Vol. 10: 173–179, 1996 | AQUATIC MICROBIAL ECOLOGY
Aquat Microb Ecol | Published May 9

Urea production and turnover following the addition of AMP, CMP, RNA and a protein mixture to a marine sediment

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ABSTRACT: The potential of adenosine 5'-monophosphate (AMP), cytidine 5'-monophosphate (CMP), 16S ribosomal RNA, and a protein (bovine serum albumin) to serve as substrates for bacterial urea production was evaluated in a defaunated, anoxic marine sediment. AMP, CMP and RNA stimulated urea production and urea turnover, but CMP to a lesser degree than AMP and RNA. The increase in urea production and turnover rates took place immediately after AMP, CMP, and RNA were added to the sediment. The rapid response in urea production and turnover rates suggests that the necessary uptake mechanisms and enzymes to utilize the substrates were present constitutively. Addition of the protein mixture did not result in any measurable changes in the urea pool size, urea turnover rate, or urea production rate during the 165 h of incubation. However, an increased and continuous net NH_4^+ production in the protein-amended sediment relative to the control sediment indicated that the added protein mixture was accessible for bacterial degradation. The results showed that purines and pyrimidines were substrates for the bacterial urea production in the marine sediment, whereas protein was not important for urea production.

KEY WORDS: Urea production · Nucleic acid · Protein · Bacteria Marine Sediment

INTRODUCTION

Urea can account for a substantial amount of the nitrogen leaving the sediment (Boucher & Boucher-Rodoni 1988, Lomstein et al. 1989, Lomstein & Blackburn 1992) and can thus be an important nitrogen source for primary production in marine environments (Remsen 1971, McCarthy et al. 1977, Sörensson & Sahlsten 1987, Price & Harrison 1988, Cochlan & Harrison 1991). However, the urea efflux from the sediment only accounts for a minor fraction of total benthic urea production (Lomstein et al. 1989, Therkildsen & Lomstein 1994), of which most is produced by bacteria (Pedersen et al. 1993). Sediment urea production is stimulated by the input of readily degradable organic material, as for instance newly sedimented phytoplankton cells, and by benthic macrofaunal activity (Lomstein et al. 1989). Bacterial urea synthesis can occur via several pathways: (1) the ornithine cycle (Meijer et al. 1990); (2) arginine degradation (Cunin et al. 1986); (3) degradation of purines and pyrimidines (Vogels & Van der Drift 1976, Busse et al. 1984, Gottschalk 1986); and (4) as a by-product in the synthesis of putrescine (Morris & Koffron 1967). The relative importance of these different pathways in marine sediment is unknown.

Novitsky & Karl (1985) found relatively high concentrations of extracellular DNA and RNA in a marine sediment. The concentrations of DNA and RNA were 224 and 124 µg g^{-1} (dry weight), respectively, in the 1.5–3.0 cm horizon in a sandy sediment, and the authors suggested that 75% of these pools were of extracellular origin. Further, a study by Novitsky (1986) showed that these nucleic acid pools were dynamic and that they were turned over rapidly. This

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implies that there is a potential for urea production from these substrates in marine sediments. It has been shown that both amino acids (Burdige 1991) and urea (Lomstein et al. 1989) are important in the production of NH_4^+ in marine sediments, and it has been suggested that urea may be an intermediate in the degradation of amino acids to NH_4^+ (Hansen et al. 1993, Pedersen et al. 1993).

A substrate addition experiment was conducted with defaunated, muddy, anoxic marine sediment to evaluate the potential of AMP (adenosine 5'-monophosphate), CMP (cytidine 5'-monophosphate), RNA (16S ribosomal RNA), and protein (bovine serum albumin) as precursors for bacterial urea production. The concentration, turnover, and production of urea and the concentration of NH_4^+ were followed in the amended and unamended sediment during a 165 h incubation.

MATERIALS AND METHODS

Sampling and sample treatment. Sediment from 1 to 8 cm depth was collected in Clarks Cove, Walpole, Maine, USA. After returning to the laboratory the sediment was passed through a 1 mm sieve to remove macrofauna. The sediment was stored in the dark under anoxic conditions at 15°C for 10 d to eliminate macro- and meiofauna. Absence of animals was confirmed microscopically at the beginning of the experiment.

The sediment was thoroughly mixed and 1053 g of undiluted sediment was transferred to each of five 1 l glass bottles. The bottles were flushed with N_2 to remove O_2, and stored overnight. Anoxic incubation conditions prevented nitrification, and thus faciliated mass balance calculations for nitrogen. The bottles were placed on a bottle roller to ensure continuous mixing of the sediment. Sampling from the bottles was performed with N_2 flushing to avoid oxygenation of the sediment. All experiments were carried out at 24°C and in the dark.

Sediment characteristics. Sediment density, water content, and concentration of total organic carbon and nitrogen were determined at the beginning and the end of the experiment. Water content was determined as the weight loss from sediment dried at 105°C for 24 h. Total organic carbon and nitrogen were determined on H_2SO_3 treated, dried and homogenized sediment in a Carlo Erba NA 1500 C/N analyzer

Addition of substrate. All substrates were obtained from Sigma Chemical Co., and used at the following final concentrations: AMP, 0.95 mM (4.75 mM N); CMP, 0.95 mM (2.85 mM N); RNA, ~1.65 mM nucleotide (~5.9 mM N); protein, ~7.3 mM N. Samples for urea and NH_4^+ concentrations and urea turnover rates were obtained at ~9 h intervals during the first 65 h of incubation. During the remaining incubation period (65 to 165 h) samples were obtained at greater intervals, and only urea and NH_4^+ concentrations were measured.

Urea and NH_4^+ concentrations. Concentrations of urea and NH_4^+ were measured after KCl extraction: 1 ml of 0.8 M KCl was added to ~7.6 g of sediment. The extraction was terminated after 10 min by centrifugation, and the supernatant was frozen for later analysis. Urea concentrations were measured using the diacetylmonoxime method (Price & Harrison 1987), and NH_4^+ concentrations by the salicylate-hypochlorite method (Bower & Holm-Hansen 1980). The KCl treatment of the sediment had no effect on the measured urea pool size.

Urea turnover. Urea turnover rates were measured using a short-term radioassay as described by Lund & Blackburn (1989). At each sampling point, 12 samples of 7.6 g were collected from each treatment, transferred to tubes, and flushed with N_2. Half of the samples were used to follow the change in urea concentration during incubation; the remaining samples were injected with ^{14}C-urea. The ^{14}C-urea incubation was modified slightly from Lund & Blackburn (1989): (1) the incubation was performed as a 3-point time course (~0, 1, 2 h), (2) the activity of the injected 10 µl of tracer was 4.5 nCi µl^{-1}, (3) the incubation was terminated by adding 1 ml 6% NaOH, (4) the scintillation fluid was Scintiverse BD (Fisher Scientific) that was pH adjusted in order to avoid CO_2 evaporation from samples (1 ml of 0.1 M NaOH was mixed with 10 ml of Scintiverse BD). Urea turnover rates were calculated by the nonsteady-state model I and the steady-state model II, respectively, described in Lund & Blackburn (1989). It was not possible to calculate the standard deviation of the urea turnover rates when they were calculated by model I, as this model involves a linear regression of dependent variables. The urea production rate was calculated as the sum of the urea turnover rate and the change in the urea concentration.

RESULTS

Sediment characteristics

The water content of the sediment was 0.57 ml g^{-1} and the density was 1.36 g cm^{-3}. Both parameters remained constant throughout the experiment. Total organic carbon and nitrogen in the sediment were 698 ± 26 µmol C cm^{-3} and 84 ± 4 µmol N cm^{-3}, respectively, at the beginning of the incubations.

Urea concentration

Urea concentrations varied between 1.5 and 3.7 µmol N l^{-1} (average 2.5 ± 0.7 µmol N l^{-1}) in the control sediment, and between 1.8 and 3.7 µmol N l^{-1} (average 2.7 ± 0.5 µmol N l^{-1}) in the protein-amended sediment (Fig. 1). During the first 24 h of incubation, urea increased in the AMP- and RNA-amended sediment to 23.9 and 33.4 µmol N l^{-1}, respectively, whereafter the urea concentrations decreased in the 2 treatments (Fig. 1). The AMP-amended sediment reached the control level after ~77 h of incubation, whereas the urea concentration in the RNA-amended sediment did not reach the control level before the end of the experiment (~165 h). There was a minor initial increase (to 6.6 µmol urea-N l^{-1}) in the CMP-treated sediment, but the urea concentration decreased to control values after ~45 h of incubation.

Urea turnover rates and kinetics

The urea turnover rates varied between 1.5 and 3.3 µmol urea-N l^{-1} h^{-1} (average 2.4 ± 0.7 µmol urea-N l^{-1} h^{-1}) in the control, and between 1.8 and 3.1 µmol urea-N l^{-1} h^{-1} (average 2.6 ± 0.4 µmol urea-N l^{-1} h^{-1}) in the protein-treated sediment (Fig. 2). Urea turnover rates in the AMP, CMP, and RNA treatments reached a maximum after ~23 h of 16.0, 6.5, and 24.2 µmol urea-N l^{-1} h^{-1}, respectively (Fig. 2). After ~65 h incubation the urea turnover rates had declined to 6.0, 0.9, and 4.6 µmol urea-N l^{-1} h^{-1}, respectively.

Urea production

Urea production rates in the AMP, CMP, and RNA treatments reached a maximum after ~23 h of 16.0, 6.5, and 24.2 µmol urea-N l^{-1} h^{-1}, respectively (Fig. 3). The integrated (Σ 0 to 65 h) urea productions were 159 and 177 µmol urea-N l^{-1}, respectively, in the control and protein-amended sediments, and 681, 238, and 852 µmol urea-N l^{-1} in the AMP-, CMP-, and RNA-treated sediments, respectively (Table 1).

NH_4^+ concentration and net NH_4^+ production

The increases of the NH_4^+ concentration in the control and the protein-amended sediment were linear throughout the incubation (Fig. 4). However, the net NH_4^+ production rate was greater in the protein-amended sediment than in the control sediment (20.3 and 5.8 µmol l^{-1} h^{-1}, respectively). There was a dramatic increase in the NH_4^+ concentration in the AMP-

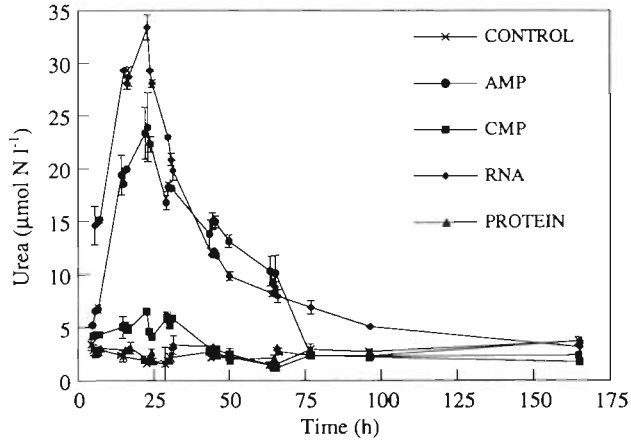

Fig. 1. Changes in the urea concentration in sediment incubations after substrate addition (± standard error)

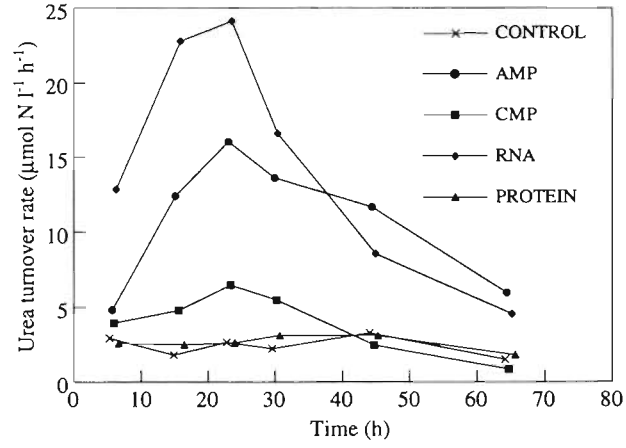

Fig. 2. Changes in the urea turnover rate in sediment incubations after substrate addition

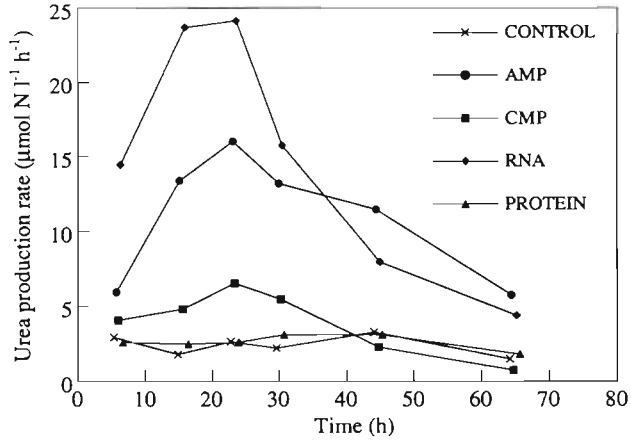

Fig. 3. Changes in the urea production rate in sediment incubations after substrate addition

Table 1. Integrated rate of urea production, urea turnover, and net NH_4^+ production in sediment incubations

Subtrate	Urea production Σ 0 to 65 h	Urea turnover Σ 0 to 65 h	Net NH_4^+ production Σ 0 to 65 h	Σ 0 to 165 h
	(µmol urea-N l^{-1})		(µmol l^{-1})	
Control	159	162	411	894
AMP	681	684	2645	3022
CMP	238	236	1575	2261
RNA	852	862	3531	4117
Protein	177	175	1266	3016

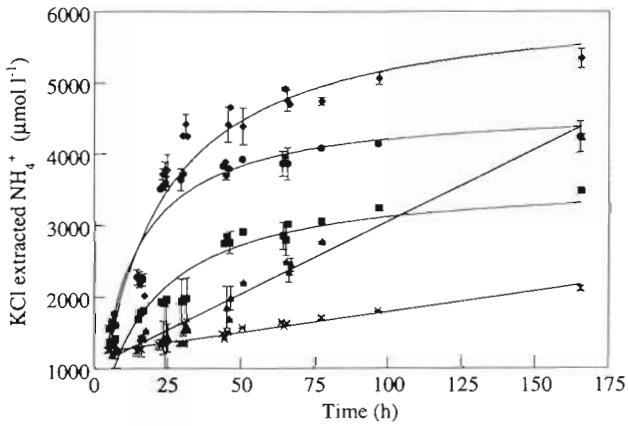

Fig. 4. Changes in the concentration of KCl-extractable NH_4^+ in sediment incubations after substrate addition (± standard error). The following curve fits were made to make the tendencies more obvious: (×) control, $y = 1218 + 5.8x$ (r = 0.98); (●) AMP, $y = 4671x/(x+11)$ (r = 0.96); (■) CMP, $y = 3649x/(x+17)$ (r = 0.93); (◆) RNA, $y = 6155x/(x+19)$ (r = 0.95); (▲) protein, $y = 1037 + 20x$ (r = 0.99)

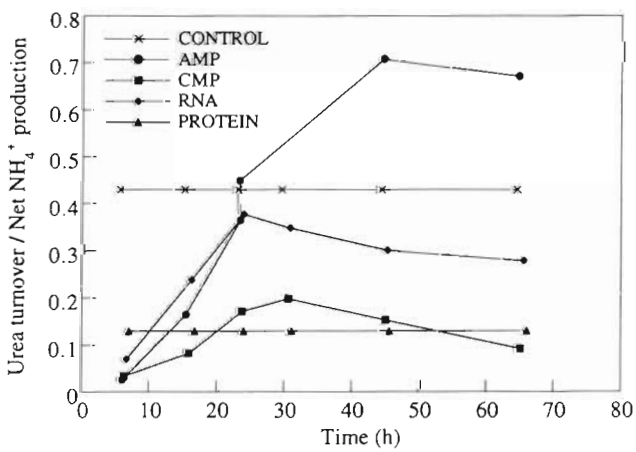

Fig. 5. Changes in the ratio between the urea turnover rate and the net NH_4^+ production rate during the first 65 h in sediment incubations. The ratio in the control sediment was calculated as the average urea turnover rate (2.4 ± 0.7 µmol N l^{-1}) divided by the constant net NH_4^+ production rate

and RNA-treated sediment during the first 30 h of incubation (Fig. 4). In the CMP-treated sediment, net NH_4^+ production was greater in the CMP sediment than in the control during the first 50 h of incubation. During the remaining part of the experiment the net NH_4^+ production rates in the AMP-, CMP-, and RNA-treated sediments were comparable to that of the control sediment (Fig. 4).

The integrated (Σ 0 to 65 h and Σ 0 to 165 h) net NH_4^+ productions were calculated as the change in the NH_4^+ pool size during the time span of interest. The initial NH_4^+ pool size was determined to have been 1218 µM in all sediments (determined from the control curve fit in Fig. 4). The integrated net NH_4^+ production (Σ 0 to 165 h) in the different treatments was 894, 3022, 2261, 4117, and 3016 µmol l^{-1} in the control, AMP-, CMP-, RNA-, and protein-amended sediments, respectively (Table 1).

The ratio between the urea turnover rate and the net NH_4^+ production rate increased from 0.03 (5.7 h) to a maximum of 0.71 (44.5 h) in AMP-treated sediment (Fig. 5). In the CMP- and RNA-treated sediments urea turnover / net NH_4^+ production increased from 0.03 (6.0 h) to 0.20 (30.3 h) and from 0.07 (6.3 h) to 0.38 (23.7 h), respectively (Fig 5). The ratio between the urea turnover rate and the net NH_4^+ production rate remained constant at 0.43 and 0.13 in the control and protein-amended sediments, respectively.

DISCUSSION

Urea production from AMP, CMP and RNA

The rapid increase in urea production and turnover after the addition of AMP, CMP, and RNA (Figs. 2 & 3) indicates that mineralization of these substrates is coupled to urea dynamics *in vitro*, and that extracellular nucleic and polynucleic acids may serve as urea precursors *in situ*. Urea production and turnover rates in the AMP, CMP and RNA treatments were within the range reported for a shallow estuary in July, where the turnover rate of urea was >350 nmol cm^{-3} d^{-1} within the upper 1 cm of the sediment (Therkildsen & Lomstein 1994) Therkildsen & Lomstein (1994) concluded that urea production was stimulated by the availability of high quality organic material (low C/N) and temperature. The most likely source of organic matter input to the sediment in the Therkildsen & Lomstein (1994) study was benthic and pelagic microalgae cells. Based on the present study it is probable that it was degradation of microalgal RNA (+DNA) that stimulated urea

production in the Therkildsen & Lomstein (1994) study. The amount of substrate RNA added in the present experiment was equivalent to a potential increase in sediment RNA+DNA due to a phytoplankton sedimentation event. Based on the content of RNA+DNA in a *Euglena* sp. cell (Smillie & Krotkov 1960), an algal carbon content of 10% of algal dry weight, an annual primary production of 300 g C m^{-2} (typical for Danish coastal waters) and an assumed 2 mm thick sediment surface zone that is affected by algal sedimentation, we calculated that the addition of RNA to the sediment in the present experiment was equivalent to an algal input of 2.9 g C m^{-2} (~1% of the annual primary production). However, the stimulation of urea production and turnover by the addition of AMP and CMP may not have been representative of natural conditions, as the input of AMP and CMP to the sediment from microalgal cells can be expected to be much lower than the addition made in the present experiment. The stimulation of urea turnover by the addition of these substrates should therefore only be considered as indicative of anaerobic bacterial urea production from these substrates.

The turnover of bacterial intracellular nucleic acids, especially RNA, may also have contributed to urea production in addition to the turnover of extracellular nucleic acids taken up by the bacterial cells. Cellular RNA is degraded continuously during exponential growth and this may lead to excretion of urea or NH_4^+. Culture experiments with the marine bacterium *Thiosphaera pantotropha* grown on a minimal medium showed that urea was excreted at a rate proportional to cell density during exponential growth, and that urea production continued after the bacteria entered the stationary phase (Pedersen et al. 1993). Similar results have been obtained in other culture studies (Therkildsen et al. unpubl.), supporting the idea that degradation of intracellular nucleic acids may contribute to urea production.

Degradation pathways of AMP, CMP, and RNA

Bacteria degrade purines (e.g. AMP) by 2 pathways under anoxic conditions, one of which gives rise to NH_4^+ (Gottschalk 1986), the other to NH_4^+ and urea (Busse et al. 1984, Kaspari & Busse 1986). Both pathways appeared functional in our experiment, as more NH_4^+ was produced within the first 65 h of incubation than could be accounted for by urea production and turnover. Urea turnover (Σ 0 to 65 h) in the AMP-treated sediment [urea turnover (AMP) – urea turnover (control)] only accounted for 23% of the corresponding net NH_4^+ production. The ratio between urea turnover and NH_4^+ production might, however, have been even lower if gross NH_4^+ production had been considered instead of net NH_4^+ production. One has to keep in mind that net NH_4^+ production did not include the fraction of NH_4^+ production that was incorporated into bacterial biomass. Blackburn & Henriksen (1983), in a study of nitrogen cycling in different types of sediments from Danish waters, found that NH_4^+ incorporation into bacterial biomass varied between 22 and 83% of gross NH_4^+ production within the upper 2 cm of the sediment. Approximately 11% of the added AMP-N was mineralized to NH_4^+ with urea as an intermediate during the first 65 h of incubation. However, this fraction was probably higher at the end of the experiment (165 h), since urea production and turnover were still elevated in the AMP-treated sediment compared to the control at the final point of urea turnover measurements (65 h). Previous investigations have shown that up to 80% of added AMP-N can be degraded through urea (Busse et al. 1984, Kaspari & Busse 1986).

Anaerobic pyrimidine degradation produces urea and NH_4^+ via an oxidative pathway, while only NH_4^+ is produced by a reductive pathway (Vogels & Van der Drift 1976, Gottschalk 1986). The reductive pathway probably dominated in the present study, as urea turnover in the CMP-treated sediment (Σ 0 to 65 h) only accounted for 6% of the integrated net NH_4^+ production. Further, only 3% of the added CMP-N was degraded via urea after 65 h incubation compared to at least 41% via NH_4^+. A theoretical maximum of 67% of the added CMP-N can potentially be degraded through urea if CMP is degraded through the oxidative pathway (Vogels & Van der Drift 1976).

Approximately 12% of the added RNA-N was degraded via urea in the RNA-treated sediment during the first 65 h of incubation. Further, the excess urea turnover (Σ 0 to 65 h) could explain 22% of the corresponding net NH_4^+ production.

The ratio between the urea turnover rate and the net NH_4^+ production rate was much lower in the AMP-, CMP-, and RNA-treated sediment during the first 65 h of the experiment (~0.04) than in the control sediment (0.4; Fig. 5). This was due to very high net NH_4^+ production rates. These high net NH_4^+ production rates were probably a result of deamination, which is the initial step in the degradation of both adenine and cytosine (Vogels & Van der Drift 1976, Busse et al. 1984, Gottschalk 1986, Kaspari & Busse 1986). Urea turnover gradually increased relative to net NH_4^+ production as degradation progressed. In the CMP-treated sediment, however, the urea turnover remained low relative to the net NH_4^+ production (0.2). This is in agreement with the predominance of the reductive degradation pathway for CMP. In contrast, the urea turnover rate could account for a greater fraction of the net NH_4^+ production rate in the AMP-treated sediment than in the control after ~30 h.

Net NH_4^+ production rates and urea concentrations in the AMP-, CMP-, and RNA-treated sediments equaled those in the control at the end of the experiment (165 h). Thus, the urea production rates at the end of the experiment were probably similar to the rate in the control. Although mineralization of the exogenous AMP, CMP, and RNA apparently had ceased, the integrated excess net NH_4^+ production (Σ 0 to 165 h) in the AMP-, CMP-, and RNA-treated sediments only accounted for 45, 48 and ~55%, respectively, of the added N. This is comparable to results obtained by Novitsky (1986), who found that 62 to 72% of added RNA was degraded in a marine sediment within 14 d and that most of the degradation took place within the first 3 to 7 d.

There are, however, several explanations for the less than 100% recovery of added substrates in the integrated net NH_4^+ production: (1) as already mentioned, part of the added N may have been incorporated into microbial biomass; (2) adsorption of substrates to sediment (e.g. Greaves & Wilson 1969, Lorenz & Wackernagel 1987); and (3) degradation to N-compounds other than urea and NH_4^+.

Protein mixture (bovine serum albumin)

The addition of a protein mixture did not result in any measurable changes in urea concentration, urea turnover or urea production (Figs. 1, 2 & 3). There was, however, an immediate increase in the concentration of KCl-extractable NH_4^+. Further, the net NH_4^+ production rate in the protein-treated sediment was about 3.5 times higher than in the control. The integrated net NH_4^+ production during the entire experiment (0 to 165 h) was equal to 29% of added protein-N and the NH_4^+ production rate was still elevated compared to the control, when the experiment was terminated. The increased and continuous NH_4^+ production during the entire time span of incubation indicates that the added protein was readily available for bacterial degradation. Further, the protein addition (7.3 mM N = 5.7 µmol-N cm^{-3}) was comparable to the content of total acid hydrolyzable amino acids (THAA-N) in a Danish coastal sediment, where THAA-N varied between 22 and 37 µmol-N cm^{-3} within the upper 6 cm (Lomstein et al. unpubl.). Thus, protein addition did not alter the ambient protein pool significantly.

Amino acid N can be metabolized to urea through the energy-requiring ornithine and citric acid cycles (Meijer et al. 1990). The degradation of the amino acid arginine, which is the precursor for urea in the ornithine cycle, can, however, lead to the formation of urea without an energy cost. Some bacteria have been found to possess a complete ornithine cycle (Gruninger & Goldman 1988). At present, the relative importance of urea production in marine sediments via the ornithine cycle or arginine degradation is unknown. However, if amino acids and proteins in general are degraded as was the bovine serum albumin in the present experiment, the urea production from protein or amino acids is of minor importance in anoxic marine sediments.

Conclusion

There was an immediate increase in urea production, urea turnover and net NH_4^+ production due to the addition of RNA, AMP and CMP to an anoxic marine sediment. This indicates that the natural bacteria population possessed the necessary uptake mechanisms and enzymes to utilize the added substrates. The stimulated urea production and turnover rates were comparable to rates obtained in surface sediment from a Danish estuary during summer and it was inferred that stimulation of *in situ* urea production may be due to RNA input from microalgal cells. Urea production and turnover were not stimulated by the addition of a bovine serum albumin protein mixture to the sediment. This indicates that urea was not an important intermediate in protein turnover.

Acknowledgements. We thank Sylvia Schnell, Peter Roslev and Cem Giray for helpful assistance during the experiments. Financial support was obtained from the Danish Environmental Protection Agency, Hav90, Grant no. 3.25 and from the Centre for Strategic Environmental Research in Marine Areas, Grant no. 4.15.

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Responsible Subject Editor: T. H. Blackburn, Aarhus, Denmark

Manuscript first received: October 24, 1995
Revised version accepted: March 22, 1996