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Denitrification in estuarine sediment stimulated by the irrigation activity of the amphipod *Corophium volutator*

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ABSTRACT: Sediment with different densities of *Corophium volutator* (Pallas), ranging from 0 to 19800 ind. m^{-2}, were incubated in laboratory microcosms, and rates of oxygen uptake, denitrification and nitrate ammonification were determined from sediment-water fluxes. The measured processes were stimulated differently by *C. volutator*; oxygen uptake, denitrification of NO_3^- from nitrification within the sediment, and denitrification of NO_3^- from the overlying water were enhanced 2-, 3- and 5-fold respectively in the presence of 19800 ind. m^{-2}. This differential stimulation was explained by the different characteristics of diffusional solute transport at the sediment-water interface and mass transfer of water into the burrows where O_2 and NO_3^- was depleted. Denitrification rates were calculated by using the ^{15}N isotope pairing technique. The applicability of the ^{15}N isotope pairing technique for measuring coupled nitrification-denitrification in bioturbated sediment was confirmed in a test incubation with different levels of $^{15}NO_3^-$ added to microcosms with 12000 *C. volutator* ind. m^{-2}

KEY WORDS: Denitrification · Bioturbation · Estuarine sediment

INTRODUCTION

Nitrogen has been identified as the most limiting nutrient for phytoplankton production in coastal seas (e.g. Ryther & Dunstan 1971) and denitrification may therefore control eutrophication by eliminating available nitrogen through NO_3^- reduction to N_2O and N_2 (e.g. Nixon et al. 1976, Seitzinger 1980). Bottom-dwelling animals affect both physical and chemical processes taking place in the sediment through burrow construction, bioturbation and irrigation (Rhoads 1974, Aller 1988, Kristensen 1988). The objective of the present study was to measure the effect of bioturbation by the amphipod *Corophium volutator* (Pallas) on denitrification in estuarine sediments.

Corophium volutator lives in 'U'-shaped burrows 2 to 6 cm deep that are continuously irrigated. The burrow represents an extension of the sediment surface, and thus of all processes taking place at the surface (Hylleberg & Henriksen 1980, Kristensen 1984). The burrow wall is often a site of high bacterial numbers and metabolic activity compared to bulk sediment (Aller & Yingst 1978, Henriksen et al. 1983, Koike & Mukai 1983, Kristensen et al. 1985, 1991, Kristensen & Blackburn 1987). Higher potential nitrification rates have been reported in *C. volutator*'s burrow wall relative to surface sediment, suggesting better conditions for nitrifiers (Henriksen et al. 1983). The same authors indicated that *C. volutator*'s excretion rates could account for 80% of the net NH_4^+ flux from the sediment and eventually constitute an important pool of NH_4^+ for nitrifying bacteria. Irrigation of the burrow increases NO_3^- transport from the water column into the sediment and thus denitrification of NO_3^- coming from the overlying water (Aller 1988, Kristensen 1988), while O_2 transport to the burrow lining may increase nitrification (Aller 1988, Kristensen 1988) and thus denitrification of NO_3^- generated within the sediment. Henriksen et al. (1980) reported, from indirect calculations, higher nitrification and denitrification rates in sediment bioturbated by *C. volutator* (6000 ind. m^{-2}) relative to non-bioturbated sediment.

The isotope pairing technique (Nielsen 1992), involving $^{15}NO_3^-$, makes possible the measurement of denitrification of NO_3^- coming from both sources: the overlying water (dw) and nitrification within the sediment (dn). Oxygen consumption, dw and dn rates were measured and compared in sediment microcosms containing different amphipod densities and with different NO_3^- concentrations in the overlying water.

MATERIAL AND METHODS

Test incubation. A small incubation series was set up to test the applicability of the denitrification assay in sediment bioturbated by *Corophium volutator*. Specimens approximately 3 to 5 mm in length were collected in a shallow mesohaline estuary (Norsminde Fjord, Denmark). The amphipods were placed in cores (13 cm long and 3.4 cm inner diameter, containing 6 cm intact sandy sediment), at a density of 12000 ind. m^{-2}. The amphipods dug immediately into the sediment. The microcosms, covered with a 340 µm net to prevent the specimens from escaping, were placed in a reservoir containing aerated *in situ* water (24‰ salinity), and kept in darkness at 13°C. After 1 d, $^{15}NO_3^-$ was added to each core to give concentrations ranging from 15 to 330 µM NO_3^- and the cores were stoppered. Incubation time was around 80 min for all the cores, ensuring a maximum oxygen depletion of 20%. Stoppers were removed at the end of the incubation and $ZnCl_2$ (240 µl 50% solution) was added to stop microbial activity. Water column and porewater was gently mixed with a stick, and samples of the slurry were stored in 12 ml glass tubes containing 2% $ZnCl_2$ closed with gastight caps. Two days later, N_2 gas was extracted from these water samples with 1 ml argon before analysis.

Main incubation. *Corophium volutator* was distributed in artificial microcosms containing homogenized and sieved (500 µm mesh) silty sediment. Three different sets of microcosms were used: 0, 6600 (normal density) and 19800 (high density) ind. *C. volutator* m^{-2}. The experiment was run at 11°C. Oxygen fluxes were measured regularly to ensure steady state conditions before the experiment was conducted (results not shown). Twelve days after setup, $^{15}NO_3^-$ was added to the reservoir to give 72 ± 2 µM NO_3^- (80 ± 1% $^{15}NO_3^-$). One day later an incubation was carried out. The cores were stoppered during a period ranging from 0.5 to 4 h (to assure maximum 20% oxygen depletion). At the end of the incubation the stoppers were removed and 30 ml water samples were taken with a 50 ml plastic syringe. Dissolved N_2 was extracted with 5 ml argon and collected in a pre-evacuated 3.4 ml Venoject blood collection tube (Risgaard-Petersen et al. 1993). Water samples for analyzing NO_3^-, $\%^{15}NO_3^-$, and $^{15}NH_4^+$ were collected and frozen. The water in the reservoir was subsequently exchanged with water containing 152 ± 7 µM NO_3^- (90% $^{15}NO_3^-$) and the microcosms were incubated again following the same steps as described above. Mortality at the end of the experiment (17 d after setup) was around 20% of the initial number of amphipods introduced. Dead amphipods were removed each day from the microcosms.

Analysis. Oxygen was measured directly in the microcosms with an oxygen microsensor provided with a guard cathode (Revsbech 1989) and NO_3^- plus NO_2^- by standard methods (Grasshoff et al. 1983) in a flow injection analyzer (Tecator, Höganäs, Sweden). The formation of ^{15}N-labelled dinitrogen pairs ($^{14}N^{15}N$ and $^{15}N^{15}N$) by denitrification was measured on an isotope ratio mass spectrometer as described by Nielsen (1992). The $\%^{15}NO_3^-$ enrichment in the water phase was analyzed on a mass spectrometer after using denitrifying bacteria cultures to transform NO_3^- to gaseous N_2 (Risgaard-Petersen et al. 1993). Accumulation of $^{15}NH_4^+$ in the water phase was analyzed by mass spectrometry after NH_4^+ in the samples was liberated as NH_3 into a headspace (after addition of an strong base), captured in a capsule containing acidified Al_2O_3, and converted to dinitrogen by heating the sample (Blackburn 1993). Due to the small concentrations, the water samples were enriched with 3 µmol unlabelled NH_4^+ to obtain sufficient N for analysis on the mass spectrometer.

Calculations. Denitrification rates were calculated by using the ^{15}N isotope pairing technique (Nielsen 1992), which assumes that addition of $^{15}NO_3^-$ does not influence denitrification of the natural $^{14}NO_3^-$ and that the NO_3^- species are uniformly mixed in the denitrification zone. The formation rates of single-labelled ($^{14}N^{15}N$) and double-labelled ($^{15}N^{15}N$) dinitrogen pairs were used to calculate dw and dn:

$$d_{15} = (^{14}N^{15}N) + 2(^{15}N^{15}N) \quad \text{(Koike \& Hattori 1978);}$$

$$d_{14} = d_{15}\frac{(^{14}N^{15}N)}{2(^{15}N^{15}N)};$$

$$dw = d_{15}\frac{100}{e_{15}};$$

$$dn = d_{14} + d_{15} - dw \quad \text{(Nielsen 1992)}$$

where e_{15} is the $\%^{15}NO_3^-$ enrichment of the reservoir water.

An analysis of variance (ANOVA) was carried out to test the significance of the differences between treatments.

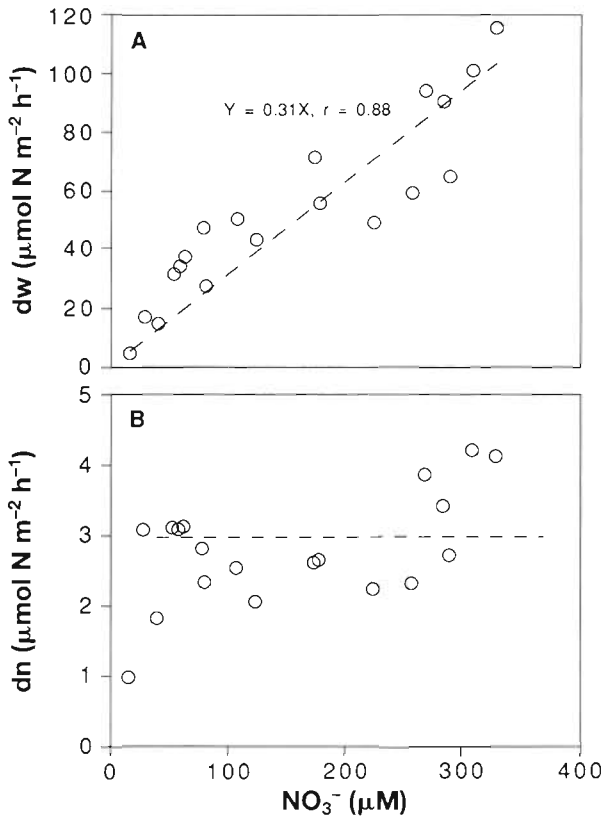

Fig. 1 *Corophium volutator*. Test incubation. Denitrification rates in intact sediment cores containing 12000 ind. m^{-2}, versus NO_3^- concentration in the overlying water. Denitrification of NO_3^- (A) coming from the overlying water (dw) and (B) produced within the sediment by nitrification (dn). Each data point represents 1 core

RESULTS

Test incubation

Fig. 1A, B shows the calculated rates of denitrification of NO_3^- coming from the overlying water (dw) and NO_3^- generated within the sediment (dn). Denitrification of NO_3^- coming from the overlying water was linearly correlated with NO_3^- concentration in the overlying water ($r = 0.88$) in the test incubation with 12000 ind. *Corophium volutator* m^{-2} (Fig. 1A). The calculated rates of dn were independent of the concentration of NO_3^- present in the overlying water at concentrations above 40 µM (slope not significantly different from zero, $p = 0.05$) (Fig. 1B). Oxygen consumption rates (not shown) were 1200 ± 200 µmol O_2 m^{-2} h^{-1}.

Main incubation

Oxygen consumption rates and denitrification rates were higher in bioturbated than in non-bioturbated

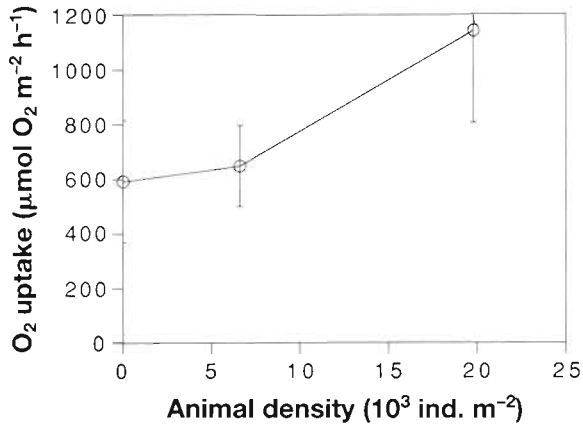

Fig. 2. *Corophium volutator*. Main incubation. Oxygen consumption rates (± SE) in cores with densities ranging from 0 to 19800 ind. m^{-2}

sediments (Figs. 2, 3 & 4). Both oxygen uptake and dn rates were significantly higher in microcosms containing 19800 ind. m^{-2} than in microcosms without amphipods ($p < 0.001$), while no statistically significant difference was found between the rates measured in microcosms containing 0 and 6600 ind. m^{-2} ($p = 0.1$) (Figs. 2 & 4). Oxygen consumption rates by *Corophium volutator*, calculated from the literature (McLusky 1969, Birklund 1977), indicated that the amphipod metabolism accounted for less than 2% of the total sediment oxygen uptake in our bioturbated microcosms. Denitrification of NO_3^- coming from the overlying water also increased with increasing NO_3^- concentration in the overlying water (from 0 to 152 µM) (Fig. 3). Apparent rates of denitrification of NO_3^- generated within the sediment were not affected by the NO_3^- concentration present in the overlying water ($p = 0.05$) but increased with amphipod density

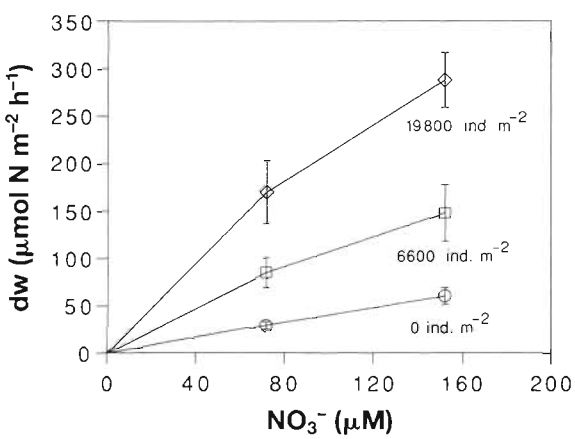

Fig. 3. *Corophium volutator*. Main incubation. Denitrification rates of NO_3^- coming from the overlying water (dw ± SE) related to NO_3^- concentration in the water phase, in artificial microcosms with densities of 0, 6600 and 19800 ind. m^{-2}

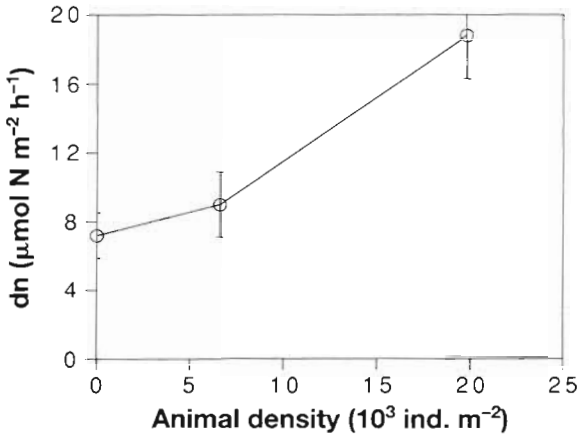

Fig. 4. *Corophium volutator*. Main incubation. Denitrification rates of NO_3^- generated within the sediment (dn ± SE) versus density

(Fig. 4). Nitrate ammonification (Fig. 5), only measured at the highest NO_3^- concentration, was also higher at greater *C. volutator* densities. Less than 5% of the $^{15}NO_3^-$ reduced was recovered as $^{15}NH_4^+$. Denitrification was shown to be the only significant NO_3^- reducing pathway in these sediments.

Oxygen consumption, dn and dw were stimulated 2-, 3- and 5-fold in sediment inhabited by *Corophium volutator* (19800 ind. m^{-2}) when compared with non-bioturbated sediment (Fig. 6).

DISCUSSION

Calculation of coupled nitrification-denitrification rates by the ^{15}N isotope pairing technique assumes uniform mixing of both NO_3^- species ($^{15}NO_3^-$ and

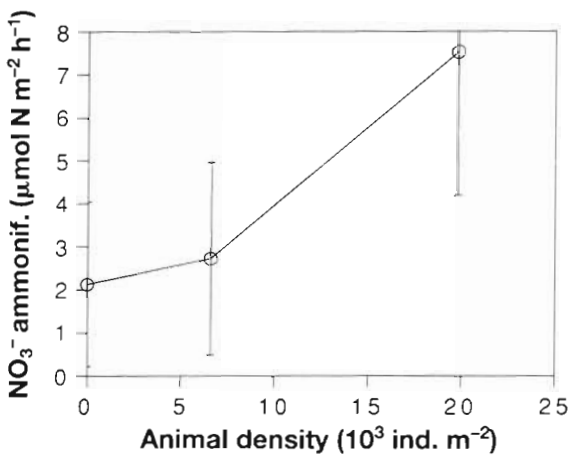

Fig. 5. *Corophium volutator*. Main incubation. Nitrate ammonification rates (± SE) versus density in microcosms containing 152 µM NO_3^- in the overlying water

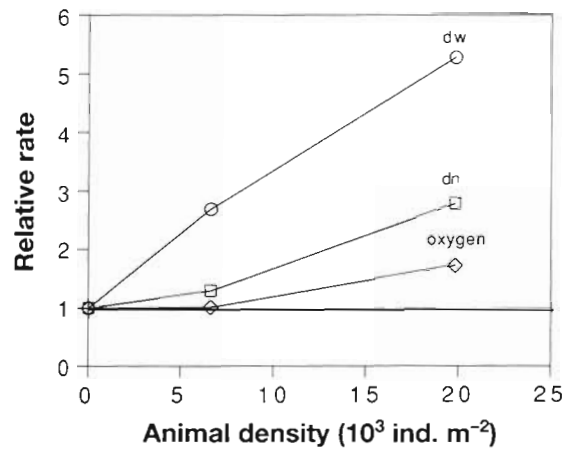

Fig. 6. *Corophium volutator*. Main incubation. Relative stimulation of O_2 uptake, denitrification of NO_3^- coming from the overlying water (dw) and denitrification of NO_3^- produced within the sediment by nitrification (dn), by amphipods at different densities. The relative stimulations are rates measured in bioturbated microcosms divided by rates measured in non-bioturbated microcosms. The thick line at a relative rate of 1 represents amphipod-free microcosms

$^{14}NO_3^-$) in the denitrification zone (Nielsen 1992). This is because production of unlabelled N_2 ($^{14}N^{14}N$) by denitrification is not measured directly but estimated from the production of single-labelled N_2 ($^{15}N^{14}N$) relative to double-labelled N_2 ($^{15}N^{15}N$). Bioturbation may create local or temporal variations in the ratio of dw to dn, thus questioning the assumption of the technique. The possible underestimation of dn can be tested in incubations with different concentrations of $^{15}NO_3^-$ (Nielsen 1992): at higher $^{15}NO_3^-$ levels, more $^{14}NO_3^-$ will be paired with $^{15}NO_3^-$ to form measurable $^{15}N^{14}N$ and less of the immeasurable $^{14}N^{14}N$ is formed. The degree of underestimation will therefore diminish at higher $^{15}NO_3^-$ levels. Too high $^{15}NO_3^-$ concentrations, however, will affect the assumed first-order kinetics of denitrification, therefore the optimal $^{15}NO_3^-$ concentration range has to be tested. The test of the present sediment with *Corophium volutator* showed that $^{15}NO_3^-$ concentrations above 40 µM did not affect the estimate of dn, an indication that the heterogeneity effect was eliminated and the estimates represented true dn rates (Fig. 1B). First-order kinetics were confirmed by the linear correlation between dw and NO_3^- concentration up to 330 µM NO_3^- (Fig. 1A). The NO_3^- concentrations of 72 and 152 µM applied in the main experiments were consequently within the optimal range of 40 to 330 µM.

Oxygen uptake, dn and dw were enhanced 2-, 3- and 5-fold in the presence of 19800 ind. m^{-2} (Fig. 6). This differential stimulation showed that the amphipod burrows could not be considered as a simple extension of the sediment surface with associated processes. The

observed pattern could be explained by differences between diffusional transport of O_2 and NO_3^- in the sediment surface and mass transport of O_2 and NO_3^- with water pumped through amphipod burrows: diffusion of NO_3^- at the sediment surface from the water column to the anoxic zone of denitrification is impeded by the presence of the oxic layer acting as a diffusion barrier. Model calculations confirmed by field studies in stream sediment have shown that the uptake of NO_3^- relative to the NO_3^- concentration therefore should be about one-third of the O_2 uptake relative to the O_2 concentration (Christensen et al. 1990). In the amphipod burrows, however, NO_3^- and O_2 should be consumed with the same efficiency provided that both NO_3^- and O_2 are depleted from burrow water as it passes through the sediment. Bioturbation by *Corophium volutator* should therefore stimulate denitrification of NO_3^- from the water 3 times more than O_2 consumption, reasonably consistent with the actually observed 4 times higher stimulation (Fig. 6). Coupled nitrification-denitrification depends on the transport of NO_3^- from the oxic nitrification zone to the anoxic denitrification zone. Microsensor studies and flux measurements have indicated that nitrification activity usually is evenly distributed in the oxic surface zone of sediment, and about half the NO_3^- produced diffuses down and is denitrified and half diffuses up into the overlying water column (Blackburn & Henriksen 1983, Jensen et al. 1993). In the burrows, NO_3^- diffusing out from the oxic nitrification zone of the walls is transported further through the burrow and is eventually denitrified; i.e. the coupling of nitrification and denitrification will be 100%. Assuming that nitrification constitutes a constant fraction of sediment oxygen consumption, bioturbation should thus stimulate nitrification and oxygen consumption to the same extent, while coupled nitrification-denitrification should increase twice as much. The measurements indeed showed 2 times more stimulation of coupled nitrification-denitrification than of oxygen consumption in the presence of 19 800 ind. m^{-2} (Fig. 6).

This study has shown that *Corophium volutator* increases not only the absolute rate of denitrification but also denitrification relative to oxygen consumption, particularly when NO_3^- from the overlying water is the prime source. *C. volutator* is one of the dominating species in the benthic fauna of Norsminde Fjord, with common densities of 6000 ind. m^{-2} and high densities up to 65 000 ind. m^{-2} (Thorson 1975), and because of riverine discharge, the water phase concentration of NO_3^- is often high (up to 600 µM) (Binnerup et al. 1992). This will indicate that different abundances of the amphipod through the same and successive years could have an important effect on nitrogen retention in the estuary.

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LITERATURE CITED

Aller, R. C. (1988). Benthic fauna and biogeochemical processes in marine sediments: the role of burrow structures. In: Blackburn, T H., Sørensen, J. (eds.) Nitrogen cycling in coastal marine environments. John Wiley and Sons, Ltd, Chichester, p. 301–338

Aller, R. C., Yingst, J. Y. (1978). Biogeochemistry of tube-dwellings: a study of the sedentary polychaete *Amphitrite ornata*. J. mar. Res. 36: 201–254

Binnerup, S. J., Jensen, K., Revsbech, N. P., Jensen, M. H., Sørensen, J. (1992). Denitrification, dissimilatory reduction of nitrate to ammonium, and nitrification in a bioturbated estuarine sediment as measured with ^{15}N and microsensor techniques. Appl. environ. Microbiol. 58: 303–313

Birklund, J. (1977). Biomass, growth and production of the amphipod *Corophium insidiosum* Crawford, and preliminary notes on *Corophium volutator* (Pallas). Ophelia 16: 187–203

Blackburn, T. H. (1993). Turnover of $^{15}NH_4^+$ tracer in sediments. In: Kemp, P., Sherr, B. F., Sherr, E. B., Cole, J. J. (eds.) Current methods in aquatic microbial ecology. Lewis Publishers, New York, p. 643–645

Blackburn, T. H., Henriksen, K. (1983). Nitrogen cycling in different types of sediments from Danish waters. Limnol. Oceanogr. 28: 477–493

Christensen, P. B., Nielsen, L. P., Sørensen, J., Revsbech, N. P. (1990). Denitrification in nitrate-rich sediments: diurnal and seasonal variation related to benthic oxygen metabolism. Limnol. Oceanogr. 35: 640–651

Grasshoff, K., Erhardt, M., Kremling, K. (1983). Methods of seawater analysis. Basel Verlag Chemie, Deerfield Beach, FL

Henriksen, K., Hansen, J. I., Blackburn, T. H. (1980). The influence of benthic infauna on exchange rates of inorganic nitrogen between sediment and water. Ophelia Suppl. 1: 249–256

Henriksen, K., Rasmussen, M. B., Jensen, A. (1983). Effect of bioturbation on microbial nitrogen transformations in the sediment and fluxes of ammonium and nitrate to the overlying water. Ecol. Bull. 35: 193–205

Hylleberg, J., Henriksen, K. (1980). The central role of bioturbation in sediment mineralization and element re-cycling. Ophelia Suppl. 1: 1–16

Jensen, K., Revsbech, N. P., Nielsen, L. P. (1993). Microscale distribution of nitrification activity in sediment determined with a shielded microsensor for nitrate. Appl. environ. Microbiol. 59: 3287–3296

Koike, I., Hattori, A. (1978). Simultaneous determination of nitrification and nitrate reduction in coastal sediments by a ^{15}N dilution technique. Appl. environ. Microbiol. 35: 853–857

Koike, I., Mukai, H. (1983). Oxygen and inorganic nitrogen contents and fluxes in burrows of the shrimps *Callianassa japonica* and *Upogebia major*. Mar. Ecol. Prog. Ser. 12: 185–190

Kristensen, E. (1984). Effect of natural concentrations on nutrient exchange between a polychaete burrow in estuarine sediment and the overlying water. J. exp. mar. Biol. Ecol. 75: 171–190

Kristensen, E. (1985). Oxygen and inorganic nitrogen exchange in a *Nereis virens* bioturbated sediment-water system. J. coast. Res. 1: 109–116

Kristensen, E. (1988). Benthic fauna and biogeochemical processes in marine sediments: microbial activities and fluxes. In: Blackburn, T. H., Sørensen, J. (eds.) Nitrogen cycling in coastal marine environments. John Wiley and Sons, Ltd, Chichester, p. 275–299

Kristensen, E., Blackburn, T. H. (1987). The fate of organic carbon and nitrogen in experimental marine sediment systems: influence of bioturbation and anoxia. J. mar. Res. 45: 231–257

Kristensen, E., Jensen, M. H., Aller, R. C. (1991). Direct measurement of dissolved inorganic nitrogen exchange and denitrification in individual polychaete (*Nereis virens*) burrows. J. mar. Res. 49: 355–377

Kristensen, E., Jensen, M. H., Andersen, T K. (1985). The impact of polychaete (*Nereis virens*) burrows on nitrification and nitrate reduction in estuarine sediments. J. exp. mar. Biol. Ecol. 85: 75–91

McLusky, D. S. (1969). The oxygen consumption of *Corophium volutator* in relation to salinity. Comp. Biochem. Physiol. 29: 743–753

Nielsen, L. P. (1992). Denitrification in sediment determined from nitrogen isotope pairing. FEMS Microbiol. Ecol. 86: 357–362

Nixon, S. W., Oviatt, C. A., Hale, S. S. (1976). Nitrogen regeneration and the metabolism of coastal marine bottom communities. In: Andersen, J. M., MacFadyen, A. (eds.) The role of terrestrial and aquatic organisms in decomposition processes. Blackwell Scientific Publications, Oxford, p. 269–283

Revsbech, N. P., Christensen, P. B., Nielsen, L. P., Sørensen, J. (1989). Denitrification in a trickling filter biofilm studied by a microsensor for oxygen and nitrous oxide. Water Res. 23: 867–871

Rhoads, D. C. (1974). Organism-sediment relations on the muddy sea floor. Oceanogr. mar. Bull. A. Rev. 12: 263–300

Risgaard-Petersen, N., Rysgaard, S., Revsbech, N. P. (1993). A sensitive assay for determination of $^{14}N/^{15}N$ isotope distribution in NO_3^-. J. Microb. Meth. 17: 155–164

Ryther, J. H., Dunstan, W. M. (1971). Nitrogen, phosphorus, and eutrophication in the coastal marine environment. Science 171: 1008–1013

Seitzinger, S. P. (1980). Denitrification and N_2O production in near-shore marine sediments. Geochim. Cosmochim. Acta 44: 1853–1860

Thorson, G. (1975). Infaunaen, den jævne havbunds dyresamfund. In: Böcher, T. W., Nielsen, C. O., Schou, A. (eds.) Danmarks Natur, Vol. III. Havet. Politikens Forlag, København, p. 82–166

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