amino acids have only a small inhibitory effect on LLβN hydrolysis, the K_i of L-leucine being about 200–300 μM.

Exoenzymes can exist either dissolved in the medium (free exoenzymes) or bound to the external side of the microorganism's membranes (cell-bound exoenzymes) (Pollock 1962). With the method described, proteolytic activity can be determined after gentle filtration of the sample through 2-, 0.8-, or 0.2-μm membranes. The first have been shown by numerous studies in seawater (Derenbach and Williams 1974; Azam and Hodson 1977) to retain most phytoplankton while most free-living bacteria pass them; the last retains all microorganisms. Although size fractionation by filtration does not provide absolute separation of algae and bacteria, it is possible by this procedure to assess with which fraction of the natural microbial community the exoenzymes are associated. As an example, the data of Fig. 2B, obtained with seawater collected in June 1981 in the eutrophic Belgian coastal zone of the North Sea, show that most exoproteases exist there as free dissolved enzymes. In the less productive waters from the eastern English channel at the same period (Fig. 2A), most exoproteases appear to be bound to particles between 0.8 and 0.2 μm and are probably linked to the external surface of bacterial cells, as has been shown for *Cytophaga* by Christison and Martin (1971).

About 50 determinations of potential exoprotease activity are available for various aquatic environments, from rather oligotrophic, as in the English Channel, to hypereutrophic, as in the heavily polluted Scheldt estuary. In some cases, the relative rate of amino acid utilization has been simultaneously determined on a parallel sample by means of a ^{14}C-labeled amino acid mixture (protein hydrolysate: Amersham) (Williams et al. 1976). The free amino acid concentrations being kept fairly constant by the microbial populations do not vary a lot in these various environments (mean concentrations are 0.48, 0.51, and 0.72 in the Scheldt estuary, the coastal North Sea, and the eastern English Channel: Billen et al. 1980; Billen 1982). Because of this the relative rate can be considered a good measure of the total rate of amino acid heterotrophic

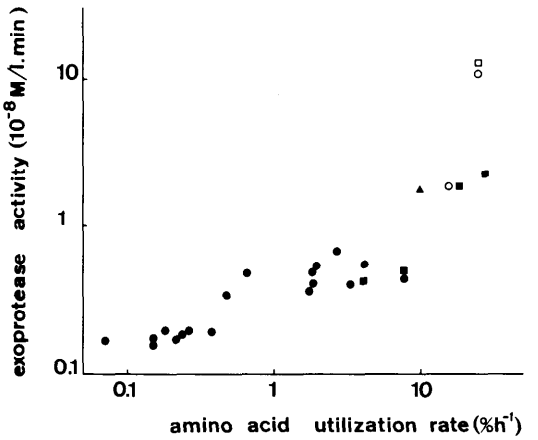

Fig. 5. Relation between exoprotease activity and relative amino acid utilization rate in various aquatic environments. ●—Coastal seas; ▲—eutrophic pond—Bois de la Cambre, Brussels; ■—Oise river, France; ○—Scheldt estuary; □—Rupel river, Belgium.

utilization. Figure 5 shows quite a good correlation between potential exoprotease activity and the rate of amino acid uptake. This observation confirms the obligate role of exoenzymatic activity in the bacterial utilization of organic nitrogen in aquatic environments.

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References

AZAM, F., AND R. E. HODSON. 1977. Size distribution and activity of marine microheterotrophs. Limnol. Oceanogr. **22:** 493–501.

BILLEN, G. 1982. Heterotrophic utilization and regeneration of nitrogen, in press. *In* J. E. Hobbie and P. J. LeB. Williams [eds.], Heterotrophic activity in the sea. Plenum.

———, C. JOIRIS, J. WIJNANT, AND G. GILLAIN. 1980. Concentration and microbiological utilization of small organic molecules in the Scheldt estuary, the Belgian coastal zone of the North Sea and the English Channel. Estuarine Coastal Mar. Sci. **11:** 279–294.

CHRISTISON, A., AND S. M. MARTIN. 1971. Isolation and preliminary characterization of an extracellular protease of *Cytophaga* sp. Can. J. Microbiol. **17:** 1207–1216.

DERENBACH, J. B., AND P. J. WILLIAMS. 1974. Autotrophic and bacterial production: Fractionation of plankton populations by differential filtration of samples from the English Channel. Mar. Biol. **25:** 263–269.

KIM, J., AND C. E. ZOBELL. 1974. Occurrence and activities of cell-free enzymes in oceanic environments, p. 368–385. *In* R. R. Colwell and R. Y. Morita [eds.], Effect of the ocean environment on microbial activities. University Park.

LITTLE, J. E., R. E. SJOGREN, AND G. R. CARSON. 1979. Measurement of proteolysis in natural waters. Appl. Environ. Microbiol. **37:** 900–908.

MEYER-RIEL, L. A. 1981. Enzymatic decomposition of proteins and carbohydrates in marine sediments: Methodology and field observations during spring. Kiel. Meeresforsch. **5:** 311–317.

POLLOCK, M. R. 1962. Exoenzymes, p. 121–178. *In* I. C. Gunsalus and R. Y. Stanier [eds.], The bacteria. v. 4. Academic.

ROGERS, H. J. 1961. The dissimilation of high molecular weight substances, p. 261–318. *In* I. C. Gunsalus and R. Y. Stanier [eds.], The bacteria. v. 2. Academic.

ROTH, M. 1965. Fluorimetric assay of some peptidases. *In* R. Ruyssen and L. Vandendriessche [eds.], Enzymes in clinical chemistry. West-Eur. Symp. Clinical Chem., v. 4. Elsevier.

WILLIAMS, P. J., T. BERMAN, AND O. HOLM-HANSEN. 1976. Amino acid uptake and respiration by marine heterotrophs. Mar. Biol. **35:** 41–47.

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